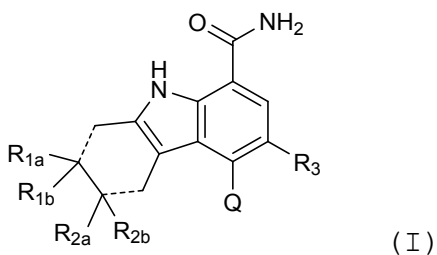


REIVINDICACIONES MODIFICADAS SOLICITUD DE PATENTE NO.

NC2017/0004517 -NOVIEMBRE 2018

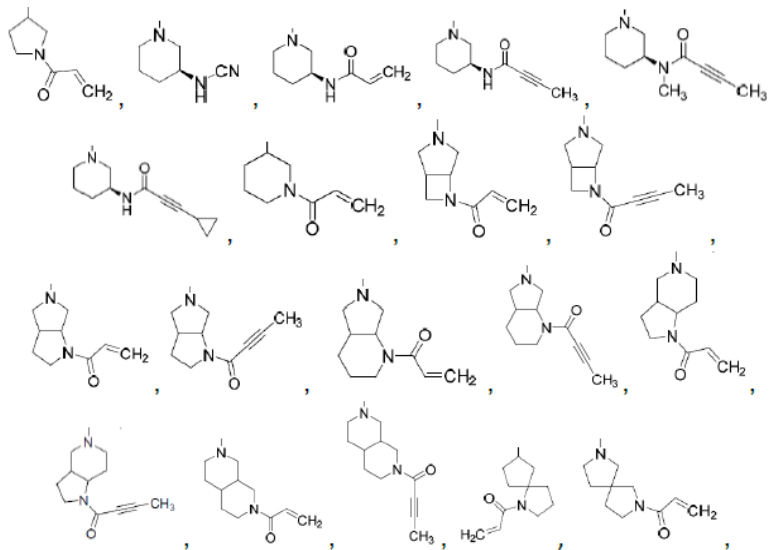
1. Compuesto de la fórmula (I)



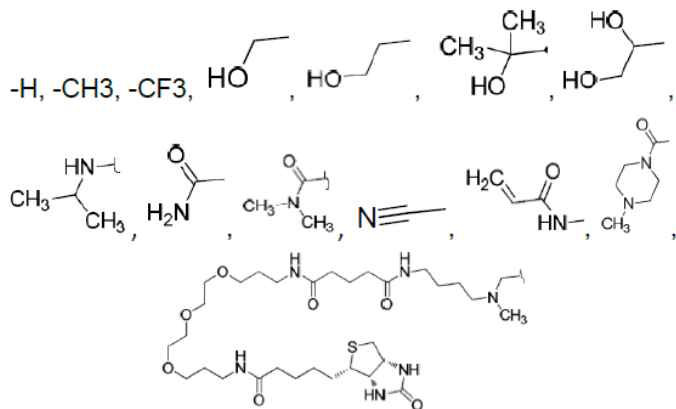
o una de sus sales farmacéuticamente aceptable, caracterizado porque:

las dos líneas de puntos representan dos enlaces sencillos o dos enlaces dobles; y R_{1b} y R_{2b} están presentes sólo si las dos líneas de puntos son dos enlaces sencillos;

Q es:



R_{1a} es:



R_{1b} , de estar presente, es H o $-CH_3$, siempre que, si R_{1a} es H entonces R_{1b} también sea H;

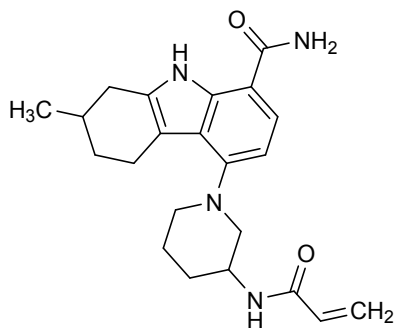
R_{1a} y R_{1b} pueden ser cada uno un grupo metilo simultáneamente;

R_{2a} es H, F, o Cl, siempre que, si R_{1a} es distinto de H entonces R_{2a} es H;

 R_{2b} es $H, F;$

R_3 es F o Cl;

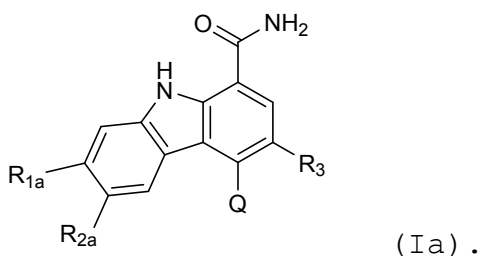
el compuesto de la fórmula (I) no es:



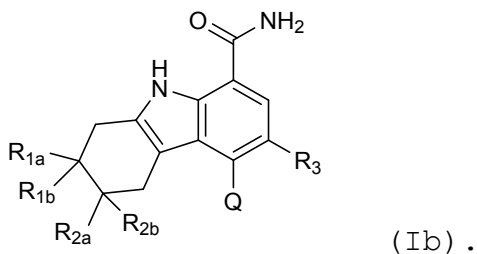
2. El compuesto de conformidad con la reivindicación 1 o una de sus sales, caracterizado porque:

R_3 es F.

3. Compuesto de conformidad con la reivindicación 1 o una de sus sales, caracterizado porque tiene la estructura de la fórmula (Ia):



4. Compuesto de conformidad con la reivindicación 1 o una de sus sales, caracterizado porque tiene la estructura de la fórmula (Ib):



5. El compuesto de conformidad con la reivindicación 1 o una de sus sales, caracterizado porque el compuesto es: (RS)-4-(3-acrilamido-2-metilfenil)-3-cloro-7-(2-hidroxipropan-2-il)-9H-carbazol-1-carboxamida (6); (RS)-5-(3-acrilamido-2-metilfenil)-6-cloro-2-(hidroximetil)-2,3,4,9-tetrahidro-1H-carbazol-8-carboxamida, mezcla de diastereómeros (8); 5-(3-acrilamido-2-metilfenil)-6-cloro-2,3,4,9-

tetrahidro-1*H*-carbazol-2,8-dicarboxamida, mezcla de diastereómeros (9); (RS)-(E)-4-(3-(but-2-enamido)-2-metilfenil)-3-cloro-7-(2-hidroxiopropan-2-il)-9*H*-carbazol-1-carboxamida (11); (2R)-6-fluoro-2-(2-hidroxiopropan-2-il)-5-(2-metil-3-(*N*-metilvinilsulfonamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, mezcla de diastereómeros (22); (2R)-6-cloro-2-(2-hidroxiopropan-2-il)-5-(2-metil-3-(*N*-metilvinilsulfonamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, mezcla de diastereómeros (23); (2R)-6-fluoro-2-(2-hidroxiopropan-2-il)-5-(3-(*N*-metilvinilsulfonamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, enantiómero simple (24); (2R)-6-cloro-2-(2-hidroxiopropan-2-il)-5-(3-(*N*-metilvinilsulfonamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, enantiómero simple (25); (RS)-3-fluoro-7-(2-hidroxiopropan-2-il)-4-(2-metil-3-(*N*-metilvinilsulfonamido)fenil)-9*H*-carbazol-1-carboxamida (30); 3-fluoro-7-(2-hidroxiopropan-2-il)-4-(3-(*N*-metilvinilsulfonamido)fenil)-9*H*-carbazol-1-carboxamida (31); 3-fluoro-7-(2-hidroxiopropan-2-il)-4-(3-(vinilsulfonamido)fenil)-9*H*-carbazol-1-carboxamida (34); (R)-6-fluoro-2-(2-hidroxiopropan-2-il)-5-(3-(*N*-metilacrilamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida (36); 3-fluoro-7-(2-hidroxiopropan-2-il)-4-(3-(*N*-metilacrilamido)fenil)-9*H*-carbazol-1-carboxamida (38); (R)-6-cloro-2-(2-hidroxiopropan-2-il)-5-(3-(*N*-metilacrilamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida (39); (R)-6-fluoro-2-(2-hidroxiopropan-2-il)-5-(3-(vinilsulfonamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida (43); (2R)-6-cloro-2-(2-hidroxiopropan-2-il)-5-(2-metil-3-

(vinilsulfonamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, mezcla de diastereómeros (44); (2*R*)-6-fluoro-2-(2-hidroxiopropan-2-il)-5-(2-metil-3-(vinilsulfonamido)fenil)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, mezcla de diastereómeros (47); 5-(3-acrilamido-2-metilfenil)-6-cloro-2-(hidroximetil)-2-metil-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, diastereómeros racémicos simples (55 y 56); 5-(3-acrilamido-2-metilfenil)-6-cloro-*N*²,*N*²-dimetil-2,3,4,9-tetrahidro-1*H*-carbazol-2,8-dicarboxamida, diastereómeros racémicos simples (57 y 58); 5-(3-acrilamido-2-metilfenil)-6-cloro-2-(2-hidroxiopropan-2-il)-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, diastereómeros racémicos simples (59 y 60); 5-(3-acrilamido-2-metilfenil)-6-cloro-2-(2-hidroxiopropan-2-il)-2-metil-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida, diastereómeros racémicos simples (61 y 62); (*S*)-5-(3-acrilamidopiperidin-1-il)-3,3,6-trifluoro-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida (66); (*S*)-4-(3-acrilamidopiperidin-1-il)-3-fluoro-9*H*-carbazol-1-carboxamida (69); (*S*)-5-(3-(but-2-inamido)piperidin-1-il)-3,3,6-trifluoro-2,3,4,9-tetrahidro-1*H*-carbazol-8-carboxamida (73); (*S*)-3-fluoro-4-(3-(*N*-metilbut-2-inamido)piperidin-1-il)-9*H*-carbazol-1-carboxamida (74); (*S*)-4-(3-(but-2-inamido)piperidin-1-il)-3-fluoro-9*H*-carbazol-1-carboxamida (75); (*S*)-4-(3-(3-ciclopropilpropiolamido)piperidin-1-il)-3-fluoro-9*H*-carbazol-1-carboxamida (76); 3-fluoro-4-((6-vinilpiridin-3-il)metil)-9*H*-carbazol-1-carboxamida (87); (*RS*)-4-(2-acriloilisoindolin-4-il)-3-fluoro-9*H*-carbazol-1-carboxamida (89); 4-(2-acriloil-1,2,3,4-tetrahidroisoquinolin-7-il)-3-fluoro-9*H*-carbazol-1-carboxamida (90);

4-(2-acriloil-1,2,3,4-tetrahidroisoquinolin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (91); 4-(1-acriloilindolin-4-il)-3-fluoro-9H-carbazol-1-carboxamida (92); 4-(1-acriloilindolin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (95); 4-(1-acriloil-1,2,5,6-tetrahidropiridin-3-il)-3-fluoro-9H-carbazol-1-carboxamida (96); (RS)-4-(1-acriloilpiperidin-3-il)-3-fluoro-9H-carbazol-1-carboxamida (97); 4-(1-acriloilpiperidin-3-il)-3-fluoro-9H-carbazol-1-carboxamida, enantiómeros simples (98 y 99); 3-fluoro-4-((2-vinilpiridin-4-il)metil)-9H-carbazol-1-carboxamida (100); 4-(1-acriloilpirrolidin-3-il)-3-fluoro-9H-carbazol-1-carboxamida (112); 4-(1-acriloilpirrolidin-3-il)-3-fluoro-9H-carbazol-1-carboxamida (113 y 114); *cis*-4-(1-(but-2-inoil)octahidro-6H-pirrolo[3,4-b]piridin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (115); *cis*-4-(1-(but-2-inoil)octahidro-6H-pirrolo[3,4-b]piridin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (116 y 117); (S)-4-(3-(but-2-inamido)piperidin-1-il)-3-fluoro-9H-carbazol-1-carboxamida (118); *cis*-4-(1-acriloiloctahidro-6H-pirrolo[3,4-b]piridin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (119); *cis*-4-(1-acriloiloctahidro-6H-pirrolo[3,4-b]piridin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (120 y 121); 3-fluoro-4-((2-vinilpirimidin-5-il)metil)-9H-carbazol-1-carboxamida (122); *cis*-4-(1-acriloilhexahidropirrolo[3,4-b]pirrol-5(1H)-il)-3-fluoro-9H-carbazol-1-carboxamida (123); *cis*-4-(1-acriloilhexahidropirrolo[3,4-b]pirrol-5(1H)-il)-3-fluoro-9H-carbazol-1-carboxamida (124 y 125); 4-(1-(but-2-inoil)octahidro-6H-pirrolo[3,4-b]piridin-6-il)-3-cloro-9H-carbazol-1-carboxamida (126); 4-((4aS,7aS)-1-(but-2-inoil)octahidro-6H-pirrolo[3,4-b]piridin-6-il)-3-cloro-9H-

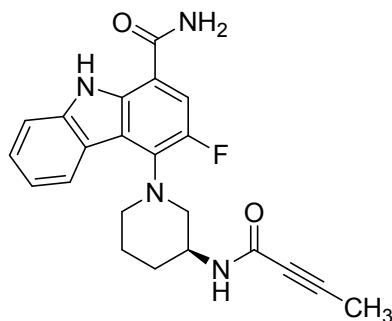
carbazol-1-carboxamida y 4-((4aR,7aR)-1-(but-2-inoil)octahidro-6H-
 pirrolo[3,4-b]piridin-6-il)-3-cloro-9H-carbazol-1-carboxamida (127 y
 128); 3-fluoro-4-((2-(prop-1-in-1-il)piridin-4-il)metil)-9H-carbazol-
 1-carboxamida (129); 5-((S)-3-(but-2-inamido) piperidin-1-il)-6-
 fluoro-2-(2-hidroxiopropan-2-il)-2,3,4,9-tetrahidro-1H-carbazol-8-
 carboxamida (130, 131, y 132); 4-(2-acriloilisoindolin-5-il)-3-fluoro-
 9H-carbazol-1-carboxamida (133); 4-(1-acriloil-2,5-dihidro-1H-pirrol-
 3-il)-3-fluoro-9H-carbazol-1-carboxamida (134); 5-(1-
 acriloilpirrolidin-3-il)-6-fluoro-2,3,4,9-tetrahidro-1H-carbazol-8-
 carboxamida (135); (R)-4-(3-(but-2-inamido)piperidin-1-il)-3-fluoro-
 9H-carbazol-1-carboxamida (136); 4-(1-(but-2-
 inoil)hexahidropirrolo[3,4-b]pirrol-5(1H)-il)-3-fluoro-9H-carbazol-1-
 carboxamida (137); 4-(1-acriloil-1,4,5,6-tetrahidropiridin-3-il)-3-
 fluoro-9H-carbazol-1-carboxamida (138); 4-(7-(but-2-inoil)-2,7-
 diazaspiro[4.4]nonan-2-il)-3-fluoro-9H-carbazol-1-carboxamida (139);
 4-(7-acriloil-2,7-diazaspiro[4.4]nonan-2-il)-3-fluoro-9H-carbazol-1-
 carboxamida (140); 4-(1-acriloiloctahidro-5H-pirrolo[3,2-c]piridin-5-
 il)-3-fluoro-9H-carbazol-1-carboxamida (141); 4-(1-(but-2-
 inoil)octahidro-5H-pirrolo[3,2-c]piridin-5-il)-3-fluoro-9H-carbazol-1-
 carboxamida (142); 4-(6-acriloil-3,6-diazabicciclo[3.2.0]heptan-3-il)-
 3-fluoro-9H-carbazol-1-carboxamida (143); 4-(6-(but-2-inoil)-3,6-
 diazabicciclo[3.2.0] heptan-3-il)-3-fluoro-9H-carbazol-1-carboxamida
 (144); 4-(7-acriloiloctahidro-2,7-naftiridin-2(1H)-il)-3-fluoro-9H-
 carbazol-1-carboxamida (145); 4-(1-acriloiloctahidro-6H-pirrolo[3,4-
 b]piridin-6-il)-3-cloro-9H-carbazol-1-carboxamida (146); 4-(1-(but-2-

inoil)indolin-4-il)-3-fluoro-9H-carbazol-1-carboxamida (147); 4-(2-(but-2-inoil)-1,2,3,4-tetrahidroisoquinolin-7-il)-3-fluoro-9H-carbazol-1-carboxamida (148); 4-(2-(but-2-inoil)-1,2,3,4-tetrahidroisoquinolin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (149); 4-(2-(but-2-inoil)isoindolin-4-il)-3-fluoro-9H-carbazol-1-carboxamida (150); 4-(1-(but-2-inoil)indolin-6-il)-3-fluoro-9H-carbazol-1-carboxamida (151); 3-fluoro-4-((6-vinilpirazin-2-il)metil)-9H-carbazol-1-carboxamida (152); 3-cloro-4-((6-vinilpirazin-2-il)metil)-9H-carbazol-1-carboxamida (153); 4-((6-etinilpiridin-3-il)metil)-3-fluoro-9H-carbazol-1-carboxamida (154); 3-cloro-4-((6-vinilpiridin-3-il)metil)-9H-carbazol-1-carboxamida (155); 4-((2-etinilpiridin-4-il)metil)-3-fluoro-9H-carbazol-1-carboxamida (156); 3-fluoro-4-((2-viniltiazol-5-il)metil)-9H-carbazol-1-carboxamida (157); 3-fluoro-4-((6-(prop-1-in-1-il)piridin-3-il)metil)-9H-carbazol-1-carboxamida (158); 3-fluoro-4-((5-vinilpirazin-2-il)metil)-9H-carbazol-1-carboxamida (159); 4-(1-acriloil-1,2,5,6-tetrahidropiridin-3-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (160); 4-(1-acriloilpiperidin-3-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (161 y 162); (S)-4-(3-acrilamidopiperidin-1-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (163); (S)-4-(3-(but-2-inamido)piperidin-1-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (164); (R)-4-(3-(but-2-inamido)piperidin-1-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (165); (S)-4-(3-(3-ciclopropilpropiolamido)piperidin-1-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (166); (S)-4-(3-cyanamidopiperidin-1-il)-3-

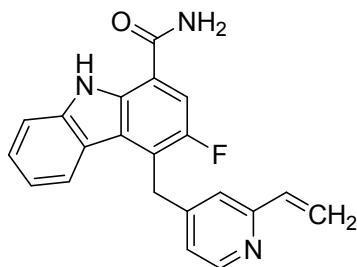
fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (167); 4-(2-acriloilisoindolin-4-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (168); 4-(1-acriloilindolin-4-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (169); 4-(1-acriloilindolin-6-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (170); 4-(1-acriloil-1-azaspiro[4.4]nonan-7-il)-3-fluoro-7-(trifluorometil)-9H-carbazol-1-carboxamida (171); (S)-4-(3-(but-2-inamido)piperidin-1-il)-3-fluoro-7-(4-metilpiperazine-1-carbonil)-9H-carbazol-1-carboxamida (172); (S)-4-(3-(but-2-inamido)piperidin-1-il)-3-fluoro-N7,N7-dimetil-9H-carbazol-1,7-dicarboxamida (173); 3-fluoro-N7,N7-dimetil-4-(2-vinilpiridin-4-il)-9H-carbazol-1,7-dicarboxamida (174); (S)-4-((1-cianopirrolidin-3-il)amino)-3-fluoro-N7,N7-dimetil-9H-carbazol-1,7-dicarboxamida (175); (S)-4-((1-cianopirrolidin-3-il)amino)-3-fluoro-7-(4-metilpiperazine-1-carbonil)-9H-carbazol-1-carboxamida (176); 4-(2-acriloil-1,2,3,4-tetrahidroisoquinolin-5-il)-7-(morfolina-4-carbonil)-9H-carbazol-1-carboxamida (177); 4-(1-acriloilindolin-6-il)-3-fluoro-7-(2-hidroxietil)-9H-carbazol-1-carboxamida (180); 4-(1-cianoindolin-6-il)-3-fluoro-7-(2-hidroxietil)-9H-carbazol-1-carboxamida (181); 4-(1-acriloiloctahidro-6H-pirrol[3,4-b]piridin-6-il)-6-cloro-3-fluoro-9H-carbazol-1-carboxamida (182); 4-(1-(but-2-inoil)octahidro-6H-pirrol[3,4-b]piridin-6-il)-6-cloro-3-fluoro-9H-carbazol-1-carboxamida (183); 5-(1-acriloil-1,2,5,6-tetrahidropiridin-3-il)-6-fluoro-2-(2-hidroxipropan-2-il)-2,3,4,9-tetrahidro-1H-carbazol-8-carboxamida (184); (R)-6-fluoro-2-(2-hidroxipropan-2-il)-5-((6-vinilpiridin-3-il)metil)-2,3,4,9-tetrahidro-

1H-carbazol-8-carboxamida (185); 5-(2-acriloil-1,2,3,4-tetrahidroisoquinolin-5-il)-6-fluoro-2-(2-hidroxiopropan-2-il)-2,3,4,9-tetrahidro-1H-carbazol-8-carboxamida (186 y 187); 6-fluoro-2-(2-hidroxiopropan-2-il)-5-((6-(prop-1-in-1-il)piridin-3-il)metil)-2,3,4,9-tetrahidro-1H-carbazol-8-carboxamida (188); 3-fluoro-4-(2-vinilpiridin-4-il)-9H-carbazol-1-carboxamida (189); 4-(7-(but-2-inoil)octahidro-2,7-naftiridin-2(1H)-il)-3-fluoro-9H-carbazol-1-carboxamida (190); 4-(1-acriloil-1,2,3,6-tetrahidropiridin-4-il)-3-fluoro-9H-carbazol-1-carboxamida (191); 4-(1-(but-2-inoil)-1,2,3,6-tetrahidropiridin-4-il)-3-fluoro-9H-carbazol-1-carboxamida (192); 3-fluoro-4-(5-(N-metilacrilamido)piridin-2-il)-9H-carbazol-1-carboxamida (193); 4-((1S,4S)-2-acriloil-2-azabicciclo[2.2.1]heptan-5-il)-3-fluoro-9H-carbazol-1-carboxamida (194); 3-fluoro-4-((2-metil-6-vinilpiridin-4-il)metil)-9H-carbazol-1-carboxamida (195); 3-fluoro-4-((2-metil-6-vinilpirimidin-4-il)metil)-9H-carbazol-1-carboxamida (196); 3-fluoro-4-((4-metil-6-vinilpirimidin-2-il)metil)-9H-carbazol-1-carboxamida (197); 3-fluoro-4-((3-fluoro-2-vinilpiridin-4-il)metil)-9H-carbazol-1-carboxamida (198); 4-(3-(1-acriloilpirrolidin-2-il)fenil)-3-fluoro-9H-carbazol-1-carboxamida (199); 4-(3-(1-(but-2-inoil)pirrolidin-2-il)fenil)-3-fluoro-9H-carbazol-1-carboxamida (200); (E)-3-fluoro-4-(3-(3-morfolino-3-oxoprop-1-en-1-il)fenil)-9H-carbazol-1-carboxamida (201); (E)-3-fluoro-4-(3-(3-oxo-3-(pirrolidin-1-il)prop-1-en-1-il)fenil)-9H-carbazol-1-carboxamida (202); o 5-(2-acriloil-1,2,3,4-tetrahidroisoquinolin-5-il)-2-(2-hidroxiopropan-2-il)-2,3,4,9-tetrahidro-1H-carbazol-8-carboxamida (203 y 204).

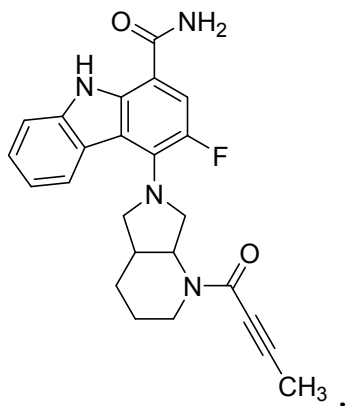
6. Compuesto de conformidad con la reivindicación 1, caracterizado porque el compuesto es:



7. Compuesto de conformidad con la reivindicación 1, caracterizado porque el compuesto es:



8. Compuesto de conformidad con la reivindicación 1, caracterizado porque el compuesto es:



9. Una composición farmacéutica, caracterizada porque comprende un compuesto de conformidad con cualquiera de las reivindicaciones 1-8 y un portador farmacéuticamente aceptable.

Developing Irreversible Inhibitors of the Protein Kinase Cysteinome

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Protein kinases are a large family of approximately 530 highly conserved enzymes that transfer a γ -phosphate group from ATP to a variety of amino acid residues, such as tyrosine, serine, and threonine, that serves as a ubiquitous mechanism for cellular signal transduction. The clinical success of a number of kinase-directed drugs and the frequent observation of disease causing mutations in protein kinases suggest that a large number of kinases may represent therapeutically relevant targets. To date, the majority of clinical and preclinical kinase inhibitors are ATP competitive, noncovalent inhibitors that achieve selectivity through recognition of unique features of particular protein kinases. Recently, there has been renewed interest in the development of irreversible inhibitors that form covalent bonds with cysteine or other nucleophilic residues in the ATP-binding pocket. Irreversible kinase inhibitors have a number of potential advantages including prolonged pharmacodynamics, suitability for rational design, high potency, and ability to validate pharmacological specificity through mutation of the reactive cysteine residue. Here, we review recent efforts to develop cysteine-targeted irreversible protein kinase inhibitors and discuss their modes of recognizing the ATP-binding pocket and their biological activity profiles. In addition, we provided an informatics assessment of the potential “kinase cysteinome” and discuss strategies for the efficient development of new covalent inhibitors.

Kinases are one of the largest gene families whose function is to catalyze the transfer of the γ -phosphate from ATP to a specific target molecule bearing nucleophilic hydroxyl or amino groups including lipids, carbohydrates, and proteins. Protein phosphorylation can induce a myriad of consequences including modulation of enzyme activity, conformation, stability, localization, and association with other proteins or small molecules (Cohen, 2002). The approximately 530 kinases present in the human genome have been named the “kinome” and include 420 serine/threonine, 94 tyrosine, 25 atypical, and 49 “pseudo kinases” that putatively lack the ability to catalyze the phosphotransfer reaction (Manning et al., 2002a, 2002b). Kinases have been found to function in virtually every biological process and pathway and perhaps not surprisingly deregulation of kinase function through environmental and genetic alterations is a hallmark of many pathological conditions (Hanahan and Weinberg, 2011). Since the mid-1980s when v-SRC was initially discovered to be a tyrosine kinase and capable of functioning as an “oncogene,” basic research and clinical investigation have implicated the deregulation of approximately 180 kinases in diverse pathology associated with metabolism, development, immunology, cancer, and infectious disease (Hunter and Sefton, 1980). The development of kinase inhibitors has attracted an enormous amount of drug discovery attention, primarily in the oncology and inflammatory diseases areas. Currently, approxi-

mately 40 kinases are actively being pursued as therapeutic targets, and 14 kinase inhibitors have received regulatory approval (Barf and Kaptein, 2012; Kontzias et al., 2012). Interestingly, by far the most significant success has been achieved in oncology by targeting mutationally activated “oncogenic” driver kinases including Bcr-Abl, EGFR, c-Kit, PDGFR, ALK, and b-RAF in tumors that are “addicted” to the constitutive activation of these kinases (Haber et al., 2011).

Currently, the vast majority of protein kinase inhibitors (PKIs) are reversible ATP competitive inhibitors, which achieve target selectivity by recognizing unique features of particular ATP-binding pockets. This class of inhibitor typically occupies the adenine-binding region of the ATP-binding pocket and extends into surrounding regions not occupied by ATP, and inhibitor binding often induces dramatic conformational rearrangements to the pocket. Compounds that bind to the ATP-binding site with the kinase assuming an active conformation, or a conformation otherwise conducive to ATP binding, are called “type I” inhibitors while those that induce a “flip” of the DFG motif that marks the start of the activation loop are called “type II” (Liu and Gray, 2006). Multiple other examples of “induced fit” have been observed providing clear evidence for the plasticity of the ATP-binding pocket (Changeux and Edelstein, 2011). However, given the high sequence conservation of the ATP-binding site and the very large number of kinases, achieving a high degree

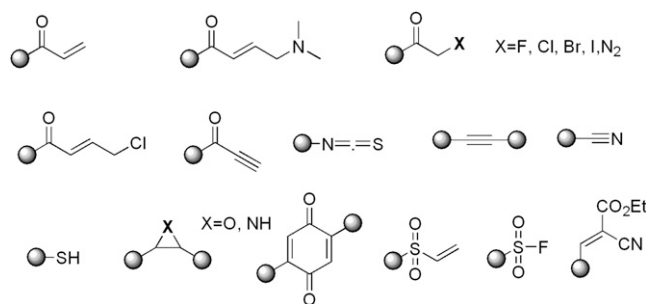


Figure 1. Electrophiles Used in Irreversible Kinase Inhibitors
Black balls represent other pharmacophore structures.

of kinase selectivity has proved extremely challenging. While there are some examples of exquisitely selective kinase ATP-competitive inhibitors such as lapatinib, which targets Her2, and SB239063, which targets p38 kinase, most inhibitors have a spectrum of targets that widens as inhibitor concentration increases (Rusnak et al., 2001; Underwood et al., 2000). There have been tremendous advances in our ability to measure kinase inhibitor selectivity across near-comprehensive panels using biochemical kinase assays, competition binding assays, and chemical proteomic approaches (Karaman et al., 2008; Liu et al., 2012b; Okerberg et al., 2005). These efforts have enabled both prospective and retrospective matching of compounds with targets and have greatly furthered our understanding of the mechanism of action of a number of inhibitors.

The majority of new kinase inhibitors are being developed in the pharmaceutical sector typically targeting kinases that have been intensively investigated previously in academia. The requirement for significant prevalidation of potential kinase targets prior to the development of inhibitors in the pharmaceutical sector has resulted in a shortage of inhibitors targeting the kinases whose biological function have received less attention. Therefore, new approaches are needed for the rapid generation of inhibitors of poorly understood kinases that can be used as pharmacological probes of mechanism. Covalent kinase inhibitors provide an ideal platform for this endeavor as we discuss further below.

Covalent kinase inhibitors have typically been developed by structure-guided incorporation of an electrophilic moiety into an inhibitor that already possesses submicromolar binding affinity to the target of interest (Potashman and Duggan, 2009). The majority of covalent inhibitors have been designed to target the highly nucleophilic thiol group of cysteine residues (Leproult et al., 2011). While a large number of kinases have cysteine residues in and around the ATP-binding site, there are no cysteine residues that are conserved among kinases that serve a key catalytic function to our knowledge. Covalent inhibitors will initially bind noncovalently, and then, if the trajectory of the reactive moiety is appropriate, covalent bond formation will take place, permanently disabling enzymatic activity. Kinase function is only restored following expression of new protein, the kinetics of which can vary dramatically for different kinases. One major advantage of covalent kinase inhibitors is that high selectivity for a given target kinase can be obtained using a combination of both noncovalent and covalent binding. An irreversible inhib-

itor that has one dominant binding mode will typically only form a covalent bond with a kinase that possesses a cysteine at a particular position in the ATP-binding site. Therefore, noncovalent recognition only needs to enable discrimination between kinases that possess an equivalently placed cysteine residue.

Cysteine residues possess an aliphatic thiol (SH), which has unique reactivity among the naturally occurring amino acids. For example, the deprotonated thiolate anion is a potent nucleophile that is exploited as the key catalytic residue in phosphatases and cysteine proteases. Cysteines also serve key non-catalytic functions such as stabilizing protein tertiary structure through formation of disulphide crosslinks and coordination of enzyme cofactors such as metals (Jacob et al., 2012). Cysteine residues can be targeted by numerous posttranslational modifications including S-nitrosylation, S-prenylation, and oxidation to sulfenic and sulfonic acids (Chalker et al., 2009). Most covalent inhibition strategies that have been explored to date target the highly nucleophilic cysteine thiolate.

A number of electrophilic “warheads” that can react with nucleophiles such as cysteine, lysine, or tyrosine have been explored in the design of irreversible kinase inhibitors. The Michael addition reaction is the most widely utilized reaction to achieve irreversible binding. Functional groups typically introduced to undergo this addition reaction include acrylamides, vinyl sulfonates, quinones, alkynyl amides, and propargylic acid derivatives (Figure 1). A second frequently employed chemistry uses nucleophilic displacement or addition to α -halo ketones, thiocyanates, alkynes, nitriles, epoxides, sulfonyl fluoride, and sometimes thiol itself. Electrophiles, such as cyclic 1,3-diketone, have also been developed that specifically react with sulfenic acids (Leonard et al., 2011). The ability to target differentially modified cysteine residues may afford further means of achieving selectivity.

Historically, there has been considerable reluctance by many organizations to pursue covalent inhibition strategies due to risks of haptenization, the most studied examples being haptens generated by reactive metabolites (Uetrecht, 2008). Unfortunately, preclinical or even clinical tests that are reliable predictors of drug safety do not currently exist. Interestingly, a recent retrospective analysis suggested that idiosyncratic toxicities have not been observed for any inhibitors administered at doses of less than 10 mg/kg (Nakayama et al., 2009). This suggests that well-designed and highly potent covalent inhibitors might have adequate safety profiles. Irreversible inhibitors are widely used in clinical practice including some of the most important medicines such as the anti-inflammatory drug aspirin and the broad class of antibacterial beta-lactam antibiotics such as penicillin. Other widely used metabolic activation mechanism-based covalent drugs include the proton pump inhibitor omeprazole and antiplatelet drug clopidogrel (Potashman and Duggan, 2009). Although it may be counterintuitive to create selectivity via a covalent mechanism, the inhibitor electrophilicity can be fine-tuned such that the reaction only occurs in the target binding site. Additionally, covalency can also provide extended pharmacodynamic duration without the need to maintain high levels of drug to achieve continuous target engagement (Smith et al., 2009).

Recently, there has been a resurgence of interest in irreversible inhibitors, and this topic has been excellently reviewed in

several publications from a historical perspective (Singh et al., 2011), from a risk-benefit perspective (Barf and Kaptein, 2012; Johnson et al., 2010), and in terms of the current irreversible inhibitors that are in preclinical or clinical development (Garuti et al., 2011; Singh et al., 2010). Leproult et al. (2011) has also provided a bioinformatic mapping of the potential cysteine containing kinases that could potentially be covalently targeted based upon available X-ray crystal structures. In this review, we summarize recent efforts to develop potent and selective irreversible PKIs and describe their modes of recognition of the ATP-binding site and a description of their biological profiles from the perspective of a medicinal chemist. We also provide an analysis of the types of approaches that can be employed to efficiently generate these inhibitors and present a bioinformatics analysis of the potentially targetable cysteines in and around the ATP-binding pocket based on a combination of Pfizer's in-house and publicly available crystal structures. This information is complementary to the previously published articles, and we encourage the interested reader to see these references for additional information.

Overview of the Currently Developed Irreversible PKIs

Although most recently reported covalent inhibitors are synthetic, a number of natural products have evolved that covalently modify cysteine residues in kinase ATP-binding sites (Liu et al., 2012a). One of the most well-characterized classes of covalent kinase inhibitors are the resorcylic acid lactones (RALs) with hypothemycin being the most well-known member (Sonoda et al., 1999). Hypothemycin was originally isolated based on its antifungal activity, and subsequent investigations demonstrated it to be a covalent protein kinase inhibitor. Covalent bond formation is achieved through reaction of its base *cis*-enone function with cysteine residues (Figure 2). Santi and co-workers used sequence alignment to identify a conserved cysteine residue immediately preceding the conserved "DFG motif" that marks the start of the kinase activation loop that is present in a number of kinases inhibited by hypothemycin including MEK1/2, ERK1/2, PDGFRs, FLT3, and VEGFRs (Schirmer et al., 2006). A cocrystallized structure of ERK2 with hypothemycin (PDB: 2E14) demonstrated a covalent bond between Cys166 of ERK2 and the *cis*-enone moiety of the inhibitor (Figure 3A) (Otori et al., 2007). The phenolic hydroxyl group of hypothemycin forms two hydrogen bonds with Met108 in the kinase hinge segment. Two additional hydrogen bonds are formed between Lys114 in the solvent exposed area and the methoxy group and between Tyr36 located in the P loop with the hydroxyl group in the marocyclic ring. Several hypothemycin analogs, including FR148083, LL-Z1640-2, and LL-782277, are believed to share the same inhibitory mechanism with TAK1 and MEK kinases (Winssinger and Barluenga, 2007). Starting from hypothemycin, a focused medicinal chemistry effort to improve its drug-like properties resulted in a variety of analogs including the structurally similar drug candidate E6201 (Barluenga et al., 2010; Goto et al., 2009; Jogireddy et al., 2009). E6201 inhibits MEK1 biochemically with a low nanomolar IC₅₀ and exhibits strong anti-inflammatory and antiproliferation activities. E6201 is currently in phase I clinical trials for advanced solid tumors and in a phase II trial for psoriasis (Table 1) (Goto et al., 2009; Muramoto et al., 2010).

The development of synthetic irreversible PKIs was initiated at Parke-Davis and Wyeth (now Pfizer) in the early 1990s with the goal of targeting EGFR with covalent inhibitors for the treatment of cancers (Singh et al., 1997; Wissner and Mansour, 2008). PD168393 was one of the first reported synthetic irreversible PKIs with a reported IC₅₀ of 2 nM against EGFR and an IC₅₀ of 114 nM against Her2 (Fry et al., 1998). PD168393 is a 4-amino-quinazoline with an acrylamide electrophile installed at the 6-position designed to target Cys797 located a few residues C-terminal to the kinase hinge binding region of EGFR. PD168393 inhibits the proliferation of EGFR and Her2-dependent cell lines A431 and SKBR3 with 95 and 15 nM EC₅₀s, respectively, while not inhibiting the proliferation of the non-EGFR and Her-dependent cell line SW620 at concentrations of up to 4 μM (Tsou et al., 2005). Further development of this scaffold resulted in a number of compounds that advanced to clinical trials including CI-1033 (clinical development terminated) and PF-00299804 (phase III trial) (Engelman et al., 2007; Smail et al., 2000). Replacement of one of the quinazoline nitrogens with a nitrile resulted in another set of compounds that advanced to clinical trials: EKB569 (phase II trial completed), HKI-272 (phase III trial), and BIBW-2992 (phase III trial) (Li et al., 2008; Yoshimura et al., 2006). All of these compounds target Cys797, a residue conserved among EGFR, Her2, and Her4, which can be visualized in the HKI-272-EGFR costructure (PDB: 2JIV). The costructure suggests that a key hydrogen bond exists between the quinoline nitrogen and Met793 in the kinase hinge segment and between Asp855 in the highly conserved DFG motif and the pyridine side chain of the inhibitor. A covalent bond is evident between the N,N-dimethyl-butenoic amide and Cys797 (Figure 2B). HKI-272 exhibits IC₅₀s of 59 and 92 nM for inhibition of Her2 and EGFR kinase activity, respectively. HKI-272 inhibits the proliferation of A431 and SKBR3 cells with EC₅₀s of 86 and 118 nM, respectively, while demonstrating selectivity over the non-EGFR-addicted cell line SW620 (730 nM). Kinome-wide selectivity profiling using KinomeScan methodology demonstrated HKI-272 to be a very selective inhibitor: in addition to EGFR and Her2, it only exhibited potent binding to MAP4K3/5 and MST3/4 (K_d < 10 nM) (Davis et al., 2011). These additional targets do not appear to have been validated in cellular assays to date.

Kinase sequence alignment indicates that there are eight non-EGFR family kinases that also possess a cysteine at the same position as Cys797 of EGFR. These eight kinases include all five members of the Tec family of kinases (BMX, BTK, ITK, TEC, and TXK), one Src family member (BLK), MKK47, and JAK3. Not surprisingly, some compounds crossreact with kinases in this group. PD168393, for example, exhibits a 1.1 μM IC₅₀ against BMX and inhibits BMX-dependent cell growth in the Ba/F3 cell system with an EC₅₀ of 0.3 μM (Hur et al., 2008). As discussed further below, more recent efforts have been expended to develop covalent inhibitors of the Tec family kinase BTK (Figure 3C).

Despite lung cancer patients that express mutant EGFR (exon 19 deletion or L858R) displaying dramatic responses to first-generation ATP-competitive inhibitors initially, all patients relapse after 12–18 months (Rosell et al., 2009). In approximately 50% of cases, resistance is the result of mutation of the so-called gatekeeper residue from a threonine to a methionine (T790M).

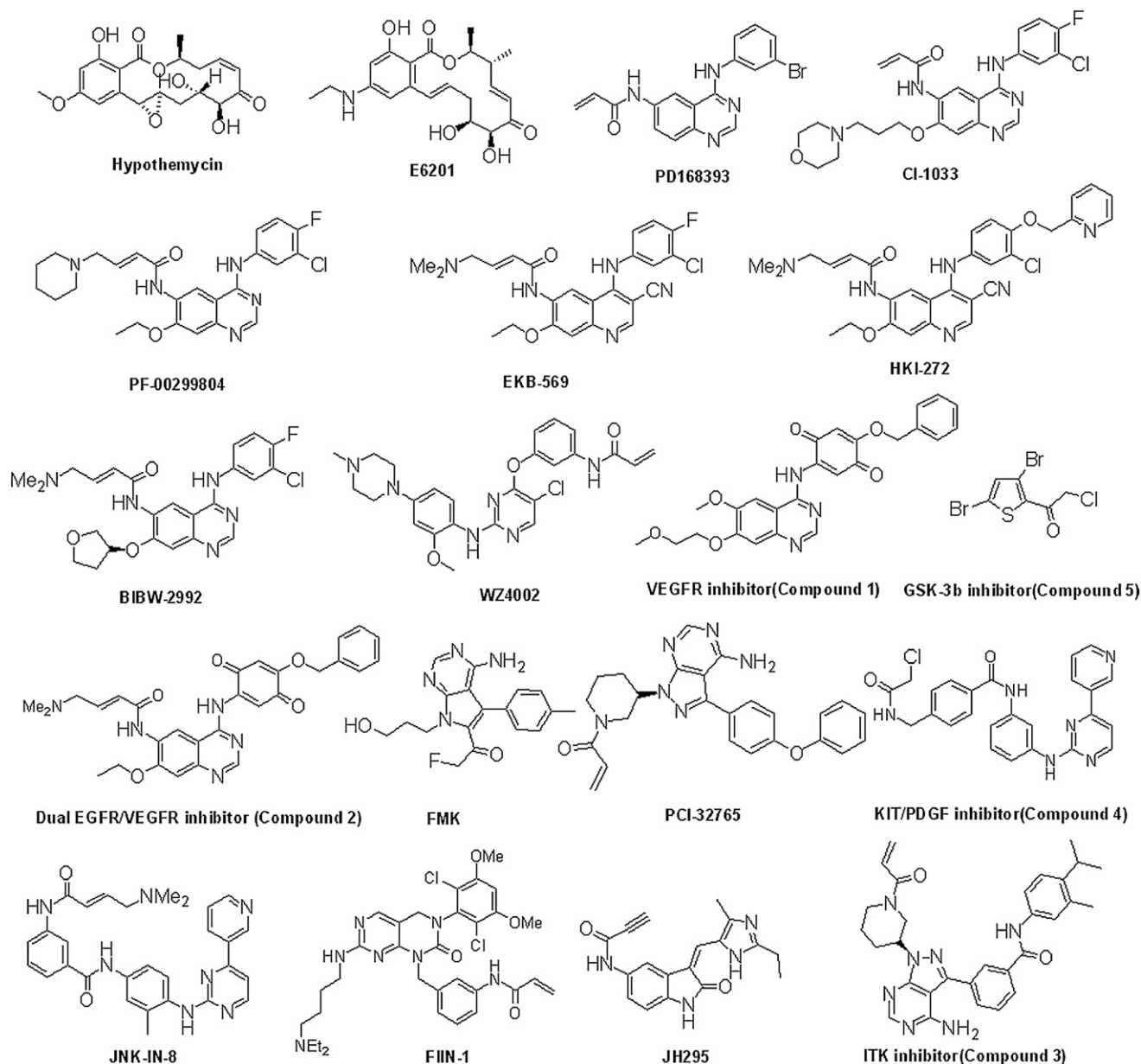


Figure 2. Representative Chemical Structures of Reported Irreversible Protein Kinase Inhibitors

Compounds were listed as reported names; those that do not have generic names are named in the order they are mentioned in the text.

This mutation is believed to induce resistance by decreasing the K_m for ATP thereby increasing the concentration of inhibitor needed to efficiently inhibit signaling (Yun et al., 2007). While the first-generation covalent inhibitors including CI-1033 and HKI-272 can inhibit activated alleles of EGFR harboring T790M, they do so at a concentration 10–100 times higher than required to inhibit the nongatekeeper mutants. To overcome this limitation, a second-generation of covalent inhibitor from the aminopyrimidine class, exemplified by WZ-4002, was discovered that potently inhibits activated alleles of EGFR harboring T790M (Zhou et al., 2009). This inhibitor also targets Cys797 but approaches the thiol group from a different trajectory relative to the first-generation quinazoline acrylamide inhibitors.

Interestingly, an interaction between the chlorine on the pyrimidine of the inhibitors with the methionine gatekeeper, an interaction that has been termed a “halogen bond,” results in the inhibitors possessing selectivity for T790M versus wild-type EGFR (Figure 3D) (Hernandes et al., 2010). WZ4002 overall possesses quite good kinase selectivity but does show activity on some Tec family kinases as expected based on the cysteine position.

The same 4-aminoquinazoline scaffold present in the first generation EGFR inhibitors was used by scientists at Wyeth to develop a covalent inhibitor of the vascular endothelial growth factor receptor (VEGFR) (compound 1) using a structure-based design approach (Figure 2) (Wissner et al., 2005). Compound 1

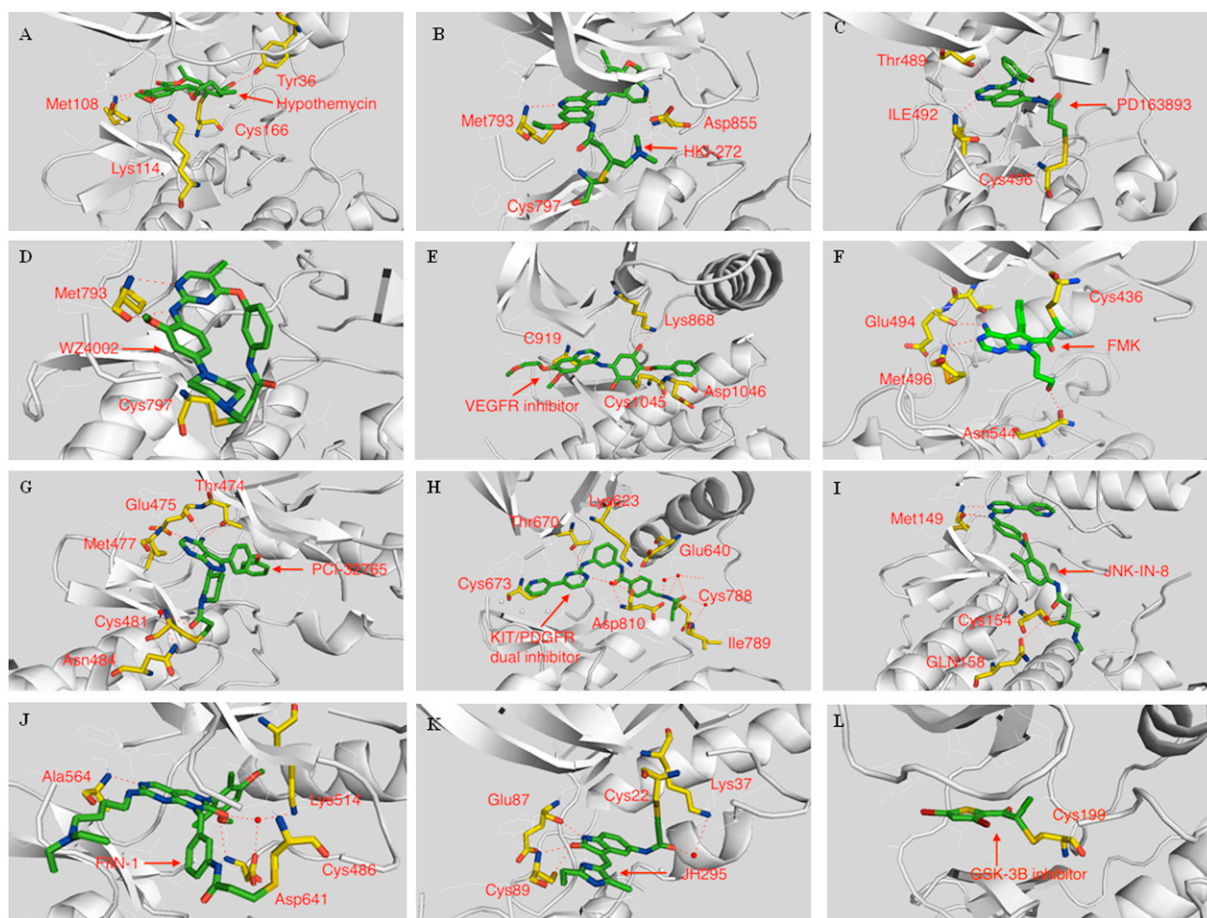


Figure 3. Representative Binding Modes of Irreversible Protein Kinase Inhibitors

(A) ERK (PDB: 2E14), (B) EGFR (PDB: 2JIV), (C) BMX (modeling-based PDB: 3SXS), (D) EGFR (PDB: 3IKA), (E) VEGFR (modeling-based PDB: 1VR2), (F) RSK2 (modeling-based PDB: 2QR7), (G) BTK (modeling-based PDB: 3GEN), (H) KIT (modeling-based PDB: 1T46), (I) JNK (PDB: 3V6S), (J) FGFR (PDB: 2FGI), (K) NEK2 (modeling-based PDB: 2JAV), (L) GSK3- β (modeling-based PDB: 1I09).

uses an electrophilic 1,4-benzoquinone to target Cys1045 located immediately before the “DFG” motif in VEGFR. Molecular modeling suggests the quinazoline N1 makes the expected hydrogen bond to the hinge region with additional hydrogen bonds formed between the inhibitor and Asp1046 and Lys868 (Figure 3E). This irreversible inhibitor exhibited a 50 nM IC_{50} for VEGFR. Further elaboration of this compound allowed the construction of a dual covalent inhibitor of VEGFR and EGFR (Compound 2) (Wissner et al., 2007). An acrylamide was installed to target Cys797 in EGFR and the quinone was maintained to target Cys1045 in VEGFR (Figure 2). The resulting compound inhibits the kinase activity of EGFR and VEGFR with IC_{50} s of 18 and 102 nM, respectively.

Another successful example of structure-guided covalent inhibitor design is the development of FMK, a fluoromethyl ketone derivatized inhibitor of RSK1, 2, 4 (Figure 2) (Cohen et al., 2005). FMK achieves selectivity for the RSKs by combining two selectivity filters: the presence of a small gatekeeper amino acid (residue T493) in Rsk and covalent bond formation with Cys463 located in the P loop, which resides on the “roof” of the ATP-binding site. There are only 11 kinases (RSK1,2,3, MSK1,2, PLK1,2,3,4, NEK2, and MEKK1) that possess a cysteine

at the same position as Cys463 in Rsk, and all of these kinases have a larger gatekeeper amino acid. FMK inhibits Rsk kinase activity with an IC_{50} of 15 nM. Molecular modeling indicates that the pyrrolpyrimidine core forms two hydrogen bonds in the hinge binding area with Met496 and Glu494 (Figure 3F). In addition, a hydrogen bond is predicted to exist between the exocyclic amino group and gatekeeper residue Thr494. The hydroxyl group in the butenol side chain forms a hydrogen bond with Asn544 that helps to fix the binding conformation. A biotin-labeled FMK probe was capable of efficiently purifying only RSK1 and RSK2 from HEK293 cell at a concentration of 1 μ M. The discovery and characterization of FMK provides a compelling example of the strength of structure-guided design of highly selective covalent kinase inhibitors.

Another group of kinases that have been successfully targeted by covalent inhibitors are the TEC family kinase and include TEC, ITK, TXK, BMX, and BTK. These kinases possess a cysteine in an identical location to Cys797 of EGFR. Researchers at Pharmacyclics developed a pyrazolopyrimidine compound, PCI-32765, that is a highly potent inhibitor of BTK (Honigberg et al., 2010). Molecular modeling predicts that the aminopyrazolopyrimidine forms three hydrogen bonds in the hinge area with Met477,

Table 1. Summary of Clinically Developed Irreversible Kinase Inhibitors

Drug Name	Primary Target	Activity IC ₅₀ (nM)	Warhead	Active Site	Clinical Stage	Developer
HKI-272	EGFR/Her2	59/92	Acrylamide	C797	Phase III	Pfizer, licensed to Puma
BIBW-2992	EGFR/Her2	14	Acrylamide	C797	Phase III	Boehringer Ingelheim
PF-00299804	Pan-EGFR	15	Acrylamide	C797	Phase III	Pfizer
CO1686	EGFR/T790M	0.5	Acrylamide	C797	Phase II	Avila/Clovis
E6201	MEK1	5.2	Enone	C297	Phase II	EiSai
PCI-32765	BTK	0.46	Acrylamide	C481	Phase III	Pharmacyclics
AVL-292	BTK	0.5	Acrylamide	C481	Phase I	Avila/Celgene

Glu475, and Thr474 (Figure 3G). The bisphenol ether is directed to the inner hydrophobic pocket, analogous to the binding mode of FMK. A water-bridged trihydrogen bond between Cys481, Asn484, and PCI-32761 also helps to fix the binding conformation. The acrylamide electrophile forms a covalent bond with Cys481. PCI-32765 inhibits BTK activity with an IC₅₀ of 0.5 nM and is also inhibitory toward several other protein kinases, including BLK, BMX, EGFR, Her2, ITK, JAK3, and TEC, that contain an analogous cysteine, but PCI-32765 does not show activity against most other protein kinases. PCI-32765 is currently in phase II clinical trials for various B-cell-related lymphomas such as DLBCL, MCL, and INHL. A compound (AVL-292, structure not disclosed) developed by Avila therapeutics (now Celgene) is believed to share the covalent mechanism as PCI-32765 and is currently in phase I clinical trials for NHL and CLL.

Researchers from Pfizer recently reported the development of the first covalent inhibitors of ITK derived from a privileged ATP-site-directed pyrazolopyrimidine (compound **3**) (Zapf et al., 2012). Starting from an acrylamide-containing screening “hit,” structure-based design was used to guide the development of a potent inhibitor that was active at nanomolar concentration in whole blood and that effectively silenced T cell receptor signaling for 24 hr.

A dual c-KIT/PDGFR inhibitor (compound **4**) (Figure 2) was rationally designed based on the systematic analysis of available X-ray structures of protein c-KIT, PDGFR, and Abl kinases (Leproult et al., 2011). Starting from the well-known type II kinase inhibitor imatinib, the methyl piperazine ring in the tail was replaced with a chloroacetamide, which resulted in a compound possessing an IC₅₀ of 788 nM for c-KIT and an IC₅₀ of 1 μM for PDGFRα, but that lost potency against Abl (IC₅₀ > 20 μM). Both c-KIT and PDGFRα possess a cysteine residue in the imatinib tail binding area. Molecular docking to PDGFR demonstrated that this new inhibitor adopted the same type II binding mode as observed for imatinib bound to ABL (Cowan-Jacob et al., 2007). One hydrogen bond was formed in the hinge binding area between the pyridine and Cys673 (Figure 3H). Several additional amino acids including Thr670, Lys623, Glu640, Asp810, and Ile789 also formed hydrogen bonds with the inhibitor. The docking model places Cys788 within 3 Å distance of the chloroacetamide; mass spectrometry has verified that Cys788 can indeed form a covalent bond with the newly designed inhibitor. Kinome-wide selectivity profiling showed that this compound only potently inhibited seven others including JNK1-3, DDR1, BRAF(V600E), and CSF1R. This irreversible PKI adopts a type II binding conformation.

The imatinib scaffold has also been elaborated to create the selective pan-JNK inhibitor, JNK-IN-8 (Zhang et al., 2012). JNK-IN-8 was discovered to inhibit JNK kinase by broad-based kinase selectivity profiling of a library of acrylamide kinase inhibitors based on the structure of imatinib using the KinomeScan approach. This discovery was perhaps not entirely unexpected as imatinib itself possesses a K_d of 5.0 and 3.1 μM for JNK1 and JNK3, respectively. JNK-IN-8 possesses distinct regiochemistry of the 1,4-dianiline and 1,3-aminobenzoic acid substructures relative to imatinib and uses an N,N-dimethyl butenoic acetamide warhead to covalently target Cys154. JNK-IN-8 potently inhibits JNK1, 2, and 3 enzymatic activity with IC₅₀s of 4.7, 18.7, and 1.0 nM, respectively, and inhibits a c-Jun, a direct phosphorylation substrate of JNK, with an EC₅₀ of 486 nM in HeLa cells. Broad-based profiling using the chemical proteomics approach termed “Kinativ” (Patricelli et al., 2007) demonstrated exclusive inhibition of JNK among the approximately 150 kinases detected using the approach. In contrast to imatinib and the covalent c-KIT/PDGFR inhibitor described above, JNK-IN-8 adopts an L-shaped type I binding conformation to access Cys 154 located toward the lip of the ATP-binding site (Figure 3I, PDB: 3V6S). This finding demonstrates how a kinase inhibitor class can adopt two completely distinct binding conformations when binding to different kinases.

A pan-FGFR1, 2, 3, and 4 covalent inhibitor FIIN-1 was developed starting from the well-established noncovalent inhibitor PD173074 using a structure-guided approach (Bansal et al., 2003; Zhou et al., 2010). FIIN-1 is a potent FGFR family covalent inhibitor that inhibits kinase activity with IC₅₀s of 9.2, 6.2, 11.9, and 189 nM for FGFR1, 2, 3, and 4 respectively. Kinome-wide selectivity profiling demonstrated that FIIN-1 is quite selective and may also bind to FLT1/4 and VEGFR but no other protein kinases. Molecular modeling suggests that a pair of hydrogen bonds is present between the amino-pyrimidine core structure and Ala564 located in the kinase hinge region (Figure 3J). Several other hydrogen bonds are predicted including a water-mediated trihydrogen bond involving Lys514, Asp641, and the carbonyl in the pyrimidine core structure and a hydrogen bond between Asp641 and the methoxy oxygen in the 2,6-dichloro-3,5-dimethoxy aniline side chain. The covalent binding takes place between Cys486 in the P loop and the acrylamide warhead.

A structure-guided approach was also used to develop an irreversible NEK2 PKI named JH295 (Henise and Taunton, 2011). Starting from a relatively nonselective oxindole-derived inhibitor SU11652, a propargyl acid warhead was installed to target Cys22 located in the P loop. JH295 is a potent NEK2 inhibitor with a biochemical IC₅₀ of 770 nM. Molecular modeling suggests

Table 2. Kinases Targeted by Covalent Inhibitors Organized by Kinase Family

AGC	CAMK	CKI	CMGC	STE	TK	TKL	Other
RSK	None reported	None reported	GSK-3 β , JNK, ERK2	MEK1	EGFR, BTK, VEGFR, FGFR, BMX, KIT, ITK	TAK1	IKK β , NEK2

that three hydrogen bonds are formed between the kinase “hinge” residues Glu87 and Cys89 and the oxindole core (Figure 3K). A water-bridged hydrogen bond involving Lys37 and propargyl amide carbonyl helps to fix the binding conformation, putting the electrophilic acetylene in close proximity to Cys22 in the glycine-rich loop (P loop).

A thienylhalomethylketone-based selective irreversible GSK3- β inhibitor was discovered using high-throughput screening (Perez et al., 2009). After optimization of the lead compound, a new GSK3 β inhibitor (compound 5) was developed with an IC₅₀ of 0.5 μ M, which exhibited selectivity over other protein kinases up to a concentration of 10 μ M was developed. Molecular modeling suggests that Cys199 in the DFG motif may react with the chloroacetate to form a covalent bond (Figure 3L).

Several natural products such as celastrol (Lee et al., 2006), 17-acetoxylolkinolide B (Yan et al., 2008), PGA1 (Rossi et al., 2000), Parthenolide (Kwok et al., 2001), and Manumycin A (Bernier et al., 2006) have been reported to irreversibly bind IKK β kinases. Irreversible binding has been indicated using mutagenesis or biochemical experiments (Liu et al., 2012a); however, crystallographic information is not yet available.

Recently, the Taunton laboratory has explored making “reversible” covalent inhibitors of RSK2 (Serafimova et al., 2012). Here, a 2-cyanoacrylate, which allows reversible addition of a cysteine thiol to an activated α , β -unsaturated ester, was introduced onto FMK (Cohen et al., 2005), a scaffold that binds to the ATP-binding site of RSK2. Competition assays and crystallographic analyses were consistent with covalent bond formation. Further investigation is required to determine whether “reversible” covalence will help mitigate pharmacology that derives from covalent modification of unintended targets.

Chemoinformatics Analysis of the Cysteinome

Many protein kinases possess cysteine residues in and around the ATP-binding site. However, to date only six distinct cysteine sites have been unequivocally demonstrated to be targeted by a covalent inhibitor: the cysteine at the lip of the ATP-binding site targeted by the covalent EGFR, BMX, and BTK inhibitors; the cysteine in the P loop region targeted by FGFR; the cysteine in the roof region of the pocket targeting by NEK2 and RSK inhibitors; the cysteine immediately preceding the DFG motif targeted by VEGFR, ERK2, and GSK3 β inhibitors; the cysteine in the solvent area targeted by JNKs inhibitors; and the cysteine located in the catalytic loop targeted by inhibitors of c-KIT and PDGFR. In total, potent and selective inhibitors exist for fewer than 20 kinases with no examples for two classes of kinases (Table 2; Figure 4B).

This analysis suggests that currently available irreversible inhibitors only target a very small fraction of the kinases that possess potentially reactive cysteine residues. Previously, we reported that a comprehensive analysis of the literature revealed accessible cysteines in the ATP-binding pocket of more than 200 protein kinases sites (Zhang et al., 2009). Recently, Leproult et al. (2011) mined available X-ray crystal structures in the public

domain and informatics analyses on different conformations of the protein kinases revealed there are 27 kinases retaining an active conformation and bearing 211 cysteine residues that can be accessible in theory; ten protein kinases retaining the DFG-out conformation and bearing 127 cysteines; and six protein kinases retaining the c-helix-out conformation and bearing 66 cysteines. These studies have provided a good starting point for developing cysteine targeted irreversible kinase inhibitors. Nonetheless, due to the database limitations, a full spectrum of the targetable cysteine has yet to be fully mapped.

In order to gain a complete picture of the accessible cysteines in the kinome and generate a “kinase cysteinome” to facilitate the systematic exploration for irreversible inhibitors, we performed an informatics study based on the kinome’s primary sequence alignment with PFAAT, a Java-based multiple sequence alignment editor and viewer designed for protein family analysis (Caffrey et al., 2007). Based on the Pfizer in-house X-ray structure database and RCSB public database, 442 protein kinases were superimposed using CLUSTAW (Thompson et al., 1994) and their ATP-binding sites were evaluated (atypical and inactive kinases were excluded). Using this program, 18 spatial cysteine positions were identified (Figure 4) in 200 unique kinases corresponding to 252 positions in total (Table 3). This estimation is only approximate as some cysteine thiols may be inaccessible due to their trajectory or posttranslational modification (nitrosylation, disulphide etc). In addition, it may also be there are kinases that possess cysteines that are distant from the ATP site in sequence space but that are in fact proximal to the ATP site due to the overall conformation of the kinase.

The 200 kinases identified bearing approachable cysteines are well distributed among the kinase subfamilies (Table 3; Figure 5). There are 27 kinases in the TK family, 8 kinases in the TKL family, 34 kinases in the STE family, 16 kinases in the CMGC family, 23 kinases in the CAMK family, 33 kinases in the AGC family, and 48 kinases in the CK1 and other families that possess an ATP site cysteine. While this primary sequence analysis is complementary to Leproult’s X-ray-based analysis, experimental validation will be required to unequivocally establish which kinases can be targeted by an irreversible PKI.

Strategies to Develop Irreversible Kinase Inhibitors

There are two major approaches for developing novel covalent inhibitors. The first is to use existing noncovalent inhibitors in conjunction with structure-guided design. The second is to create libraries of potentially covalent kinase inhibitors in conjunction with broad-based kinase profiling to identify new covalent inhibitors. While we discuss both methods separately, in practice, both are complementary and are typically used in an interwoven fashion.

Structure-Guided Design of Covalent Inhibitors

The early reported “human-inspired” covalent inhibitors were constructed by modifying a known noncovalent inhibitor with a reactive electrophile at a position predicted to be in proximity

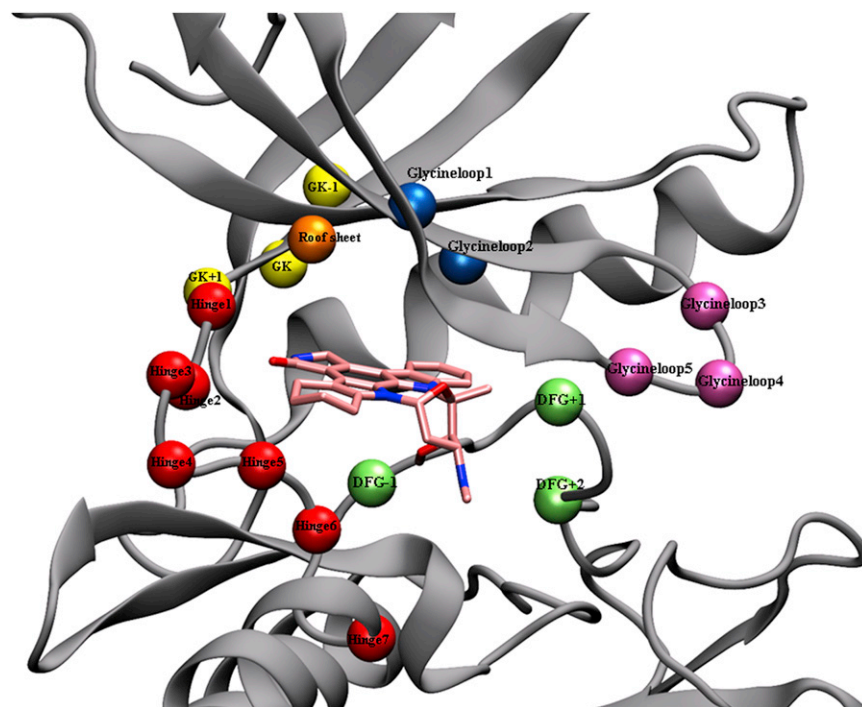


Figure 4. Representative Positions of Accessible Cysteines in the Active Site

1YVJ, kinase domain used for depiction (cyan, staurosporine). The various colored circles indicate relative positions of Cys residues depicted on top of 1YVJ. Red, hinge region; yellow, gatekeeper and neighboring residues; blue, glycine loop closer to the ATP site; mauve, flexible region of glycine loop; green, activation loop (DFG area); orange, roof region.

to a reactive cysteine residue. Examples include the first-generation covalent EGFR inhibitor derived from PD168393, the FGFR inhibitor FIIN-1, the VEGFR inhibitor (compound **1**), the NEK2 inhibitor JH295, the RSK inhibitor FMK, and the KIT/PDGFR inhibitor (compound **4**). With the massive expansion in the number of kinase-ligand complex structures available in the public domain, there is a wealth of starting points for this approach. The major challenge with this approach is finding templates that bind noncovalently in the micromolar range and that exhibit selectivity among all the kinases with an equivalently positioned cysteine. Successful covalent bond formation can then allow selectivity to be achieved relative to all kinases that do not possess an appropriately positioned cysteine residue. A further challenge is to obtain a scaffold that presents a suitable “platform” for installation of an electrophilic moiety at the correct trajectory relative to the nucleophilic cysteine. The less flexibility there is between the ATP site recognition element and the electrophile, the higher the chances that only a single cysteine residue will be targeted. In this scenario, the ideal compound is one whose initial binding mode to the kinase positions the electrophile in a geometry that will allow for rapid bond formation.

The development of FIIN-1 provides a good example of this structure-guided design approach (Zhou et al., 2010). After a survey of the known ATP-competitive FGFR inhibitors including Chir258, Su5402, SU6668, NP603, and PD173074, PD173074 was chosen based upon its potency, selectivity, and availability of cocrystal structure with FGFR. Inspection of the structure reveals that Cys486 in the P loop (this residue needed to be modeled in because it was mutated in the X-ray structure) of FGFR1 is an approachable nucleophile that is located approximately 10 Å away from the pyridine nitrogen of PD173074. A modeling study suggested that attaching a phenyl group bearing a meta-acrylamide to the 1-nitrogen of the pyrimido

[4,5]pyridimine might be a reasonable approach. Compound **6** exhibited good selectivity when screened against 402 kinases using the KinomeScan approach, but its cellular activity against FGFR-dependent cell lines was moderate at 1.5 μM. The loss of 300-fold in cellular activity relative to PD173074 suggested that no covalent bond formation was taking place. Further elaboration involved elongation of the phenyl warhead by one more carbon to afford compound **7**, which improved the EC₅₀ to 400 nM. Further elaboration of the structure by incorporation of the previously used 2,6-dichloro-3,5-dimethoxyphenyl group to the core scaffold afforded FIIN-1, which exhibited an EC₅₀ of 14 nM for inhibiting FGFR-dependent cell growth. Although FIIN-1 is a covalent inhibitor, its cellular EC₅₀ is very similar to a corresponding noncovalent analog (FRIN), which possesses a propyl amide in place of the acrylamide (Figure 6). This suggests that the scaffold has sufficiently potent noncovalent binding to achieve the degree of target engagement necessary to initiate apoptosis in the tel-FGFR2 transformed Ba/F3 cells used in the study. The major disadvantage of this structure-guided approach is that it often fails, which unfortunately goes unreported in the literature.

Selectivity Profiling Data Orientated Design of Irreversible Inhibitors

A large number of kinase inhibitors are developed based upon serendipitous observations of cross reactivity observed for established kinase inhibitors. Examination of large-scale kinase profiling data sets has revealed that each compound class has a particular constellation of kinases that it can efficiently target. These constellations can range in size from very small such as lapatinib-related compounds, which primarily only target the EGFR family, to exceedingly large such as staurosporine-related compounds. By combining information regarding the constellation of targets that can be addressed noncovalently with the subset of these targets that may possess an appropriately positioned cysteine, one can generate new potential covalent inhibitors. The development of JNK-IN-8 provides an instructive example (Zhang et al., 2012). Examination of broad-based profiling of imatinib reveals that the compounds can bind to Abl, c-Kit, PDGFR, DDR1, and DDR2 with relatively high affinity and to JNK1,2,3 and Raf with moderate affinity. Both c-Kit and PDGFR possess a cysteine that looks like it could be accessed by replacing the piperazine ring of imatinib, which was realized by the development of compound **4** (Leproult et al., 2011).

Table 3. Detailed Classification of Cysteine

Position	Subposition	Kinases
Gatekeeper region	GK	MOK
	GK+1	SgK494
	GK-1	MAP2K4 MKK3 MAP2K6 RNaseL KHS1 KHS2 GCK HPK1 MEK2 MEK1 MAP2K5
DFG region	DFG+1	MAP3K8 MOS MAP3K4 PINK1
	DFG+2	PKCz PKCi AKT1 AKT2 AKT3 PKCg SGK1F SGK2 ROCK1 ROCK2 WART1 NDR1 NDR2 WART2 PAK4 PAK5 PAK6 PAK2 PAK3 PAK1 PRK1 PRK2 PKN2 PKCt PKCi PKCe PKCd PKCb PKCa DMPK MRCK1 MRCKb DMPK2 P70S6K SGK3 MOK SgK496 P70S6Kb IRE2 IRE1 MELK PINK1
	DFG-1	PBK TGFbR2 CDKL3 CDKL2 PRP4 MNK2 MNK1 ERK7 CDKL5 TAK1 ERK2 ERK1 NLK NIK MAPKAPK5 PKD2 PKD3 PKD1 GSK3b GSK3a Fused MAP2K4 MKK3 MAP2K6 MEK2 MEK1 MAP2K5 ZAK RSK1_Domain2 RSK3_Domain2 RSK4_Domain2 RSK2_Domain2 AAK1 BIKE FLT1 FLT4 KDR PDGFRb PDGFRa CDKL1 CDKL4 GAK SPEG Obscn MAP2K7 Kit FLT3 RSK1 RSK2 RSK3 RSK4
Glycine-rich loop region	Glycine loop	WNK4 WNK1 WNK2 WNK3 HER3
	Glycine loop 1	ZAK
	Glycine loop 2	SgK496 MEKK1 PLK2 PLK3 PLK1 RSK1_Domain2 RSK3_Domain2 RSK4_Domain2 RSK2_Domain2 NEK2 MSK2_Domain2 MSK1_Domain2
	Glycine loop 3	SgK493
	Glycine loop 5	FGFR1 FGFR2 FGFR3 FGFR4
Hinge binding region	Hinge1	FGFR4 TTK MAPKAPK2 MAPKAPK3 P70S6Kb
	Hinge2	IKKa IKKb LKB1 NEK4 Obscn_Domain2 ROR2 Wee1 Wee1B AAK1 BIKE SLK Lmr3 Lmr1 Lmr2 NEK9 BRAF RAF1 ARAF HRI FLT1 FLT4 KDR PDGFRb Kit PDGFRa CSFR FLT3 IRE2 IRE1 CLIK1L CLIK1 MYT1 CDKL1 CDKL4 PKR GCN2 PEK GAK SPEG_Domain2 LZK DLK ZC4 ZC1 ZC2 ZC3 MYO3A MYO3B CHK1 TAO2 TAO1 TAO3 SPEG NEK11 MAP3K4 STK33 Obscn CDK5 CDK9 CDK10 TLK1 TLK2 PLK4 TBK1 IKKi ULK3 MELK PKG1 PKG2 FAK NEK5 NEK3 NEK1 ULK1 ULK2 MST1 MST2 LOK HUNK RNaseL KHS1 KHS2 GCK HPK1 PLK2 PLK3 PLK1 NEK2
	Hinge3	Ron FGR SgK494 Kit CSFR FLT3
	Hinge4	SgK110 BubR1 LKB1 TBK1
	Hinge5	PINK1 EphB3
	Hinge6	MAP2K7 TEC TXK ITK BTK BMX BLK HER2 EGFR HER4 JAK3
	Hinge7	JNK1 JNK2 JNK3
Roof region	Roof sheet	HER3

Boldface indicates that kinases have been irreversibly targeted by small molecule inhibitors.

However broad-based profiling of these types of inhibitors revealed that they are in fact also inhibitors of JNK1,2,3. Examination of the JNK X-ray structures revealed that Cys116 in JNK1/2 and Cys154 in JNK3 could be targeted if imatinib used a binding conformation crystallographically observed for Syk (PDB: 1XBB). Further structure-activity-guided optimization of the linker arm between the pyridylpyrimidine ATP-pharmacophore of the compound and the acrylamide resulted in the identification of JNK-IN-8 (Figure 7).

Our endeavors to identify an irreversible JNK inhibitor started from the rational design of a type II irreversible inhibitor of KIT and PDGFR. Kinome-wide selectivity profiling serendipitously demonstrated that JNKs are one of the most potently inhibited enzymes by this class of molecule. A key consideration with this broad-based screening approach is the choice of kinase profiling technology. Currently, there are several different technologies available: competitive binding based KinomeScan developed by DiscoverX (previously Ambit Biosciences) that can provide more than 450 kinases and related mutants assay (Fabian et al., 2005; Karaman et al., 2008); activity-based FRET facilitated SelectScreen technology developed by LifeScience (previously Invitrogen) that can test more than 220 different

kinases and mutants (Lebakken et al., 2009); proteomic-technology-based Kinativ developed by ActivX that can provide data on up to up to 400 different kinases in a single cell type depending on the kinase expression (Patricelli et al., 2007); and the traditional radiometric activity-based assays provided from multiple commercial and noncommercial sources (Table 4). The Kinativ approach is a chemical proteomics-based technology that uses a biotin-tagged acyl phosphate ATP/ADP probe that can acylate the conserved catalytic lysines and other lysines located in proximity of the ATP-binding cleft (Patricelli et al., 2011) By performing competition assays in cells or lysates with the inhibitor of interest, it is possible to monitor which kinases are protected from labeling. Because this is one of the only kinase profiling technologies that can be done following treatment of live cells where the inhibitor is exposed to kinases under more “native” conditions, it is ideally suited to the profiling of covalent inhibitors.

Conclusions

Potent and selective kinase inhibitors are highly desirable reagents for use as probes to pharmacologically interrogate kinase biology or for use as potential therapeutics. Despite the

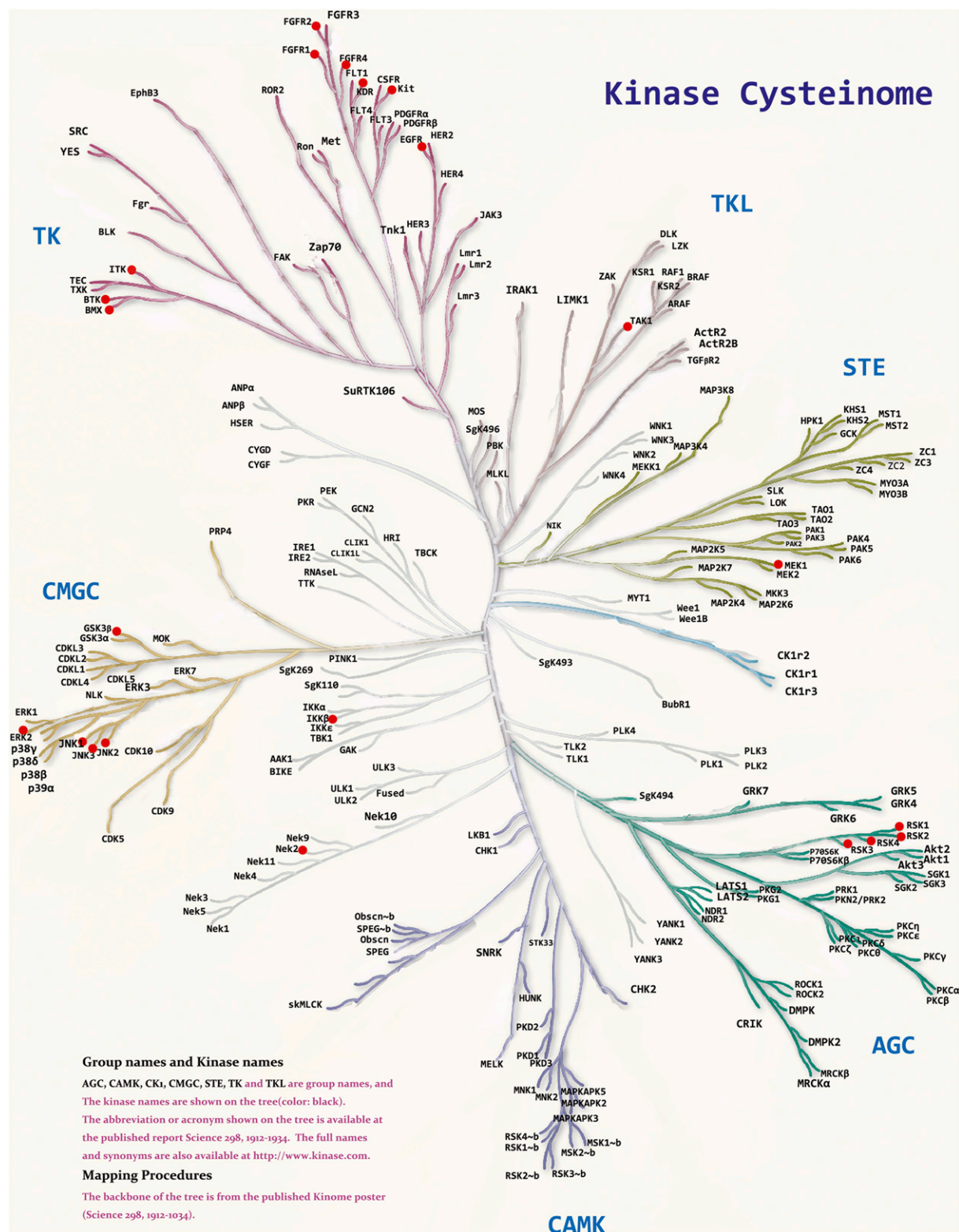


Figure 5. Distribution of the Cysteinome in the Kinome Tree

Red dots represent kinases that have been proved to be targeted irreversibly by small molecule inhibitors.

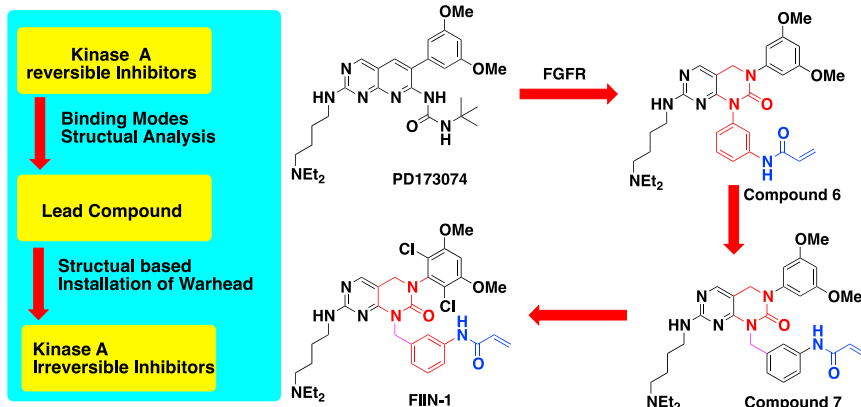


Figure 6. Schematic Representation of Reversible PKI Scaffold Base Approach to Develop Irreversible Inhibitors

great utility of irreversible kinase inhibitors to fulfill both of these goals, there currently exist only a small number of well-characterized covalent inhibitors. However, recently there has been a resurgence of interest in covalent inhibitors driven by a number of factors. First is the realization that suitably designed, potent, and selective inhibitors can achieve clinical efficacy and safety. Second is the clear potential to use structure-based design and broad-based selectivity profiling to rapidly generate and characterize lead compounds against a large number of additional kinase targets (Serafimova et al., 2012).

Informatic and structural analysis of the cysteome has identified approximately 200 distinct kinases with cysteines in close proximity to the ATP-binding cleft. Furthermore, it is likely that additional kinases will possess cysteines that are spatially proximal to the ATP cleft despite being distant in the primary sequence. Despite this abundance of targets, approximately only 20 kinases have been reported to be targeted by an irreversible inhibitor. There is thus tremendous opportunity for development of new inhibitors. From a research perspective, the inhibitors will be valuable tools to help understand the biological function of the roughly one-third of the kinome whose function is poorly understood or for which inhibitors with useful levels of selectivity do not exist. Cysteine-directed covalent inhibitors

have some key advantages relative to noncovalent inhibitors when they are to be used as pharmacological tools for investigating the biological function of kinases. Most irreversible inhibitors will require covalent bond formation with a particular cysteine residue. Therefore, it is possible to create a mutant form of the kinases that is resistant to the effects of the inhibitor by mutating the reactive cysteine to a serine or an alanine. This inhibitor-resistant mutant form of the kinase can then be reintroduced to the biological system of interest by transient or stable expression and the degree to which it can “rescue” the biological effects elicited by the covalent inhibitor can be used to establish the functional selectivity of the compound. This is an extremely powerful control experiment because the ubiquity of ATP-binding sites in biological systems always provides for the potential of unanticipated off-target effects for kinase inhibitors. In addition, it is possible to make noncovalent versions of the inhibitors that can be used to establish the requirement for covalent bond formation to achieve potent inhibition. From a therapeutic perspective, estimates suggest 180 kinases may represent attractive targets for the development of new therapeutics. However, to date there exist only 11 kinases that are targeted by FDA-approved kinase inhibitors, which suggests that there exists considerable opportunity for new covalent inhibitor drugs. The advantage of covalent inhibitors from a therapeutic standpoint is the potential to achieve durable target suppression without the necessity of maintaining high continuous drug exposure.

There are a number of key research directions that would extend the potential of covalent inhibitors both as pharmacological “tools” and as potential therapeutics. The greatest challenge

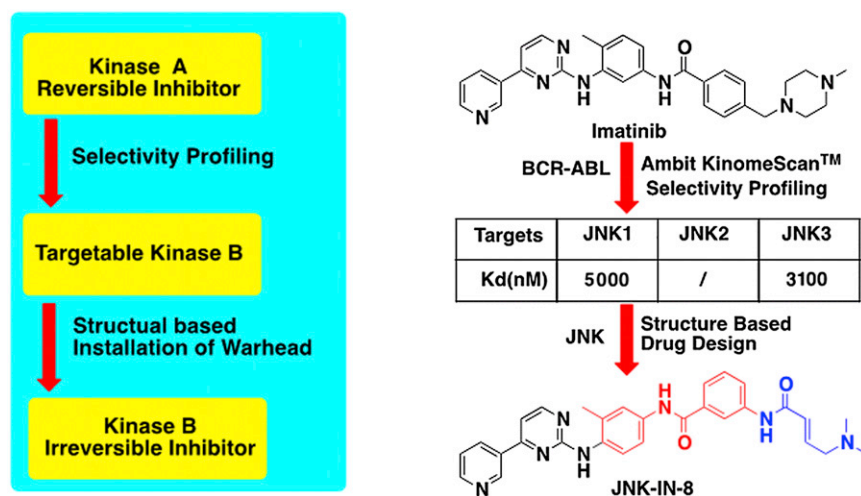


Figure 7. Schematic Representation of Flowchart for Profiling Data-Based Approach

Table 4. Commercially Available Kinase Inhibitor Selectivity Profiling Services

Companies	Website	Technology	Kinase Detected
ActivX Biosciences	http://www.activx.com	Kinativ	400
Bellbrook Labs	http://www.bellbrooklabs.com	Transcreeper	
Carna Biosciences	http://www.carnabio.com	QuickScout	305
DiscoverRx	http://www.discoverx.com	KinomeScan	451
Invitrogen	http://www.invitrogen.com	SelectScreen	224
MDS Pharma Services	http://www.mdsp.com	IMAP	155
Millipore	http://www.millipore.com	KinEASY FP	
MRC Protein Phosphorylation Unit	http://www.kinase-screen.mrc.ac.uk	(³³ P-ATP) filter-binding assay	137
ProKinase	http://www.prokinase.com	ADP Glo	239
Reaction Biology Corporation	http://www.reactionbiology.com	HotSpot	440

is to devise new strategies to improve inhibitor selectivity. The most obvious way to achieve this is to improve inhibitor potency through optimization of reversible binding (K_i) and efficiency of covalent bond formation (k_{inact}). Unlike noncovalent inhibitors, which can be optimized by monitoring K_i or IC_{50} values, such measurements are not accurate means for establishing potency for covalent inhibitors as they depend upon the incubation time between the protein and inhibitor. Therefore, considerable caution should be exercised when comparing the IC_{50} s of different irreversible inhibitors, and a more appropriate method to assess inhibition is the “specificity constant” k_{inact}/K_i —the larger this value, the more efficient the molecule is at inhibiting the protein, as it takes into account both the initial equilibrium of the noncovalent complex and the rate of inactivation through the formation of a covalent bond (Schirmer et al., 2006). Unfortunately, there is a paucity of such information in the scientific literature for irreversible inhibitors.

For example, these values can be used to delineate structure-activity (and selectivity) relationships across a range of covalent inhibitors. Prospectively then, optimization of both binding affinity and the resulting rate of reaction with the target residue in the kinase will ultimately lead to the development of highly efficient, and selective, inhibitors. When the covalent inhibitors are used in cellular assays or in vivo, further considerations become important. First, the cellular environment possesses a large number of potentially reactive cysteine residues and modification of the desired target always occurs competitively with these additional off-targets. A second consideration is that a covalent inhibitor only inactivates the enzyme-target until protein synthesis generates new protein. Proteins are synthesized at a range of rates, and therefore it is important to determine the time it takes to recover protein function after exposure to the covalent inhibitor (Johnson et al., 2010; Singh et al., 2011).

A second technological breakthrough would be to develop new classes of “electrophilic warheads” that are specifically “tuned” to a thiol based on its pKa. For example, it may be possible to develop “mechanism-based” covalent inhibitors where the electrophile is only unleashed upon binding the kinase target. This could be achieved by designing an electrophile whose reactivity is enhanced due to a protein-induced conformational change to the inhibitor. Another significant challenge is to devise methods to better annotate the subset of the proteome that is reactive to a given covalent inhibitor class (i.e., the “protein reactome”) and

to establish targets associated with toxicology in various organs. We anticipate that a large number of new covalent kinase inhibitors will be developed in the near future that will provide the means to obtain new therapeutic insights.

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REFERENCES

- Bansal, R., Magge, S., and Winkler, S. (2003). Specific inhibitor of FGF receptor signaling: FGF-2-mediated effects on proliferation, differentiation, and MAPK activation are inhibited by PD173074 in oligodendrocyte-lineage cells. *J. Neurosci. Res.* 74, 486–493.
- Barf, T., and Kaptein, A. (2012). Irreversible protein kinase inhibitors: balancing the benefits and risks. *J. Med. Chem.* 55, 6243–6262. <http://dx.doi.org/10.1021/jm3003203>.
- Barluenga, S., Jogireddy, R., Koripelly, G.K., and Winssinger, N. (2010). In vivo efficacy of natural product-inspired irreversible kinase inhibitors. *ChemBioChem* 11, 1692–1699.
- Bernier, M., Kwon, Y.K., Pandey, S.K., Zhu, T.N., Zhao, R.J., Maciuk, A., He, H.J., Decabo, R., and Kole, S. (2006). Binding of manumycin A inhibits IκappaB kinase beta activity. *J. Biol. Chem.* 281, 2551–2561.
- Caffrey, D.R., Dana, P.H., Mathur, V., Ocano, M., Hong, E.J., Wang, Y.E., Somaroo, S., Caffrey, B.E., Potluri, S., and Huang, E.S. (2007). PFAAT version 2.0: a tool for editing, annotating, and analyzing multiple sequence alignments. *BMC Bioinformatics* 8, 381.
- Chalker, J.M., Bernardes, G.J., Lin, Y.A., and Davis, B.G. (2009). Chemical modification of proteins at cysteine: opportunities in chemistry and biology. *Chem. Asian J.* 4, 630–640.
- Changeux, J.P., and Edelstein, S. (2011). Conformational selection or induced fit? 50 years of debate resolved. *F1000 Biol. Rep.* 3, 19.
- Cohen, M.S., Zhang, C., Shokat, K.M., and Taunton, J. (2005). Structural bioinformatics-based design of selective, irreversible kinase inhibitors. *Science* 308, 1318–1321.
- Cohen, P. (2002). The origins of protein phosphorylation. *Nat. Cell Biol.* 4, E127–E130.
- Cowan-Jacob, S.W., Fendrich, G., Floersheimer, A., Furet, P., Liebetanz, J., Rummel, G., Rheinberger, P., Centeleghe, M., Fabbro, D., and Manley, P.W. (2007). Structural biology contributions to the discovery of drugs to treat chronic myelogenous leukaemia. *Acta Crystallogr. D Biol. Crystallogr.* 63, 80–93.
- Davis, M.I., Hunt, J.P., Herrgard, S., Ciceri, P., Wodicka, L.M., Pallares, G., Hocker, M., Treiber, D.K., and Zarrinkar, P.P. (2011). Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* 29, 1046–1051.

- Engelman, J.A., Zejnullahu, K., Gale, C.M., Lifshits, E., Gonzales, A.J., Shimamura, T., Zhao, F., Vincent, P.W., Naumov, G.N., Bradner, J.E., et al. (2007). PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. *Cancer Res.* 67, 11924–11932.
- Fabian, M.A., Biggs, W.H., 3rd, Treiber, D.K., Atteridge, C.E., Azimioara, M.D., Benedetti, M.G., Carter, T.A., Ciceri, P., Edeen, P.T., Floyd, M., et al. (2005). A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* 23, 329–336.
- Fry, D.W., Bridges, A.J., Denny, W.A., Doherty, A., Greis, K.D., Hicks, J.L., Hook, K.E., Keller, P.R., Leopold, W.R., Loo, J.A., et al. (1998). Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosine kinase inhibitor. *Proc. Natl. Acad. Sci. USA* 95, 12022–12027.
- Garuti, L., Roberti, M., and Bottegoni, G. (2011). Irreversible protein kinase inhibitors. *Curr. Med. Chem.* 18, 2981–2994.
- Goto, M., Chow, J., Muramoto, K., Chiba, K., Yamamoto, S., Fujita, M., Obaishi, H., Tai, K., Mizui, Y., Tanaka, I., et al. (2009). E6201 [(3S,4R,5Z,8S,9S,11E)-14-(ethylamino)-8, 9,16-trihydroxy-3,4-dimethyl-3,4,9,19-tetrahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione], a novel kinase inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-1 and MEK kinase-1: in vitro characterization of its anti-inflammatory and antihyperproliferative activities. *J. Pharmacol. Exp. Ther.* 331, 485–495.
- Haber, D.A., Gray, N.S., and Baselga, J. (2011). The evolving war on cancer. *Cell* 145, 19–24.
- Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674.
- Henise, J.C., and Taunton, J. (2011). Irreversible Nek2 kinase inhibitors with cellular activity. *J. Med. Chem.* 54, 4133–4146.
- Hernandes, M.Z., Cavalcanti, S.M., Moreira, D.R., de Azevedo Junior, W.F., and Leite, A.C. (2010). Halogen atoms in the modern medicinal chemistry: hints for the drug design. *Curr. Drug Targets* 11, 303–314.
- Honigberg, L.A., Smith, A.M., Sirisawad, M., Verner, E., Loury, D., Chang, B., Li, S., Pan, Z., Thamm, D.H., Miller, R.A., and Buggy, J.J. (2010). The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Natl. Acad. Sci. USA* 107, 13075–13080.
- Hunter, T., and Sefton, B.M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* 77, 1311–1315.
- Hur, W., Velentza, A., Kim, S., Flatauer, L., Jiang, X., Valente, D., Mason, D.E., Suzuki, M., Larson, B., Zhang, J., et al. (2008). Clinical stage EGFR inhibitors irreversibly alkylate Bmx kinase. *Bioorg. Med. Chem. Lett.* 18, 5916–5919.
- Jacob, C., Battaglia, E., Burkholz, T., Peng, D., Bagrel, D., and Montenarh, M. (2012). Control of oxidative posttranslational cysteine modifications: from intricate chemistry to widespread biological and medical applications. *Chem. Res. Toxicol.* 25, 588–604.
- Jogireddy, R., Dakas, P.Y., Valot, G., Barluenga, S., and Winssinger, N. (2009). Synthesis of a resorcylic acid lactone (RAL) library using fluororous-mixture synthesis and profile of its selectivity against a panel of kinases. *Chemistry* 15, 11498–11506.
- Johnson, D.S., Weerapana, E., and Cravatt, B.F. (2010). Strategies for discovering and derisking covalent, irreversible enzyme inhibitors. *Future Med. Chem.* 2, 949–964.
- Karaman, M.W., Herrgard, S., Treiber, D.K., Gallant, P., Atteridge, C.E., Campbell, B.T., Chan, K.W., Ciceri, P., Davis, M.I., Edeen, P.T., et al. (2008). A quantitative analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* 26, 127–132.
- Kontzias, A., Laurence, A., Gadina, M., and O'Shea, J.J. (2012). Kinase inhibitors in the treatment of immune-mediated disease. *F1000. Med-Rep.* 4, 5.
- Kwok, B.H., Koh, B., Ndubuisi, M.I., Elofsson, M., and Crews, C.M. (2001). The anti-inflammatory natural product parthenolide from the medicinal herb *Feverfew* directly binds to and inhibits IkappaB kinase. *Chem. Biol.* 8, 759–766.
- Lebakken, C.S., Riddle, S.M., Singh, U., Frazee, W.J., Eliason, H.C., Gao, Y., Reichling, L.J., Marks, B.D., and Vogel, K.W. (2009). Development and application of a broad-coverage, TR-FRET-based kinase binding assay platform. *J. Biomol. Screen.* 14, 924–935.
- Lee, J.H., Koo, T.H., Yoon, H., Jung, H.S., Jin, H.Z., Lee, K., Hong, Y.S., and Lee, J.J. (2006). Inhibition of NF-kappa B activation through targeting I kappa B kinase by celastrol, a quinone methide triterpenoid. *Biochem. Pharmacol.* 72, 1311–1321.
- Leonard, S.E., Garcia, F.J., Goodsell, D.S., and Carroll, K.S. (2011). Redox-based probes for protein tyrosine phosphatases. *Angew. Chem. Int. Ed. Engl.* 50, 4423–4427.
- Leproult, E., Barluenga, S., Moras, D., Wurtz, J.M., and Winssinger, N. (2011). Cysteine mapping in conformationally distinct kinase nucleotide binding sites: application to the design of selective covalent inhibitors. *J. Med. Chem.* 54, 1347–1355.
- Li, D., Ambrogio, L., Shimamura, T., Kubo, S., Takahashi, M., Chirieac, L.R., Padera, R.F., Shapiro, G.I., Baum, A., Himmelsbach, F., et al. (2008). BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* 27, 4702–4711.
- Liu, Y., and Gray, N.S. (2006). Rational design of inhibitors that bind to inactive kinase conformations. *Nat. Chem. Biol.* 2, 358–364.
- Liu, J., Hu, Y., Waller, D.L., Wang, J., and Liu, Q. (2012a). Natural products as kinase inhibitors. *Nat. Prod. Rep.* 29, 392–403.
- Liu, Q., Kirubakaran, S., Hur, W., Niepel, M., Westover, K., Thoreen, C.C., Wang, J., Ni, J., Patricelli, M.P., Vogel, K., et al. (2012b). Kinome-wide selectivity profiling of ATP-competitive mammalian target of rapamycin (mTOR) inhibitors and characterization of their binding kinetics. *J. Biol. Chem.* 287, 9742–9752.
- Manning, G., Plowman, G.D., Hunter, T., and Sudarsanam, S. (2002a). Evolution of protein kinase signaling from yeast to man. *Trends Biochem. Sci.* 27, 514–520.
- Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002b). The protein kinase complement of the human genome. *Science* 298, 1912–1934.
- Muramoto, K., Goto, M., Inoue, Y., Ishii, N., Chiba, K., Kuboi, Y., Omae, T., Wang, Y.J., Gusovsky, F., and Shirota, H. (2010). E6201, a novel kinase inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase-1: in vivo effects on cutaneous inflammatory responses by topical administration. *J. Pharmacol. Exp. Ther.* 335, 23–31.
- Nakayama, S., Atsumi, R., Takakusa, H., Kobayashi, Y., Kurihara, A., Nagai, Y., Nakai, D., and Okazaki, O. (2009). A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab. Dispos.* 37, 1970–1977.
- Ohor, M., Kinoshita, T., Yoshimura, S., Warizaya, M., Nakajima, H., and Miyake, H. (2007). Role of a cysteine residue in the active site of ERK and the MAPKK family. *Biochem. Biophys. Res. Commun.* 353, 633–637.
- Okerberg, E.S., Wu, J., Zhang, B., Samii, B., Blackford, K., Winn, D.T., Shreder, K.R., Burbaum, J.J., and Patricelli, M.P. (2005). High-resolution functional proteomics by active-site peptide profiling. *Proc. Natl. Acad. Sci. USA* 102, 4996–5001.
- Patricelli, M.P., Szardenings, A.K., Liyanage, M., Nomanbhoy, T.K., Wu, M., Weissig, H., Aban, A., Chun, D., Tanner, S., and Kozarich, J.W. (2007). Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* 46, 350–358.
- Patricelli, M.P., Nomanbhoy, T.K., Wu, J., Brown, H., Zhou, D., Zhang, J., Jagannathan, S., Aban, A., Okerberg, E., Herring, C., et al. (2011). In situ kinase profiling reveals functionally relevant properties of native kinases. *Chem. Biol.* 18, 699–710.
- Perez, D.I., Conde, S., Pérez, C., Gil, C., Simon, D., Wandosell, F., Moreno, F.J., Gelpi, J.L., Luque, F.J., and Martínez, A. (2009). Thiénylhalomethylketones: Irreversible glycogen synthase kinase 3 inhibitors as useful pharmacological tools. *Bioorg. Med. Chem.* 17, 6914–6925.
- Potashman, M.H., and Duggan, M.E. (2009). Covalent modifiers: an orthogonal approach to drug design. *J. Med. Chem.* 52, 1231–1246.

- Rosell, R., Moran, T., Queralt, C., Porta, R., Cardenal, F., Camps, C., Majem, M., Lopez-Vivanco, G., Isla, D., Provencio, M., et al.; Spanish Lung Cancer Group. (2009). Screening for epidermal growth factor receptor mutations in lung cancer. *N. Engl. J. Med.* 361, 958–967.
- Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M.G. (2000). Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of I κ B kinase. *Nature* 403, 103–108.
- Rusnak, D.W., Lackey, K., Affleck, K., Wood, E.R., Alligood, K.J., Rhodes, N., Keith, B.R., Murray, D.M., Knight, W.B., Mullin, R.J., and Gilmer, T.M. (2001). The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol. Cancer Ther.* 1, 85–94.
- Schirmer, A., Kennedy, J., Murli, S., Reid, R., and Santi, D.V. (2006). Targeted covalent inactivation of protein kinases by resorcylic acid lactone polyketides. *Proc. Natl. Acad. Sci. USA* 103, 4234–4239.
- Serafimova, I.M., Pufall, M.A., Krishnan, S., Duda, K., Cohen, M.S., Maglathlin, R.L., McFarland, J.M., Miller, R.M., Frödin, M., and Taunton, J. (2012). Reversible targeting of noncatalytic cysteines with chemically tuned electrophiles. *Nat. Chem. Biol.* 8, 471–476.
- Singh, J., Dobrusin, E.M., Fry, D.W., Haske, T., Whitty, A., and McNamara, D.J. (1997). Structure-based design of a potent, selective, and irreversible inhibitor of the catalytic domain of the erbB receptor subfamily of protein tyrosine kinases. *J. Med. Chem.* 40, 1130–1135.
- Singh, J., Petter, R.C., and Kluge, A.F. (2010). Targeted covalent drugs of the kinase family. *Curr. Opin. Chem. Biol.* 14, 475–480.
- Singh, J., Petter, R.C., Baillie, T.A., and Whitty, A. (2011). The resurgence of covalent drugs. *Nat. Rev. Drug Discov.* 10, 307–317.
- Small, J.B., Rewcastle, G.W., Loo, J.A., Greis, K.D., Chan, O.H., Reyner, E.L., Lipka, E., Showalter, H.D., Vincent, P.W., Elliott, W.L., and Denny, W.A. (2000). Tyrosine kinase inhibitors. 17. Irreversible inhibitors of the epidermal growth factor receptor: 4-(phenylamino)quinazoline- and 4-(phenylamino)pyrido[3,2-d]pyrimidine-6-acylamides bearing additional solubilizing functions. *J. Med. Chem.* 43, 1380–1397.
- Smith, A.J., Zhang, X., Leach, A.G., and Houk, K.N. (2009). Beyond picomolar affinities: quantitative aspects of noncovalent and covalent binding of drugs to proteins. *J. Med. Chem.* 52, 225–233.
- Sonoda, H., Omi, K., Hojo, K., Nishida, K., Omura, S., and Sugita, K. (1999). Suppression of oncogenic transformation by hypothemycin associated with accelerated cyclin D1 degradation through ubiquitin-proteasome pathway. *Life Sci.* 65, 381–394.
- Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Tsou, H.R., Overbeek-Klumpers, E.G., Hallett, W.A., Reich, M.F., Floyd, M.B., Johnson, B.D., Michalak, R.S., Nilakantan, R., Discifani, C., Golas, J., et al. (2005). Optimization of 6,7-disubstituted-4-(arylamino)quinoline-3-carbonitriles as orally active, irreversible inhibitors of human epidermal growth factor receptor-2 kinase activity. *J. Med. Chem.* 48, 1107–1131.
- Uetrecht, J. (2008). Idiosyncratic drug reactions: past, present, and future. *Chem. Res. Toxicol.* 21, 84–92.
- Underwood, D.C., Osborn, R.R., Kotzer, C.J., Adams, J.L., Lee, J.C., Webb, E.F., Carpenter, D.C., Bochnowicz, S., Thomas, H.C., Hay, D.W., and Griswold, D.E. (2000). SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. *J. Pharmacol. Exp. Ther.* 293, 281–288.
- Winssinger, N., and Barluenga, S. (2007). Chemistry and biology of resorcylic acid lactones. *Chem. Commun. (Camb.)* (1), 22–36.
- Wissner, A., and Mansour, T.S. (2008). The development of HKI-272 and related compounds for the treatment of cancer. *Arch. Pharm. (Weinheim)* 341, 465–477.
- Wissner, A., Floyd, M.B., Johnson, B.D., Fraser, H., Ingalls, C., Nittoli, T., Dushin, R.G., Discifani, C., Nilakantan, R., Marini, J., et al. (2005). 2-(Quinazolin-4-ylamino)-[1,4]benzoquinones as covalent-binding, irreversible inhibitors of the kinase domain of vascular endothelial growth factor receptor-2. *J. Med. Chem.* 48, 7560–7581.
- Wissner, A., Fraser, H.L., Ingalls, C.L., Dushin, R.G., Floyd, M.B., Cheung, K., Nittoli, T., Ravi, M.R., Tan, X., and Loganzo, F. (2007). Dual irreversible kinase inhibitors: quinazoline-based inhibitors incorporating two independent reactive centers with each targeting different cysteine residues in the kinase domains of EGFR and VEGFR-2. *Bioorg. Med. Chem.* 15, 3635–3648.
- Yan, S.S., Li, Y., Wang, Y., Shen, S.S., Gu, Y., Wang, H.B., Qin, G.W., and Yu, Q. (2008). 17-Acetoxyjorkinolid B irreversibly inhibits I κ B kinase and induces apoptosis of tumor cells. *Mol. Cancer Ther.* 7, 1523–1532.
- Yoshimura, N., Kudoh, S., Kimura, T., Mitsuoka, S., Matsuura, K., Hirata, K., Matsui, K., Negoro, S., Nakagawa, K., and Fukuoka, M. (2006). EKB-569, a new irreversible epidermal growth factor receptor tyrosine kinase inhibitor, with clinical activity in patients with non-small cell lung cancer with acquired resistance to gefitinib. *Lung Cancer* 51, 363–368.
- Yun, C.H., Boggon, T.J., Li, Y., Woo, M.S., Greulich, H., Meyerson, M., and Eck, M.J. (2007). Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell* 11, 217–227.
- Zapf, C.W., Gerstenberger, B.S., Xing, L., Limburg, D.C., Anderson, D.R., Caspers, N., Han, S., Aulabaugh, A., Kurumbail, R., Shukya, S., et al. (2012). Covalent inhibitors of interleukin-2 inducible T cell kinase (itk) with nanomolar potency in a whole-blood assay. *J. Med. Chem.* 55, 10047–10063. <http://dx.doi.org/10.1021/jm301190s>.
- Zhang, J., Yang, P.L., and Gray, N.S. (2009). Targeting cancer with small molecule kinase inhibitors. *Nat. Rev. Cancer* 9, 28–39.
- Zhang, T., Inesta-Vaquera, F., Niepel, M., Zhang, J., Ficarro, S.B., Machleidt, T., Xie, T., Marto, J.A., Kim, N., Sim, T., et al. (2012). Discovery of potent and selective covalent inhibitors of JNK. *Chem. Biol.* 19, 140–154.
- Zhou, W., Ercan, D., Chen, L., Yun, C.H., Li, D., Capelletti, M., Cortot, A.B., Chirieac, L., Iacob, R.E., Padera, R., et al. (2009). Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* 462, 1070–1074.
- Zhou, W., Hur, W., McDermott, U., Dutt, A., Xian, W., Ficarro, S.B., Zhang, J., Sharma, S.V., Brugge, J., Meyerson, M., et al. (2010). A structure-guided approach to creating covalent FGFR inhibitors. *Chem. Biol.* 17, 285–295.

Doctor José Luis Salazar

Director de Nuevas Creaciones (C)

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E.

S.

D.

Referencia: Expediente núm. **NC2017/0004517**

Solicitud de Patente de Invención titulada: *"Inhibidores de las tirosina cinasas de bruton derivados de la 2, 3, 4, 9-tetrahidro-1H-carbazol-8-carboxamida y 9H-carbazol-1-carboxamida, composiciones que los comprenden y métodos de elaboración"*

Solicitante: Bristol-Myers Squibb Company

Asunto: **RESPUESTA A OFICIO No. 10043. EXAMEN DE FONDO**

JUAN PABLO CONCHA DELGADO, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término legal:

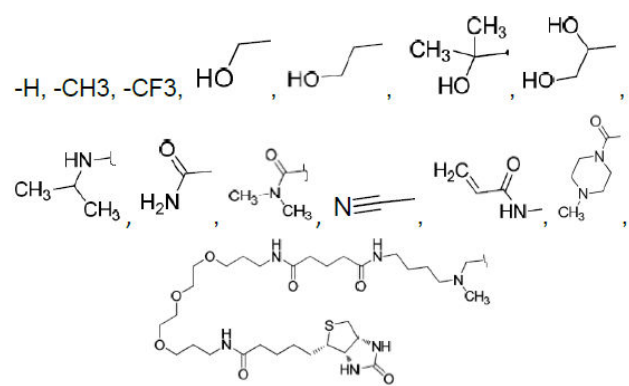
1. Repuesta a las objeciones del Examinador

1.1 Reivindicaciones

1.1.1. Menciona el examinador que las reivindicaciones 1 y 2 no son claras porque para el radical R_{1a} proponen el grupo 2,2-dimetilo; y es parecer del examinador que posiblemente se refiere a que los radicales R_{1a} y R_{1b} pueden ser cada uno un grupo metilo simultáneamente. Adicionalmente, afirma el examinador que estas reivindicaciones para el radical R_{1a} proponen el grupo 1,2-dihidroetilo; y es parecer de éste que posiblemente el sustituyente correcto es 1,2-dihidroxietilo.

Con respecto a la anterior objeción sobre la claridad de las reivindicaciones 1 y 2, el solicitante presenta un nuevo capítulo reivindicatorio en donde las definiciones de R_{1a} se

modificaron siguiendo las sugerencias del examinador de la tabla de la página 7 del Oficio N° 10043 como sigue:

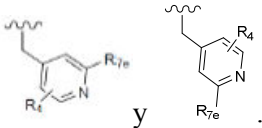


De esta manera, solicito que se de por superada la presente objeción.

1.1.2. Según el examinador, la reivindicación 1 no es clara porque la expresión "una de sus sales" no delimita apropiadamente el objeto de la solicitud.

A este respecto, se ha reemplazado la expresión "una de sus sales" por la expresión "una sal farmacéuticamente aceptable de los mismos". La presente modificación le otorga mayor claridad a las reivindicaciones, y por lo tanto se entiende superada la presente objeción.

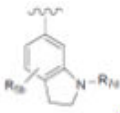
1.1.3. Menciona el examinador que las reivindicación 1 no es clara, toda vez que presenta



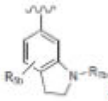
una modalidad del radical Q repetido que corresponde a

En cuanto la anterior objeción, el radical Q, se limitó a los sustituyentes sugeridos por el examinador en la tabla de las páginas 7-9 del Oficio N° 10043 de esta forma se da por superada la anterior observación.

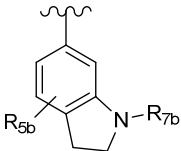
1.1.4. Menciona el examinador que la reivindicación 1 no es clara porque una de las

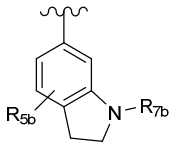


modalidades del radical Q es sin embargo, el "disclaimer" (b) especifica que Q

no es , nótese la diferencia entre los sustituyentes del átomo de N, en el primero es R_{7d} mientras que en el segundo es R_{7b}.

En cuanto a la objeción, respetuosamente aclaro al examinador que el "disclaimer" (b)(i)

especifica que Q no es  si las dos líneas discontinuas representan dos enlaces simples. Sin embargo, cuando las dos líneas discontinuas son dos enlaces dobles, entonces

Q puede ser .

Adicionalmente, aclaramos a su Despacho que el sustituyente R_{7b}, debe ser R_{7d}.

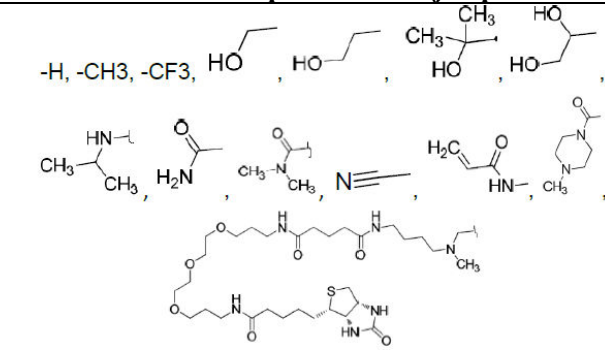
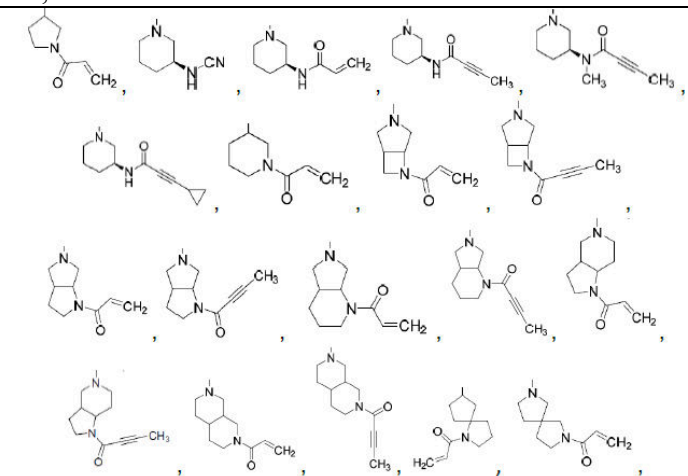
1.1.5. Afirma el examinador que las reivindicaciones 1 y 2 no son concisas porque las definiciones de los radicales R_{2a}, R_{2b}, R₃, R₄, R_{5a}, R_{5b}, R_{6c}, R_{7c}, R_{7d}, R_{7e}, R₁₂ y R₁₃ abarcan una amplia variabilidad estructural carente de sustento descriptivo.

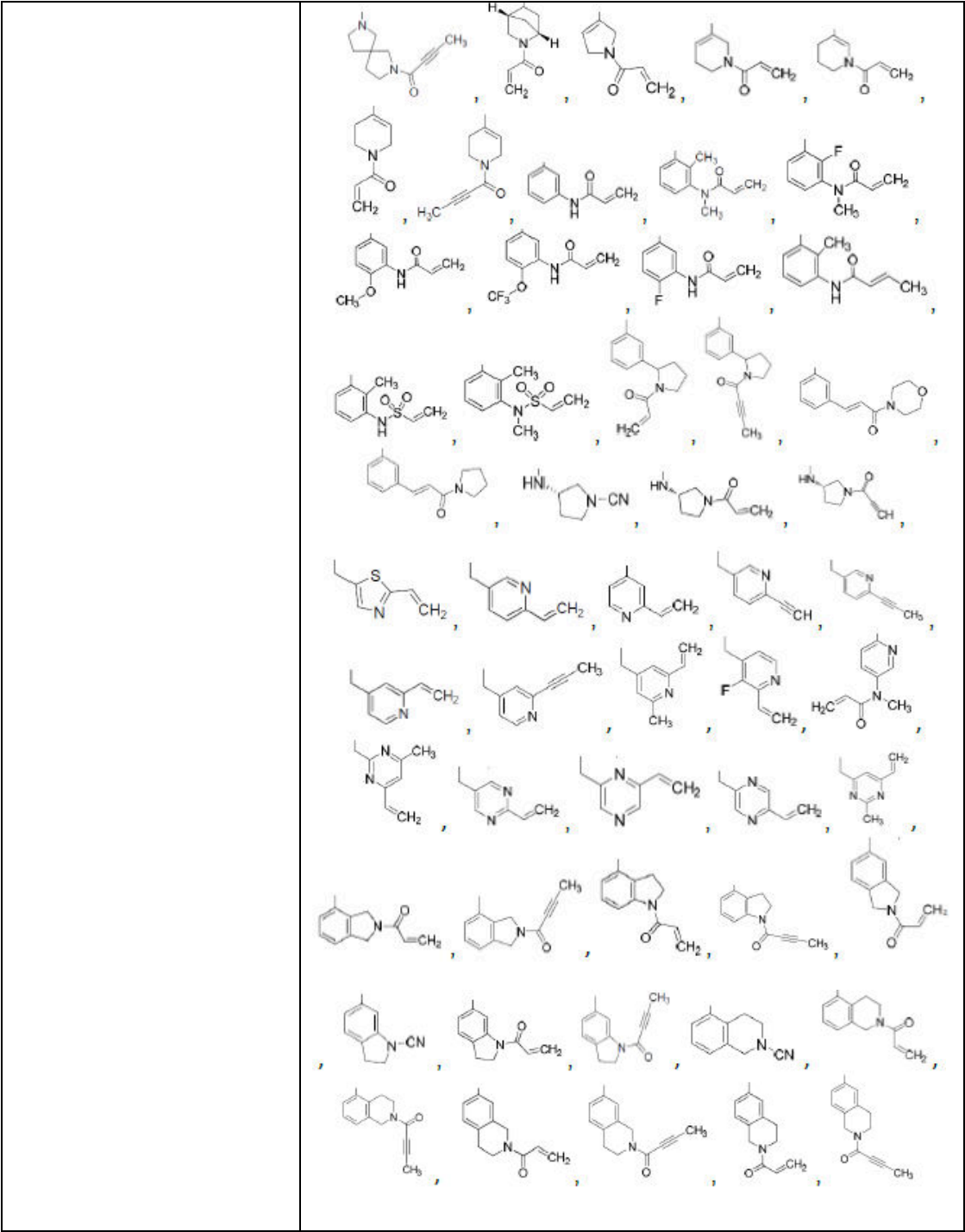
Del mismo modo, afirma el examinador que los grupos alquilo C1-4 para los radicales R₁₂ y R₁₃ carecen de sustento descriptivo. La amplia variabilidad involucra drásticas modificaciones sobre la stereoquímica y la superficie electrónica de los compuestos de la fórmula (I) y abarca un indeterminado número de compuestos de los cuales no es posible establecer que todos estos dan lugar a una solución al problema técnico planteado.

Finalmente, el examinador menciona que lo reivindicado corresponde a una extrapolación para un elevando número de compuestos reclamados. Esto, en tanto que, según el examinador, lo que se observa como evidencia técnica es proporcionada por un grupo muy limitado de agentes evaluados con solo algunas modalidades de los sustituyentes propuestos.

Con la única intención de dar por superadas las anteriores objeciones, y así continuar con el trámite de la presente solicitud, el solicitante ha decidido aceptar la sugerencia del

examinador, y limitar la solicitud a una razonable generalización del tipo de compuestos que han sido sintetizados y probados. La definiciones se muestran en la tabla a continuación:

Sustituciones	Soporte en los ejemplos
R1a	
R1b	-H, -CH3
R2a	-H, -F, Cl
R2b	-H, -F
R3	Cl, F
Q	



Por su parte, el sustituyente R_3 se ha modificado para que sea leído “ R_3 es F o Cl”. Adicionalmente, la reivindicación 10 se ha modificado para incluir solo los ejemplos en donde R_3 es F o Cl.

1.2 Novedad

El examinador reconoce la novedad de la presente invención. Por lo tanto, el requisito a sustentar es el nivel inventivo.

1.3 Nivel inventivo

De acuerdo con las observaciones del examinador, el nivel inventivo de la presente solicitud se encuentra afectado por las enseñanzas contenidas en los documentos **D1** (WO2010/080481 "*Carbazole Carboxamide Compounds Useful As Kinase Inhibitors*") **D2** (WO2009/158571 "*Heteroaryl Compounds and uses thereof*").

Particularmente, el examinador advierte que el documento **D1** se considera el estado de la técnica más cercano a la invención definida en la reivindicación 1, porque divulga compuestos derivados de carbazol con actividad inhibitoria de proteínas quinasas, entre las cuales se incluye Btk y otras quinasas de la familia Tec. Los compuestos de esta anterioridad comparten el grupo farmacológico de la presente solicitud y menciona el radical A en donde sugiere de manera general que puede ser carbociclo C3-10, arilo mono o bicíclicos, heterociclo de 5 a 14 miembros que contienen 1 a 4 heteroátomos, heteroarilo de 5 a 10 miembros que contienen de 1 a 4 heteroátomos seleccionados de N, O y S, los cuales pueden estar sustituidos por grupos -NR₁₁C(=O)-R₁, donde R₁₁ puede ser alquilo C1-6 y R₁ puede ser alquenilo C2-6.

La diferencia entre la invención, definida en la reivindicación 1 y el compuesto divulgado de D1 consiste en que la presente solicitud tiene múltiples modalidades específicas del radical Q entre las que se encuentran los grupos acrilamidofenil, aminoacriloilpirrolidin, acrilolpirrolidin, acrilolisoindolin, acritetrahidroisoquinolin, acrilindolin.

El efecto que logra incluir estas modalidades del radical Q consiste en la obtención de compuestos con actividad inhibitoria de proteínas cinasas Ntk.

Sin embargo, compuestos que contienen en su estructura grupos tales como acrilamidofenil, aminoacriloilpirrolidin, acrilolpirrolidin, acrilolisoindolin, acritetrahidroisoquinolin, acrilindolin, ya estaban divulgados en el documento D2 que se refiere a compuestos

inhibidores de Btk útiles para el tratamiento de desórdenes asociados con la actividad Btk.

En la descripción no pueden observarse ensayos realizados para determinar la actividad farmacológica como el ensayo de inactivación de Btk de sangre entera humana, en el cual se observa que no existe un compuesto de referencia con el cual comparar los resultados obtenidos, adicionalmente, este ensayo sólo se realizó con un compuesto lo que no permite conocer la capacidad de inactivación Btk del resto de los compuestos que la solicitante desea reivindicar. Por otro lado, los ensayos de evaluación de potencia para Btk y Ramos FLIPR son igualmente divulgados en los documentos D1 y D2 enseñando IC_{50} menores de 10nM para el primer ensayo y IC_{50} menores a 20nM y menores a 10nM en el segundo ensayo, todos estos resultados son similares a los obtenidos en varios compuestos de la presente solicitud. Finalmente, en el capítulo descriptivo se menciona el ensayo de diálisis por disociación de Btk recombinante humana, no obstante, se evidencia que dicho ensayo sólo se realizó con dos compuestos lo cual no permite determinar el porcentaje de recuperación de la inhibición de Btk (inhibición irreversible) para el resto de los compuestos que la solicitante desea reivindicar; es de resaltar que el documento D2 también realiza un ensayo para determinar qué tan fuerte es la inhibición de los compuestos en ella enseñados encontrando resultados similares a los de la presente solicitud (inhibición irreversible).

En consecuencia, la persona del oficio normalmente versada en la materia incluiría grupos tales como acrilamidofenil, aminoacrilolpirrolidin, acrilolpirrolidin, acrilolisoinindolin, acril tetrahidroisoquinolin, acrilindolin descritos en el documento D2 en los compuestos divulgados en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

La reivindicación 2 es afectada por los documentos D1 y D2 de la misma manera en que es afectada la reivindicación 1.

La reivindicación 3 es afectada por el documento D2 toda vez que enseña que el radical R_{6a} puede ser H y que el radical R7a puede ser $-C(O)CH=CH(R_{11})$

Las reivindicaciones 6 y 7 son afectadas por los documentos D1 y D2 de la misma manera en la que es afectada la reivindicación 1

Las reivindicaciones 8 y 9 son afectadas por el documento D2 el cual divulga modalidades del radical Q de la presente solicitud.

La reivindicación 14 es afectada por los documentos D1 y D2 los cuales enseñan composiciones farmacéuticas que contienen vehículos o diluyentes farmacéuticamente aceptables

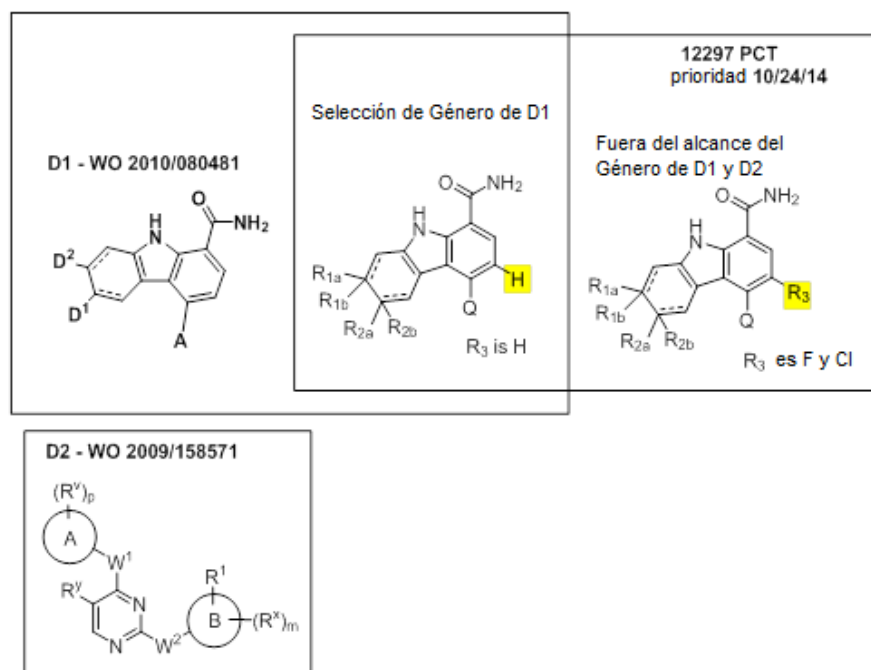
Las reivindicaciones 1 - 3, 6 - 9 y 14 cumplen el requisito de novedad, pero no cumplen con el requisito de nivel inventivo.

2. Argumentos en favor del nivel inventivo de la presente invención con respecto a D1 y D2

El nivel inventivo de una solicitud se encuentra definido en el Artículo 18 de la Decisión 486 y se establece cuando *“si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”*.

Contrario a lo que plantea el Examinador, los documentos del arte previo citados no afectan el nivel inventivo de la presente solicitud por cuanto no es posible derivar la presente invención a partir de las enseñanzas presentadas en los documentos D1 y D2

El siguiente diagrama muestra la relación entre las presentes reivindicaciones y los documentos D1 y D2:



Cabe señalar que los compuestos de las presentes reivindicaciones en los que R₃ es F o Cl están fuera del alcance de D1 y D2 (véanse las reivindicaciones 4 y 5 pendientes), que con las modificaciones efectuadas dicha limitación se encuentra en la reivindicación 1.

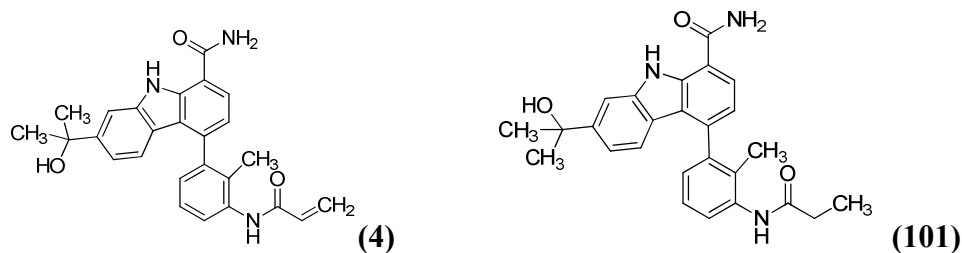
Los compuestos de la presente solicitud se diseñaron para unirse covalentemente a la enzima Btk e inhibir irreversiblemente la enzima Btk (ver descripción, desde la página 2 a la página 6). Los compuestos con la capacidad de unirse covalentemente a una enzima diana ofrecen varias ventajas en comparación con los compuestos de enlace no covalentes, incluida la oportunidad de obtener una mayor potencia (ver los tres artículos adjuntos: Barf et al., *Journal of Medicinal Chemistry* 55, 6243-6262 (2012); Kalgutkar, et al., *Expert Opinion on Drug Discovery* 7(7) 561-581 (2012); Liu et al., *Chemistry & Biology*, 20, 146-159 (2013)). Como se discutió en el artículo de Barf, la potencia mejorada para los compuestos de enlace covalente irreversible se relaciona, en parte, con el efecto farmacodinámico, que es impulsado por la tasa de recambio de la enzima diana para los inhibidores irreversibles, y no por la farmacocinética del fármaco para inhibidores reversibles (ver figura 1 en Barf y el texto que la acompaña).

El mecanismo, para que un compuesto se enlace covalentemente en la cavidad de la enzima de una enzima seleccionada, el compuesto debe tener una forma tridimensional específica para "encajar" en la cavidad de la enzima; y debe tener un grupo "ojiva" en inglés

"warhead", un grupo reactivo capaz de reaccionar con un grupo funcional complementario en la estructura de la proteína de la enzima. Además, el grupo "ojiva" en inglés "warhead" unido al compuesto debe estar unido en una ubicación específica para que esté correctamente orientada para permitir el contacto próximo con el grupo funcional complementario de la estructura de la proteína para la formación del enlace covalente. En particular, los compuestos de la presente solicitud se diseñaron para inhibir la enzima Btk al formar un enlace covalente con el residuo de cisteína 481 (cisteína 481) ubicado dentro de la cavidad de la enzima. Se espera que la unión covalente al objetivo Btk aumente la potencia y permita una menor dosificación para lograr el efecto terapéutico deseado.

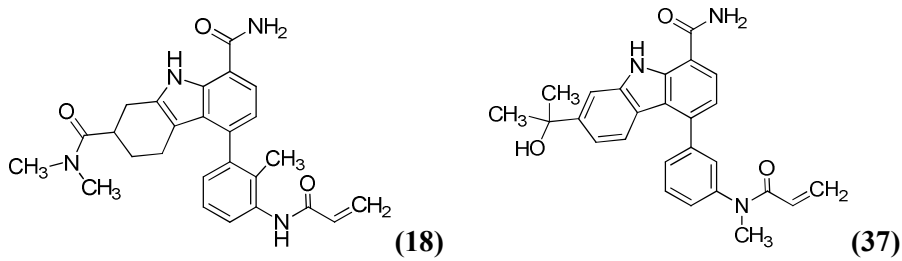
Los compuestos del presente género, como se ejemplifica por los compuestos del ejemplo, se encontró que generalmente tienen una actividad mejorada en comparación con los compuestos descritos en D1 y D2. Se encontró que ciertos compuestos ejemplificados tienen valores de actividad de inhibición de Btk IC_{50} sub-nanomolar.

El examinador reconoció que el efecto técnico se demostró mediante una comparación del ejemplo 4 y el ejemplo comparativo 101:



El ejemplo 4 es capaz de enlazarse covalentemente (R_{7a} es $-C(O)CH=CH_2$). En el ejemplo comparativo 101, el grupo "ojiva" en inglés "warhead" se reemplazó con el grupo saturado $-C(O)CH_2CH_3$. Los valores de Btk IC_{50} , como se reportó en las Tablas 9 y 10, son 0,46 nM y 7,3 nM para el Ejemplo 4 y el Ejemplo comparativo 101, respectivamente (aproximadamente un aumento de 15 X en la actividad para el Ejemplo 4).

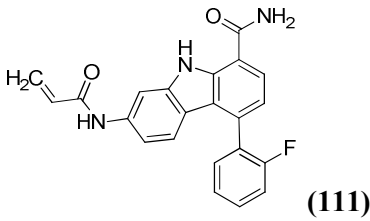
Además, se hace notar a su Despacho que la Tabla 12 incluye datos que comparan el porcentaje de recuperación de la inhibición de Btk para el Ejemplo comparativo 101 con los Ejemplos 18 y 37:



Ejemplo	Valor (nM) Btk IC ₅₀	% de recuperación de inhibición Btk
18	0.2	4.5
37	0.16	3.5
101	7.3	101

Estos resultados muestran que los Ejemplos 18 y 37, que tienen el grupo funcional $R_{7a} = -C(O)CH=CH_2$, se une a la enzima con menos del 5% de recuperación de la inhibición de Btk, lo que indica que estos compuestos forman enlaces covalentes con Btk recombinante humano y que la unión a Btk recombinante humano era irreversible. En contraste, en esta medida, se encontró que el Ejemplo Comparativo 101 tenía una recuperación completa (101%) de la actividad de inhibición de Btk, lo que indica que la unión al Btk humano recombinante era reversible. Estos resultados son consistentes con el mecanismo de la inhibición irreversible de Btk por la formación de un enlace covalente entre el compuesto inhibidor y la enzima Btk. Además, estos resultados respaldan la ventaja de que la unión covalente puede proporcionar inhibidores de Btk de mayor potencia.

El Ejemplo comparativo 111, que corresponde al Ejemplo 57-10 en D1, tiene un grupo "ojiva" en inglés "warhead" reactivo unido a una ubicación diferente en la molécula:



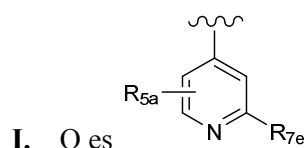
Este compuesto tiene el grupo "ojiva" en inglés "warhead" $-C(O)CH=CH_2$ unido al núcleo de carbazol y no al grupo lateral A. El valor de Btk IC₅₀ reportado para el Ejemplo comparativo 111 en la descripción en la Tabla 10 fue de 30nM. En contraste, los compuestos del ejemplo en los que A es fenilo y el grupo "ojiva" en inglés "warhead" está

unido al grupo fenilo, mostraron valores de actividad de 3nm o menos. (Ver Tabla 9, valores Btk IC₅₀ para los Ejemplos 1-62, 64 y 88). Esto evidenció que la ubicación del el grupo "ojiva" en inglés "warhead" de enlace covalente fue importante para lograr una mayor potencia. No es suficiente que la molécula tenga un grupo "ojiva" en inglés "warhead", sino la ubicación de ese grupo en un anillo lateral (grupo A) es importante para obtener una mayor potencia.

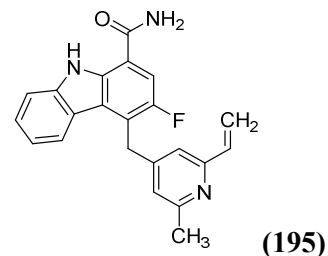
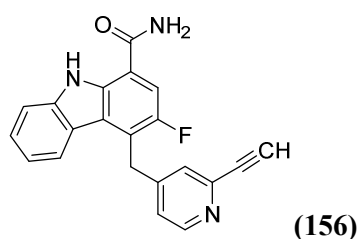
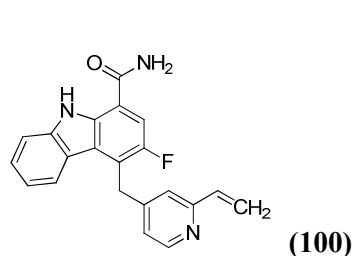
El artículo, *Developing Irreversible Inhibitors of the Protein Kinase Cysteine*, Liu et al., Chemistry & Biology Review 20, 146-159 (2013), describe otros grupos "ojiva" en inglés "warhead" adecuados utilizados como inhibidores de la proteína cinasa irreversible dirigidos a la cisteína (ver Figura 1). Los grupos electrofílicos ojiva ejemplificados (y las estructuras químicas representativas descritas en las Figuras 2, 6-7) muestran que los presentes compuestos son una extrapolación razonable de los Ejemplos 4, 18 y 37.

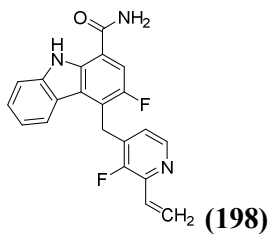
Además, la Tabla 9 muestra datos de actividad, por ejemplo, compuestos que tienen otros grupos A con grupos ojiva laterales , lo que indica un aumento de las potencias para los compuestos del presente género.

Varias comparaciones entre los compuestos del presente género y ejemplos comparativos son las siguientes:

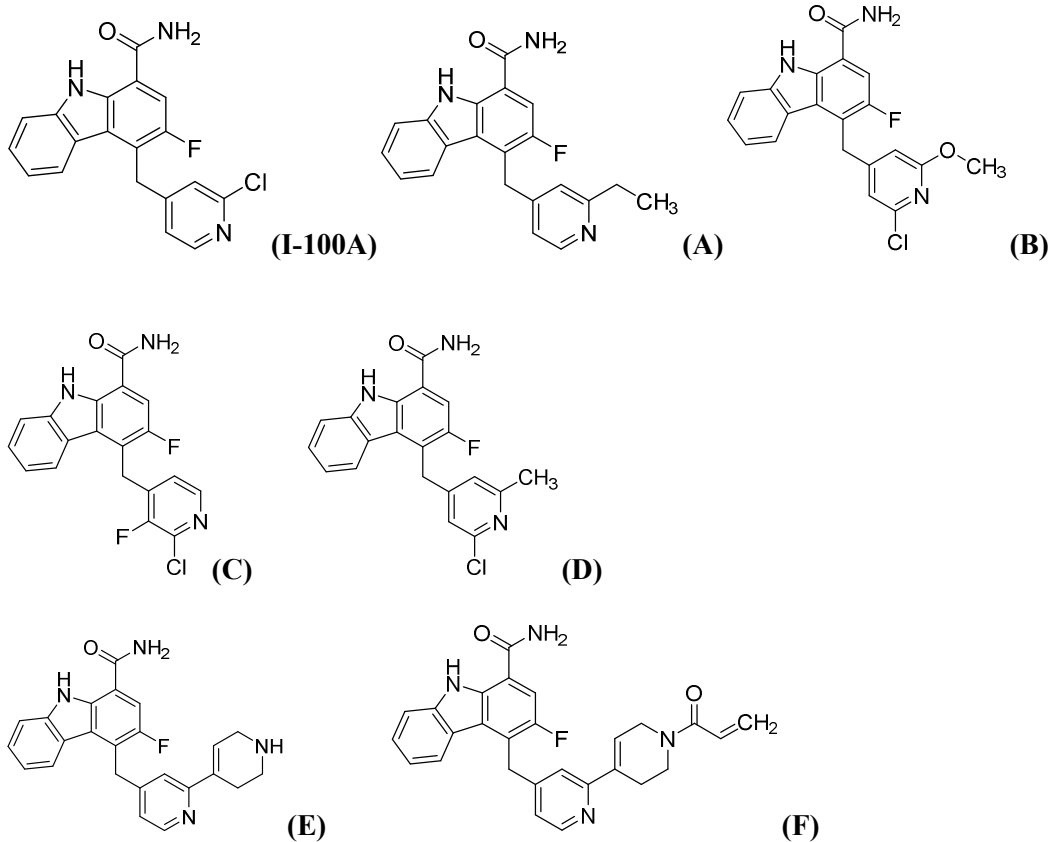


Ejemplos de compuestos:



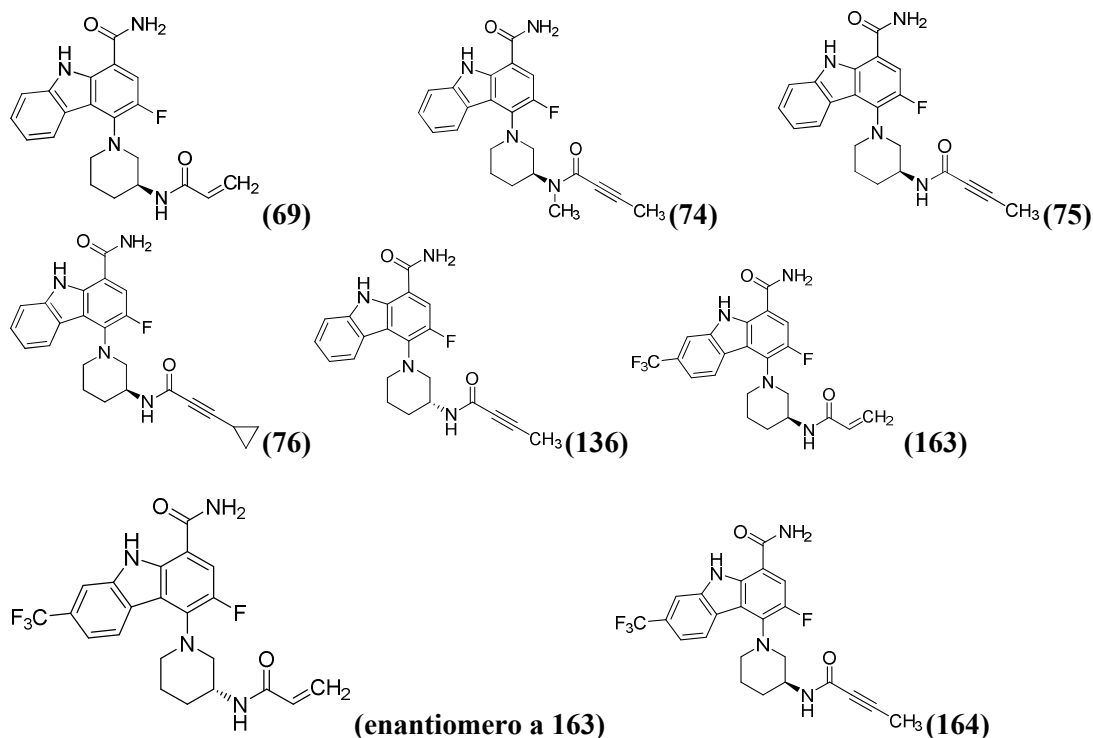
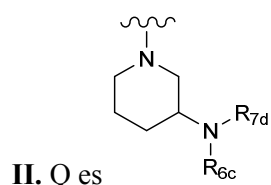


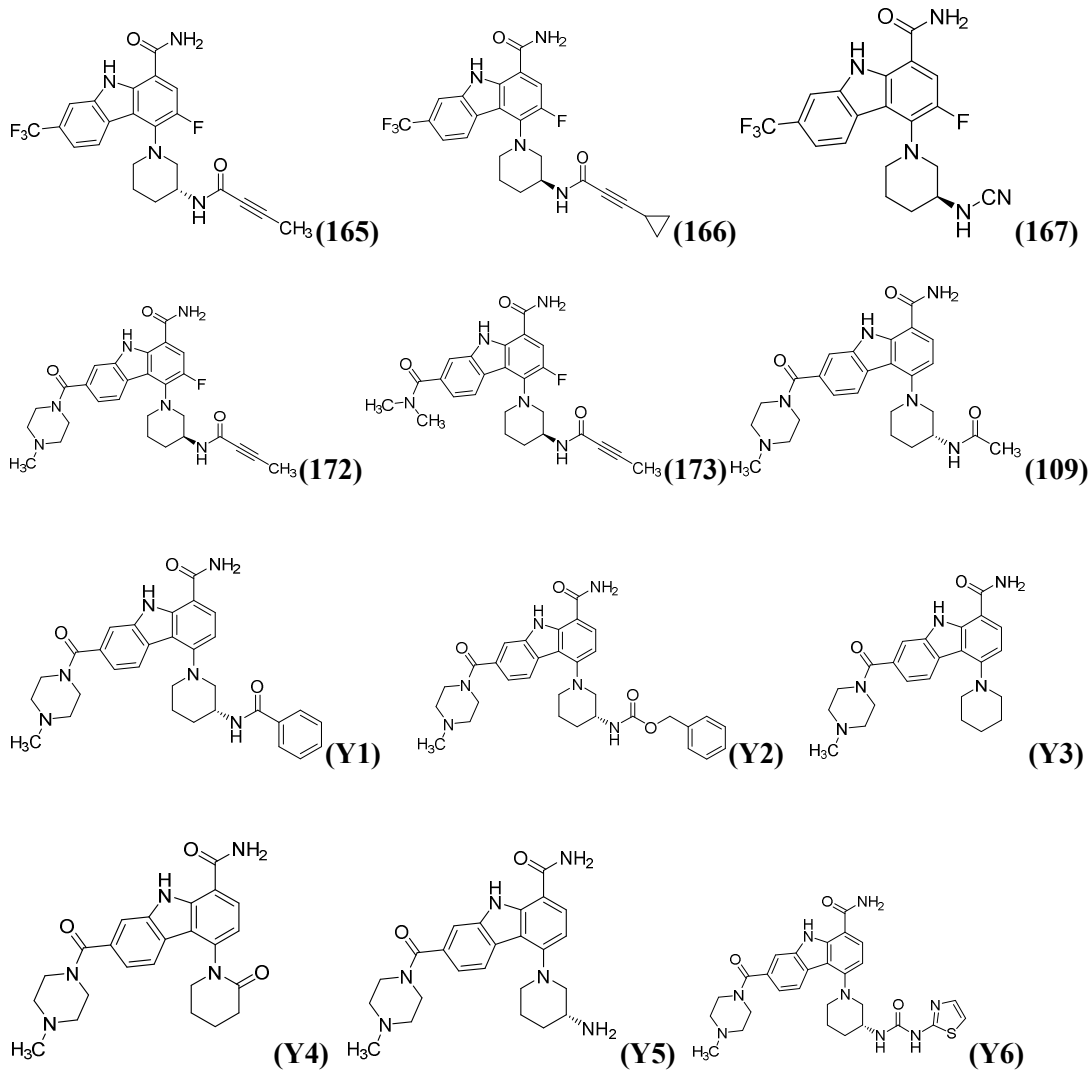
Compuestos comparativos:



Compuesto	R	Btk IC ₅₀ (nM)
Ejemplo 100	-CH=CH ₂	0.12
Ejemplo 156	-C≡CH	0.3
Ejemplo 195	-CH=CH ₂ , -CH ₃	0.3
Ejemplo 198	-CH=CH ₂ , F	2.2
Intermedio 100A	Cl	213
Compuesto comparativo A	CH ₂ CH ₃	209
Compuesto comparativo B	Cl, -OCH ₃	1370
Compuesto comparativo C	F, Cl	602
Compuesto comparativo D	Cl, -CH ₃	636
Compuesto comparativo E	tetrahidropiridinil	933
Compuesto comparativo F	tetrahidropiridinil-C(O)CH=CH ₂	255

En la tabla anterior, los Ejemplos 100, 156, 195 y 198, que tienen grupos ojiva en inglés "warhead" de enlace covalente (R_{7e}), tenían valores de Btk de 2.2 nM o menos. En contraste, el Intermediario 100A y los Compuestos Comparativos A hasta F tenían valores Btk de 209 nM y superiores. El Compuesto comparativo F, que tenía un grupo reactivo separado del grupo piridinilo por un grupo espaciador tetrahidropiridinilo, tenía un valor Btk IC_{50} de 255 nM, lo que indica que el grupo "ojiva" en inglés "warhead" unido al compuesto debe estar unido en una ubicación específica para que sea orientado adecuadamente para permitir el contacto próximo con el grupo funcional complementario de la estructura de la proteína para la formación del enlace covalente. (También, ver los comentarios anteriores con respecto al Ejemplo Comparativo 111.)





Compuestos	NR _{6a} R _{7d}	BTK IC ₅₀ (nM)
Ejemplo 69	-NHC(O)CH=CH ₂	0.073
Ejemplo 74	-N(CH ₃)C(O)C≡CCH ₃	0.21
Ejemplo 75	-NHC(O)C≡CCH ₃	0.067
Ejemplo 76	-NHC(O)C≡C(ciclopropil)	0.052
Ejemplo 136	-NHC(O)C≡CCH ₃	0.8
Ejemplo 163	-NHC(O)CH=CH ₂	0.1
Enantiomero a Ejemplo 163	-NHC(O)CH=CH ₂	5.4
Ejemplo 164	-NHC(O)C≡CCH ₃	0.2
Ejemplo 165	-NHC(O)C≡CCH ₃	2.0
Ejemplo 166	-NHC(O)C≡C(ciclopropil)	0.2
Ejemplo 167	-NHCN	8.0
Ejemplo 172	-NHC(O)C≡CCH ₃	0.1
Ejemplo 173	-NHC(O)C≡CCH ₃	0.2
Ejemplo comparativo 109	-NHC(O)CH ₃	243
Compuesto comparativo Y1	-NHC(O)(fenil)	91

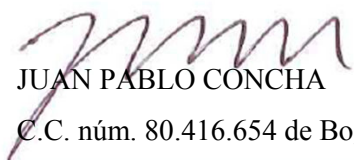
Compuesto comparativo Y2	NHC(O)OCH ₂ fenil)	58.6
Compuesto comparativo Y3	No substituyente	46.6
Compuesto comparativo Y4	=O	511
Compuesto comparativo Y5	-NH ₂	1066
Compuesto comparativo Y6	-NHC(O)NH(tiazolil)	17

Los datos en la tabla anterior muestran que los compuestos de los ejemplos mostrados en la tabla tenían valores Btk IC₅₀ de 8.0 nM o menos, incluidos los Ejemplos 69, 74-76, 136, 163, 164-166 y 172-173, que tenían valores de 2.9 nM o menos. En comparación, los ejemplos comparativos tenían valores de 17 o valores mayores.

En conclusión, los compuestos modificados de la presente invención son inventivos en vista de las referencias D1 y D2. En consecuencia, la presente invención cumple con los requisitos de novedad y de nivel inventivo y de esta manera es susceptible de ostentar el título de patente de invención.

Teniendo en cuenta los anteriores argumentos, respetuosamente solicito que se acepten las razones expuestas en el presente documento y en consecuencia, se otorgue el privilegio de patente de invención para el invento que se tramita en este expediente.

Atentamente,


JUAN PABLO CONCHA
C.C. núm. 80.416.654 de Bogotá
T.P. núm. 80.677 del C. S. de la J.

- Anexos:
- Capítulo reivindicatorio modificado conformado por 9 reivindicaciones
 - Pago de examen de patentabilidad.
 - Copia de los artículos: Barf et al., *Journal of Medicinal Chemistry* 55, 6243-6262 (2012); Kalgutkar, et al., *Expert Opinion on Drug Discovery* 7(7) 561-581 (2012); Liu et al., *Chemistry & Biology*, 20, 146-159 (2013))

EXPERT OPINION

1. Introduction
2. Toxicity concerns with covalent drugs
3. Design consideration for "safe" covalent drugs
4. Assessing the chemical (and off-target) reactivity of covalent drugs
5. Mitigating toxicity risks with covalent drug candidates
6. Expert opinion

informa
healthcare

Drug discovery for a new generation of covalent drugs

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Pharmacokinetics, Dynamics, and Metabolism Department, Pfizer Worldwide Research and Development, Cambridge, MA, USA

Introduction: The design of target-specific covalent inhibitors is conceptually attractive because of increased biochemical efficiency through covalency and increased duration of action that outlasts the pharmacokinetics of the agent. Although many covalent inhibitors have been approved or are in advanced clinical trials to treat indications such as cancer and hepatitis C, there is a general tendency to avoid them as drug candidates because of concerns regarding immune-mediated toxicity that can arise from indiscriminate reactivity with off-target proteins.

Areas covered: The review examines potential reason(s) for the excellent safety record of marketed covalent agents and advanced clinical candidates for emerging therapeutic targets. A significant emphasis is placed on proteomic techniques and chemical/biochemical reactivity assays that aim to provide a systematic rank ordering of pharmacologic selectivity relative to off-target protein reactivity of covalent inhibitors.

Expert opinion: While tactics to examine selective covalent modification of the pharmacologic target are broadly applicable in drug discovery, it is unclear whether the output from such studies can prospectively predict idiosyncratic immune-mediated drug toxicity. Opinions regarding an acceptable threshold of protein reactivity/body burden for a toxic electrophile and a non-toxic electrophilic covalent drug have not been defined. Increasing confidence in proteomic and chemical/biochemical reactivity screens will require a retrospective side-by-side profiling of marketed covalent drugs and electrophiles known to cause deleterious toxic effects via non-selective covalent binding.

Keywords: covalent, electrophile, enzyme, glutathione, inhibitor, nucleophile, pharmacodynamics, pharmacokinetics, protein, toxicity

Expert Opin. Drug Discov. (2012) 7(7):561-581

1. Introduction

In recent years, the pursuit of irreversible, covalent inhibitors as drug candidates has gained renewed impetus in the pharmaceutical industry [1-3]. Potential advantages of covalent inhibitors over conventional reversible inhibitors include the possibility of achieving increased biochemical potency and isoform selectivity through targeted covalent modification of a specific amino acid residue in the active site of the receptor or enzyme. The desired pharmacologic effect can be achieved at lower drug concentrations/doses, since covalent modification will limit competition with high endogenous ligand/substrate concentrations. Furthermore, in scenarios where the biochemical mechanism of target inhibition involves substrate buildup, covalent inhibitors are attractive because they prevent the achievement of mass-action equilibrium between the inhibitor and substrate. Covalent inhibition causes complete neutralization of the pharmacologic target, and activity can only be recovered by *de novo* protein synthesis. Therefore, as long as protein resynthesis is not too fast, systemic drug exposure required to inactivate the target would be minimal,

Article highlights.

- Covalent inhibitors offer an advantage over conventional reversible inhibitors with respect to increased biochemical potency and greater isoform selectivity via targeted covalent modification of a specific amino acid residue in the active site of the receptor or enzyme. Covalent inhibition can also overcome clinical resistance resulting from weak or transient binding of reversible inhibitors to mutant forms of the target enzymes.
- The pharmacodynamic effects achieved from covalent modification usually outlast measurable drug exposure in circulation. Hence, systemic drug exposure required to neutralize the target is minimal, which eliminates the need for an agent with sustained systemic exposure.
- Several new covalent drugs have been recently approved or are in advanced clinical trials to treat indications such as cancer, hepatitis C, and obesity.
- Guidelines for the rational design for covalent drugs are described with a special emphasis on warhead selection.
- Potential strategies to evaluate molecular selectivity and toward mitigation of overt and covert (idiosyncratic) toxicity risks are discussed.
- Validations of experimental (and computational) tools to derisk toxicologic concerns with covalent drugs are future areas of research in the field.

This box summarizes key points contained in the article.

which eliminates the need for sustained systemic exposure (long half-life agents), and decreases the potential for off-target toxicities and drug–drug interactions (DDIs). Thus, in cases where complete and sustained inhibition of the pharmacologic target is required, covalent inhibitors may be the preferred choice as drug candidates.

Against the backdrop of these advantages, it is noteworthy to point out that covalent inhibitors are also known to overcome clinical resistance resulting from weak or transient binding of reversible inhibitors to mutant forms of the target enzymes. For example, covalent modification of epidermal growth factor receptor (EGFR) by PF-00299804 (dacomitinib) and HKI-272 (neratinib) (Figure 1) results in clinical efficacy against tumors that have developed resistance to the currently marketed reversible EGFR inhibitors (e.g., gefitinib and erlotinib). The overarching success of the covalent drug strategy is evident from the fact that several covalent drugs have been approved or are in advanced clinical trials to treat indications such as cancer, hepatitis C, and obesity (Figure 1).

2. Toxicity concerns with covalent drugs

Despite promising efficacy across a broad range of disease areas, there is a general tendency in the pharmaceutical industry to avoid covalent inhibitors as drug candidates. As illustrated in Figure 1, all covalent drugs possess a strategically placed electrophilic functional group [4]. Small molecules that are reactive enough to covalently modify their targets, the thinking goes, could be promiscuous, and react with

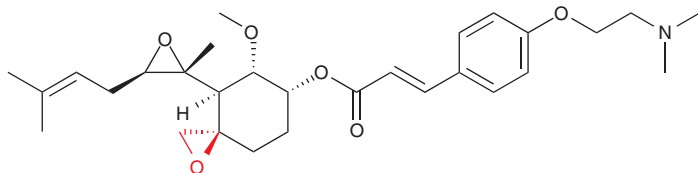
other macromolecules, DNA bases and/or the endogenous antioxidant glutathione (GSH), resulting in some form of idiosyncratic adverse drug reactions (IADRs).

2.1 Idiosyncratic adverse drug reactions (IADRs)

Reliably predicting the occurrence of IADRs for *all* drug candidates (irrespective of whether the pharmacologic interactions are covalent or non-covalent in nature) represents a significant challenge in drug discovery/development. The term “idiosyncratic” implies that little is known about the underlying mechanism and that the toxicity is generally unpredictable. Many IADRs possess an immune component and occur in only a subset of patients either acutely or as a delayed response. The observations that certain IADRs (e.g., hypersensitivity associated with the antiretroviral agent abacavir) are linked to specific human leukocyte antigen genes provide compelling evidence for the immune-mediated component of these toxicities [5]. IADRs can manifest as a rare and sometimes life-threatening reaction (e.g., hepatotoxicity, epidermal necrolysis, and agranulocytosis) in drug-treated patients that cannot be explained based on the primary pharmacology of the drug. For instance, nefazodone is used to treat depression, but can cause fatal hepatotoxicity. IADRs are, by definition, difficult to reproduce in the human population, and there are few, if any, generally applicable animal models for examining these toxicities [6]. Because the frequency of occurrence in patients is very low, these reactions are often not detected until the drug has gained broad exposure in a large patient population. The precise mechanisms of IADRs remain unclear; however, it is believed that the majority of these reactions are caused by immunogenic conjugates formed from the reaction of an electrophilic reactive metabolite of a drug with cellular proteins resulting in direct cellular dysfunction or an immune response via the formation of a hapten.

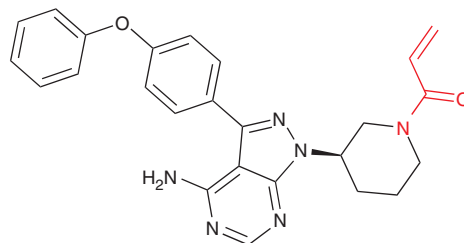
2.2 Reactive metabolites as a causative factor for IADRs

Much of the groundwork of the reactive metabolite theory was laid by James and Elizabeth Miller who elegantly demonstrated that the carcinogenic and hepatotoxic activity of aminoazo dyes resulted from their metabolic activation (also referred to as bioactivation) to reactive electrophiles [7]. Over the decades, bioactivation of xenobiotics, including drugs, has been clearly implicated in causing DNA modification and mutations relevant to cancer [8]. The extension of the reactive metabolite concept to idiosyncratic drug toxicity stems from pioneering work conducted at the 1970s, which demonstrated the cytochrome P450 (CYP)-mediated oxidation of the anti-inflammatory agent acetaminophen to an electrophilic quinone-imine intermediate, capable of depleting GSH levels and/or indiscriminately adducting to hepatic proteins as principal causative factors underlying the liver injury associated with this drug [9,10]. Although there has been much controversy over the years on the role of



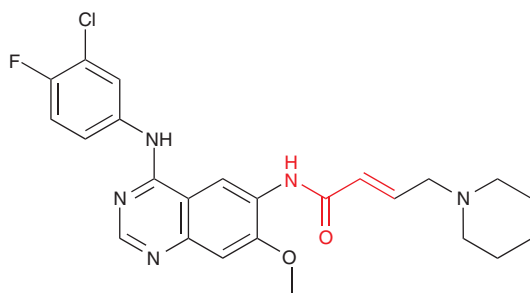
ZGN-433 (Beloranib) (Zafgen Pharma)

Biochemical target: **Methionine aminopeptidase 2**
 Electrophilic site: **Spiro-epoxide (covalent interaction with catalytic histidine)**
 Indication: **Obesity**
 Daily dose - **3 to 6 mg twice weekly, intravenous**
 Clinical status: *End of phase Ib (poised to enter phase II)*



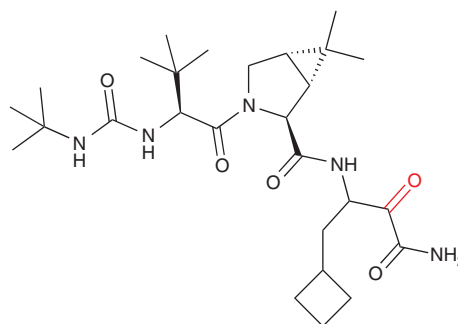
PCI-32765 (Pharmacyclics)

Biochemical target: **Bruton's tyrosine kinase**
 Electrophilic site: **α,β -unsaturated acrylamide (covalent interaction with cysteine)**
 Indication: **Cancer**
 Daily dose - **420 mg QD, oral**
 Clinical status: *Phase II*



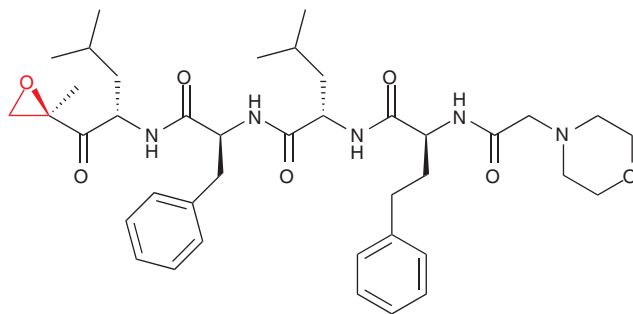
Dacomitinib (Pfizer)

Biochemical target: **HER-1 (EGFR), HER-2, HER-4 tyrosine kinases**
 Electrophilic site: **α,β -unsaturated acrylamide (covalent interaction with cysteine)**
 Indication: **Cancer**
 Daily dose - **45 mg QD, oral**
 Clinical status: *Phase III*



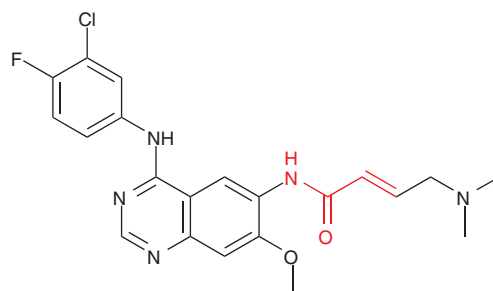
Boceprevir (Victrelis(R)) (Merck)

Biochemical target: **Hepatitis C virus protease**
 Electrophilic site: **carbonyl (reversible hemiketal with catalytic serine)**
 Indication: **Hepatitis C**
 Daily dose - **2400 mg QD, oral**
 Clinical status: *Launched*



Carfilzomib (Onyx)

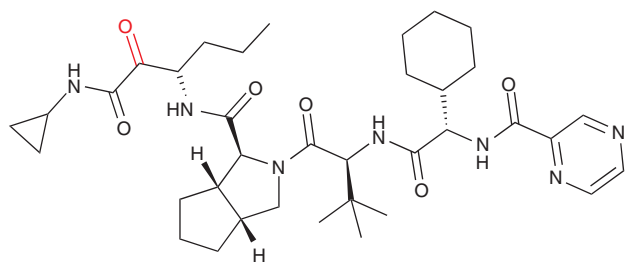
Biochemical target: **Proteasome**
 Electrophilic site: **epoxide (alkylation of catalytic serine)**
 Indication: **Multiple myeloma**
 Initial dose - **20 mg/m², intravenous**
 Clinical status: *Phase II*



Afatinib (Tamtovok) (Boehringer Ingelheim)

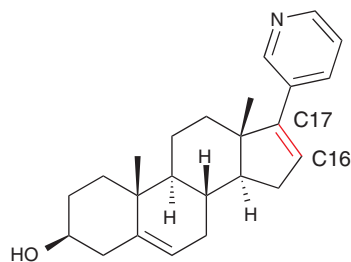
Biochemical target: **HER-2, EGFR tyrosine kinases**
 Electrophilic site: **α,β -unsaturated acrylamide (covalent interaction with cysteine)**
 Indication: **Cancer**
 Daily dose - **40 – 50 mg QD, oral**
 Clinical status: *Phase III*

Figure 1. Representative illustrations of new covalent drugs in various stages of clinical development. Electrophilic motifs are indicated in red.



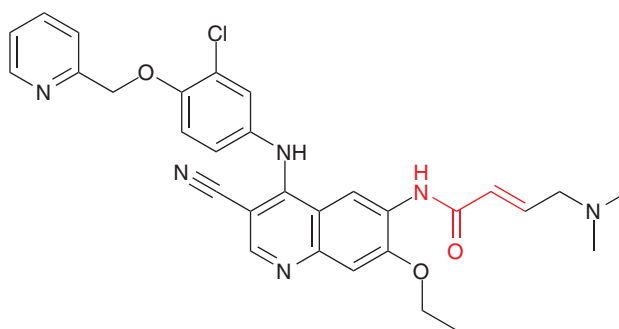
Telaprevir (Incivek™) (Vertex)

Biochemical target: **Hepatitis C virus protease**
 Electrophilic site: **carbonyl (reversible hemiketal with catalytic serine)**
 Indication: **Hepatitis C**
 Daily dose - **2250 QD, oral**
 Clinical status: *Launched*



Abiraterone (Zytiga) (Johnson & Johnson)

Biochemical target: **CYP17A1**
 Electrophilic site: **mechanism-based inactivation (epoxidation of C16 – C17 bond)**
 Indication: **Castration-resistant prostate cancer**
 Daily dose - **1000 QD, oral**
 Clinical status: *Launched*



Neratinib (Wyeth/Pfizer)

Biochemical target: **HER-2, EGFR tyrosine kinases**
 Electrophilic Site: **α,β -unsaturated acrylamide (covalent interaction with cysteine)**
 Indication: **Cancer**
 Daily dose - **240 mg QD, oral**
 Clinical status: *Phase III*

Figure 1. Representative illustrations of new covalent drugs in various stages of clinical development (continued). Electrophilic motifs are indicated in red.

electrophilic reactive metabolites in the pathogenesis of drug-related toxicity [11], their formation in the course of drug metabolism has been viewed as a risk factor in drug development, either through direct tissue damage or through haptenization of proteins that may elicit an IADR.

Association of toxicity with the chemical/biochemical reactivity of electrophilic molecules is evident in structure-toxicity relationships on a series of heterocyclic sulfonamide-based carbonic anhydrase inhibitors. The clinical development of 2-sulfamoylbenzo[d]thiazol-6-yl pivalate (1), a prodrug form of 6-hydroxybenzothiazole-2-sulfonamide (2), (Figure 2) was suspended due to a significant incidence of allergy in a 3-month ocular safety study in rabbits [12]. Subsequent evaluation in a guinea pig model for dermal-sensitization potential revealed that the compounds were potent allergens. A number of related 6-substituted-benzothiazole-2-sulfonamides (Figure 2)

were found to share this attribute [12]. It was presumed that the allergic reactions were likely mediated via non-specific protein arylation resulting in an immune-mediated allergic response, since the sulfonamide moiety in these derivatives was readily displaced (half-life < 1 h) by GSH (Figure 2) under physiologic conditions (pH 7.4 phosphate buffer, 37 °C) [12,13]. Support for this hypothesis was evident from analogous studies with the corresponding deaza analogs of benzothiazoles, i.e., benzothienophene-2-sulfonamides, which were practically inert toward reaction with GSH (Figure 2) and were also devoid of allergic side reactions in animals [12]. In contrast with the observation on benzothienophene-2-sulfonamides, the corresponding benzofuran- and indole-2-sulfonamide derivatives were found to react with GSH (Figure 2), at a three- to sevenfold greater rate than the corresponding benzothienophene-2-sulfonamides [14]. Consistent with the *in vitro* findings on GSH reactivity,

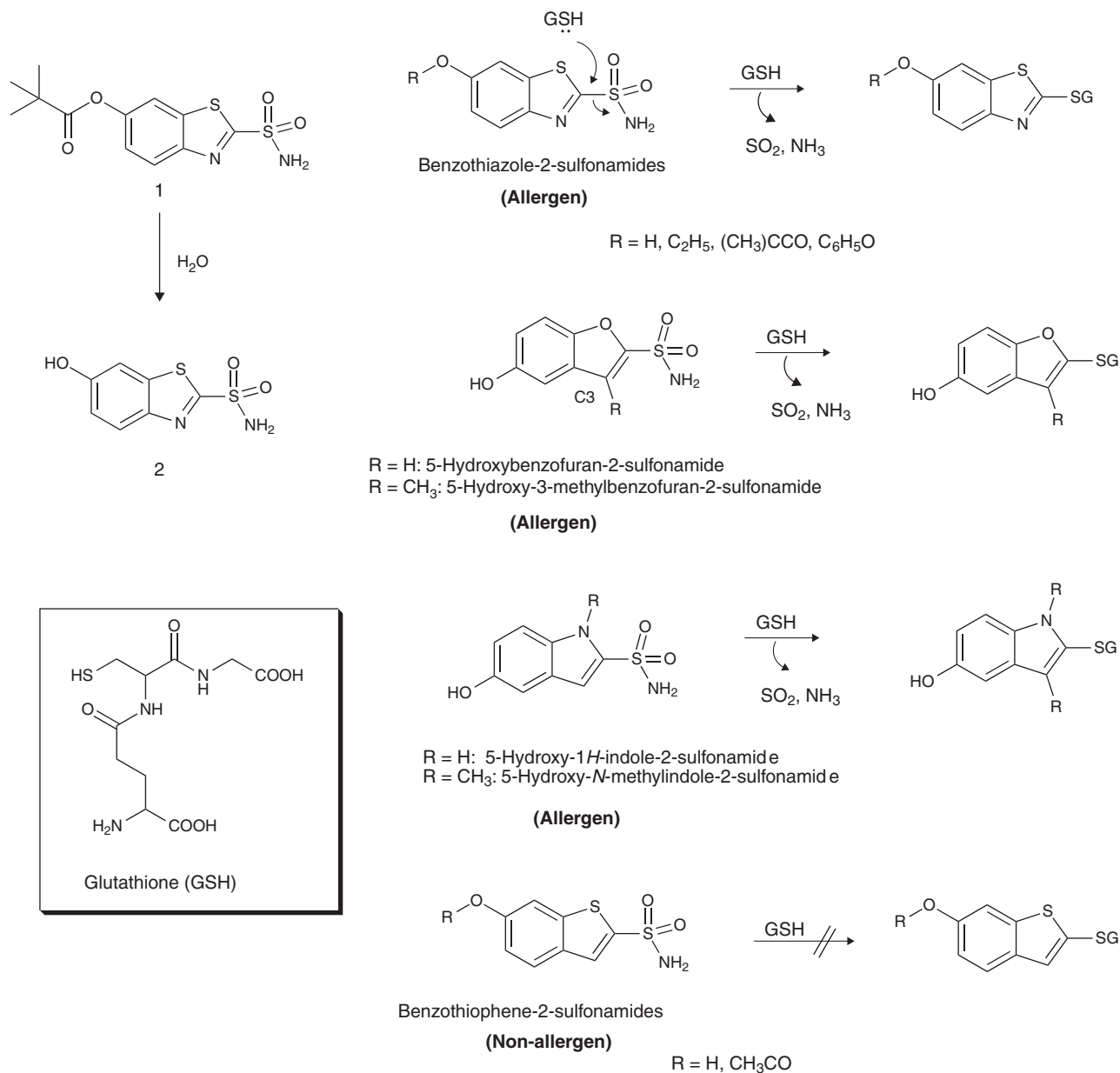


Figure 2. Relationship between GSH reactivity of electrophilic heterocyclic sulfonamides and allergic reactions in animals.

representative members of the benzofuran- and indole-2-sulfonamide series also elicited moderate to strong allergic responses in the guinea pig dermal sensitization assay [14]. While a straightforward linear correlation between GSH reactivity and sensitization potential was not apparent in these studies, it seems clear that a compound is likely to induce an allergic response if some threshold level of electrophilicity is exceeded.

While there is significant concern with respect to IADR risks with covalent drugs, it is important to note that dozens of such drugs have been in the market for decades. They include aspirin; the world's most widely used medicine with

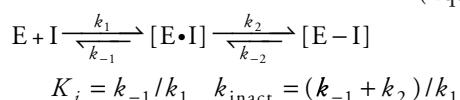
an estimated 80 billion tablets being consumed in the United States alone; penicillin and related β -lactam antibiotics; and recently developed blockbusters such as clopidogrel (Plavix[®]) and esomeprazole (Nexium[®]). The drugs treat a broad range of ailments, and many have minimal side effects, even when taken for years. In fact, approximately 30% of the marketed drugs whose biochemical targets are enzymes are known to act by irreversible inhibition [15]. Although the majority of covalent drugs target enzymes, there are examples of compounds that target other protein classes. For example, clopidogrel modifies P2Y₁₂, a G-protein-coupled receptor. In almost all cases, the marketed covalent drugs were not

discovered by rational design. Their chemical mechanisms came to light after the molecules were designed or discovered, sometimes years after they were introduced in the market. The earliest example is aspirin, which was marketed over hundred years ago. The biochemical mechanism of aspirin's anti-inflammatory effects, which were discovered in the 1970s, involves acetylation of an active site serine residue in prostaglandin synthetase isoforms [16,17]. Inactivated prostaglandin synthetases can no longer bind the fatty acid substrate arachidonic acid, leading to inhibition of proinflammatory prostaglandin production. Details on the mechanism of covalent interactions with marketed covalent drugs have been the subject of a recent review by Potashman and Duggan [1].

3. Design consideration for "safe" covalent drugs

The biochemical mechanism of a target-specific covalent inhibitor can be described by the general two-step mechanism depicted in equation 1. The first step involves a rapid non-covalent binding of the inhibitor [I] to the target receptor or enzyme [E], leading to reversible inhibition, and placement of the electrophile in proximity to a specific nucleophile on the protein. The second step is conversion of the initial enzyme inhibitor [E•I] complex to one [E-I] in which the inhibitor covalently attaches to the enzyme resulting in irreversible inhibition. In scenarios in which bond formation is effectively irreversible, k_{-2} will essentially be zero.

(Equation 1)



From a medicinal chemistry perspective, successful design of a covalent drug requires careful optimization of both the reversible (non-covalent) binding affinity (reflected in K_i) and the reactivity of the electrophilic functional group (reflected in k_2). The design strategy typically involves three key steps: i) the identification of a catalytic (or non-catalytic) nucleophilic amino acid (e.g., cysteine or serine) that is rare in that protein family, ii) the identification of a reversible inhibitor whose binding mode has been well established with the protein target, and iii) the incorporation of an electrophilic functionality (usually referred to as the "warhead"), which is strategically positioned in the reversible inhibitor to react specifically with the "rare" nucleophilic residue in the target protein. The opportunity to start with reversible inhibitors that display inherent affinity (as reflected by a low K_i) toward the target protein enables less reactive warheads to be utilized in the covalent inhibitor design, thus minimizing potential off-target reactivity with other proteins. The non-covalent binding affinity of the inhibitor must be high enough for selectivity against the target and to achieve a long enough residence time for covalent interaction [18-20]. Because the attachment of the electrophilic warhead does not dramatically impact the physicochemical attributes of the initial drug-like,

reversible inhibitor scaffold, the odds of quickly identifying a clinical candidate with the desired biopharmaceutical properties are likely to increase significantly.

3.1 Selection of electrophilic warheads for irreversible, covalent inhibition

Virtually all warheads that are incorporated into covalent inhibitors interact with their target proteins via an alkylation or acylation reaction. Alkylating substituents such as halomethyl-, diazomethyl- and acyloxymethyl-ketones, epoxides, aziridines, conjugated enones (α,β -unsaturated carbonyl derivatives), vinylsulfones or acrylonitriles (Figure 3) exhibit very little utility in covalent inhibitor design because of their high chemical reactivity [21]. Despite their high reactivity, certain α,β -unsaturated carbonyl groups have proven to be a popular choice as warheads to target cysteine residues in proteins.

3.1.1 α,β -Unsaturated carbonyls

On the basis of Pearson's Hard and Soft, Acids and Bases principle [22], α,β -unsaturated carbonyl derivatives can be viewed as soft electrophiles that preferentially react in a 1,4-fashion with soft nucleophilic anionic thiolates found in the amino acid cysteine (see Figure 3). The electrophilicity at the β -position is governed by the substitution pattern around the carbonyl group; higher electrophilicity of α,β -unsaturated aldehydes and α,β -unsaturated esters generally translates into higher reactivity rates, often leading to mutagenicity, skin sensitization or liver toxicity [23-25]. α,β -Unsaturated-carboxylic acids, -amides, and -ketones are typically less prone to such pathogenic behavior.

As evident in Figure 1, α,β -unsaturated carbonyls (in the form of an acrylamide substituent) are a common structural motif in covalent inhibitors of the EGFR family of tyrosine kinases [26-28]. As such, the success of the covalent drug strategy in the EGFR program has relied heavily on: i) the initial identification of Cys773 in the ATP binding site of the EGFR kinase that is rare in the protein kinase family, and ii) the modeling of selective scaffolds (e.g., 4-anilinoquinazoline and 4-anilinoquinoline-3-carbonitrile) or selective, reversible inhibitors (e.g., erlotinib, gefitinib and lapatinib depicted in Figure 3) to the EGFR binding site to identify suitable positions to append a soft electrophilic center for efficient covalent interaction with Cys773 in 1:1 stoichiometry. These efforts led to the discovery of the clinical candidate CI-1033 (canertinib) (Figure 3), which advanced to Phase II clinical trials for cancer treatment [26]. The acrylamide (or substituted acrylamide) proved to be the electrophile of choice across several scaffolds; the motif imparted potency and selectivity against EGFR in biochemical and cellular assays, and demonstrated potent oral activity. In contrast with canertinib, which is an unsubstituted acrylamide (Figure 3), neratinib, afatinib, and dacomitinib (depicted in Figure 1) are substituted acrylamides with a pendant tertiary amine substituent at the β -position. The presence of a general basic center at the β -position is thought to enhance selectivity (through complementary ion-pair interactions with

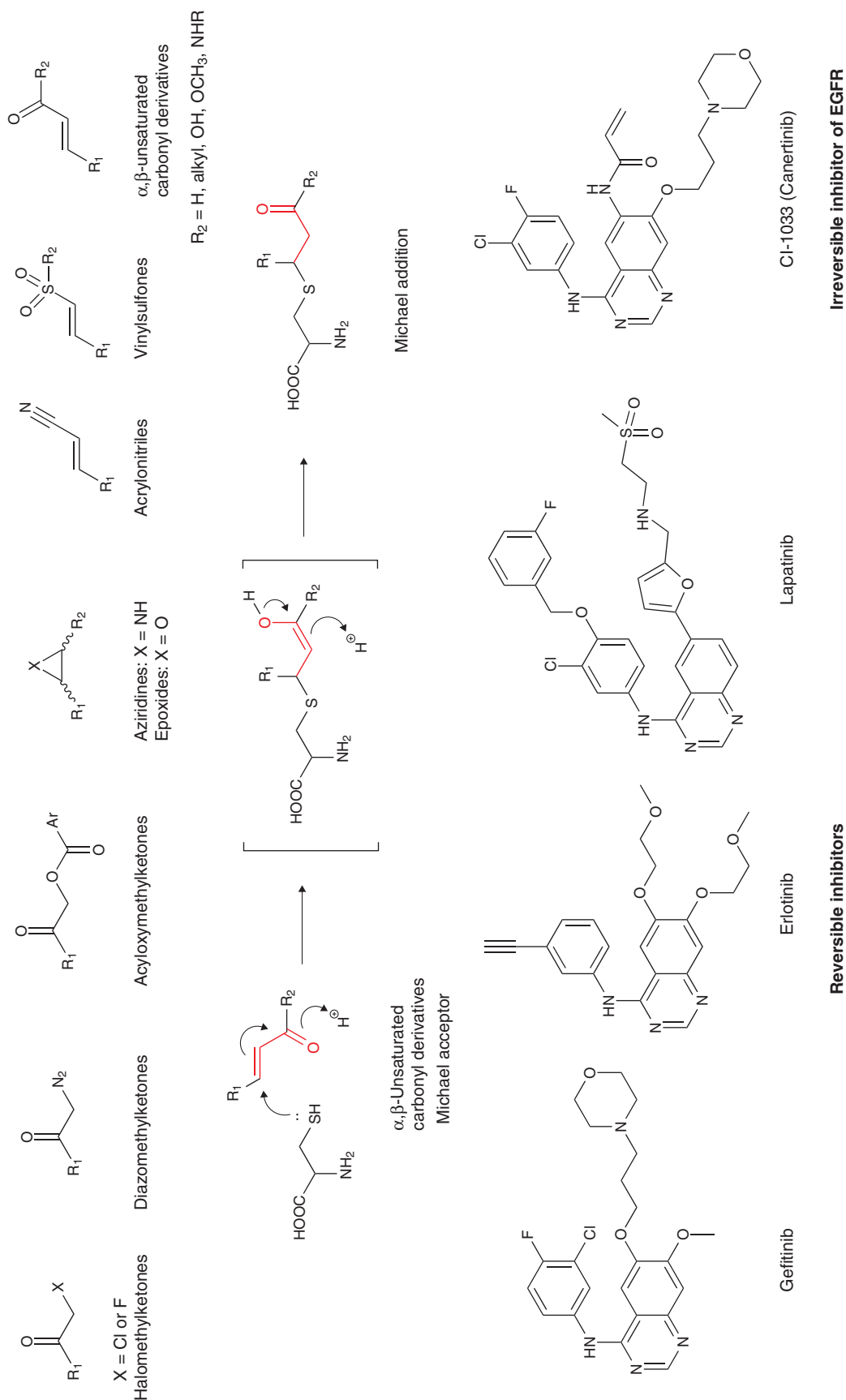


Figure 3. Highly reactive electrophilic functional groups: Utility of the α,β -unsaturated carbonyl in the design of the first irreversible EGFR inhibitor canertinib.

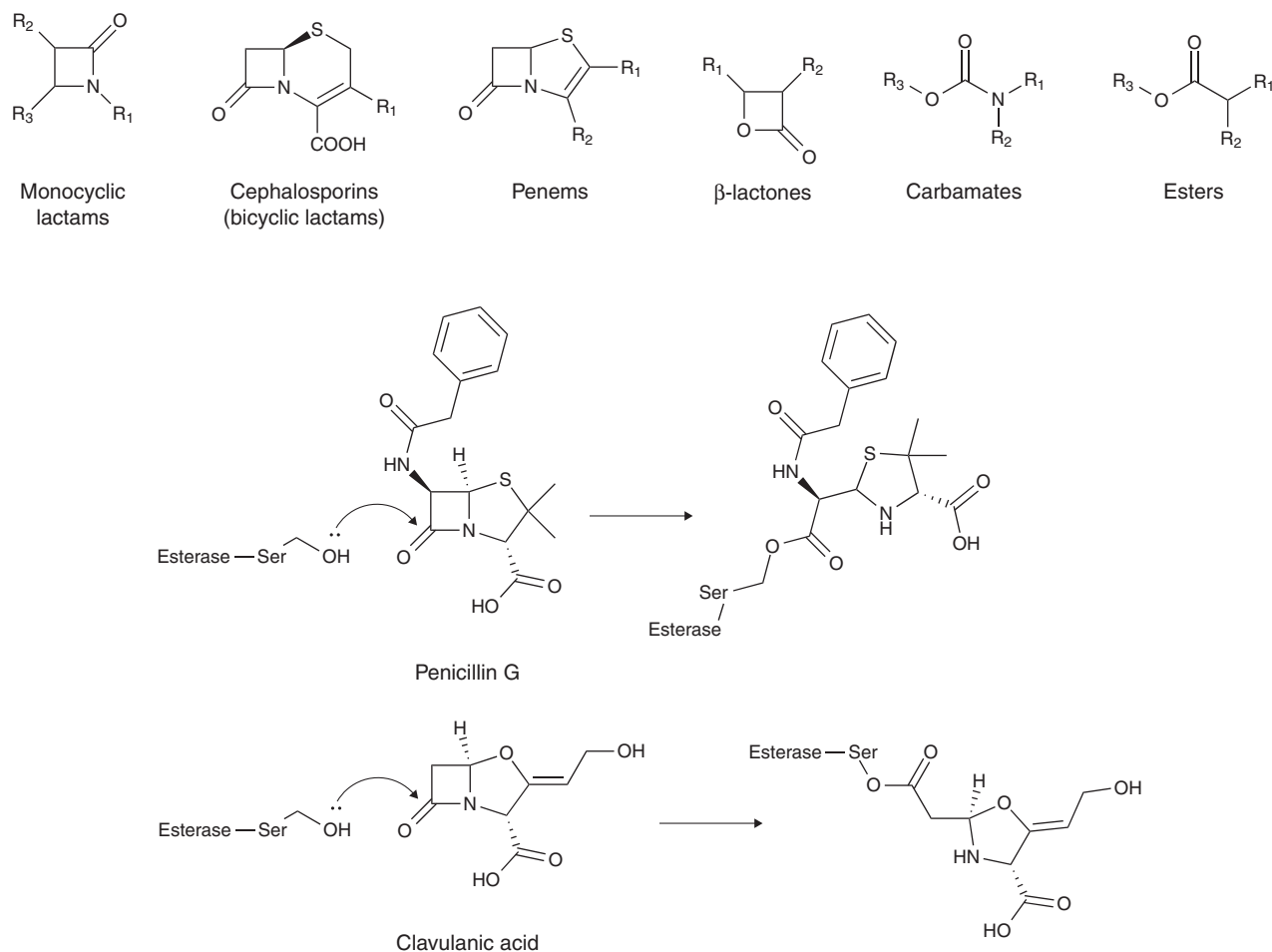


Figure 4. Commonly used acylating agents as warheads and mechanism of pharmacologic action of β -lactam antibiotics.

active site residue(s) in EGFR) and accelerate bond formation (via deprotonation of the cysteinyl thiol) between the warhead and the cysteine residue. The unique benefits of the “targeted covalent inhibitors” of EGFR tyrosine kinase is their exceptional potency in overcoming endogenous ATP competition and selectivity that is largely orthogonal to that afforded by conventional, non-covalent inhibitors. As mentioned earlier, sustained potency in the face of resistance mutations has also emerged as an important attribute of this mechanism. The consequence is complete inhibition of EGFR signaling and more durable inhibition of the target until the return of its activity, which is dependent upon protein resynthesis. From a toxicologic perspective, these compounds appear to exhibit low chemical reactivity with GSH and exhibit low reactivity with dithiothreitol in enzyme assays, suggesting that their reactivity toward nonspecific thiols is low [3].

3.1.2 Enzyme acylators

Acylating substituents such as monocyclic β -lactams, cephalosporins, penems, β -lactones, carbamates, and esters have also been utilized to generate selective covalent drugs (Figure 4).

Several antibiotics (e.g., amoxicillin, ampicillin, and clavulanic acid) containing a β -lactam nucleus are selective covalent modifiers of serine-type D-alanine-D-alanine carboxypeptidase and β -lactamase enzymes. Irreversible acylation of the active site serine to form kinetically stable serine-ester-linked irreversible adducts results in inactivation of the bacterial resistance mechanism (Figure 4) [29,30]. Although β -lactam drugs are generally well tolerated, they are also frequently associated with IADRs such as drug allergy and anaphylaxis, which are thought to originate from indiscriminant acylation of lysine and cysteine residues on proteins resulting in an immune response against the β -lactam-protein adduct [31–33]. In fact, one of the principal metabolites of amoxicillin in humans is derived from the hydrolysis of the β -lactam ring [34].

A subtle variation to the β -lactam scaffold is seen in orlistat (XenicalTM), a β -lactone derivative that blocks the intestinal absorption of fats by selectively acylating an active site serine residue in a number of gastric and pancreatic lipases (including triacyl glycerol lipase) (Figure 5) [35,36]. In addition, the tempered electrophilicity (and hydrolytic stability) of the carbamate functional group can also provide a privileged

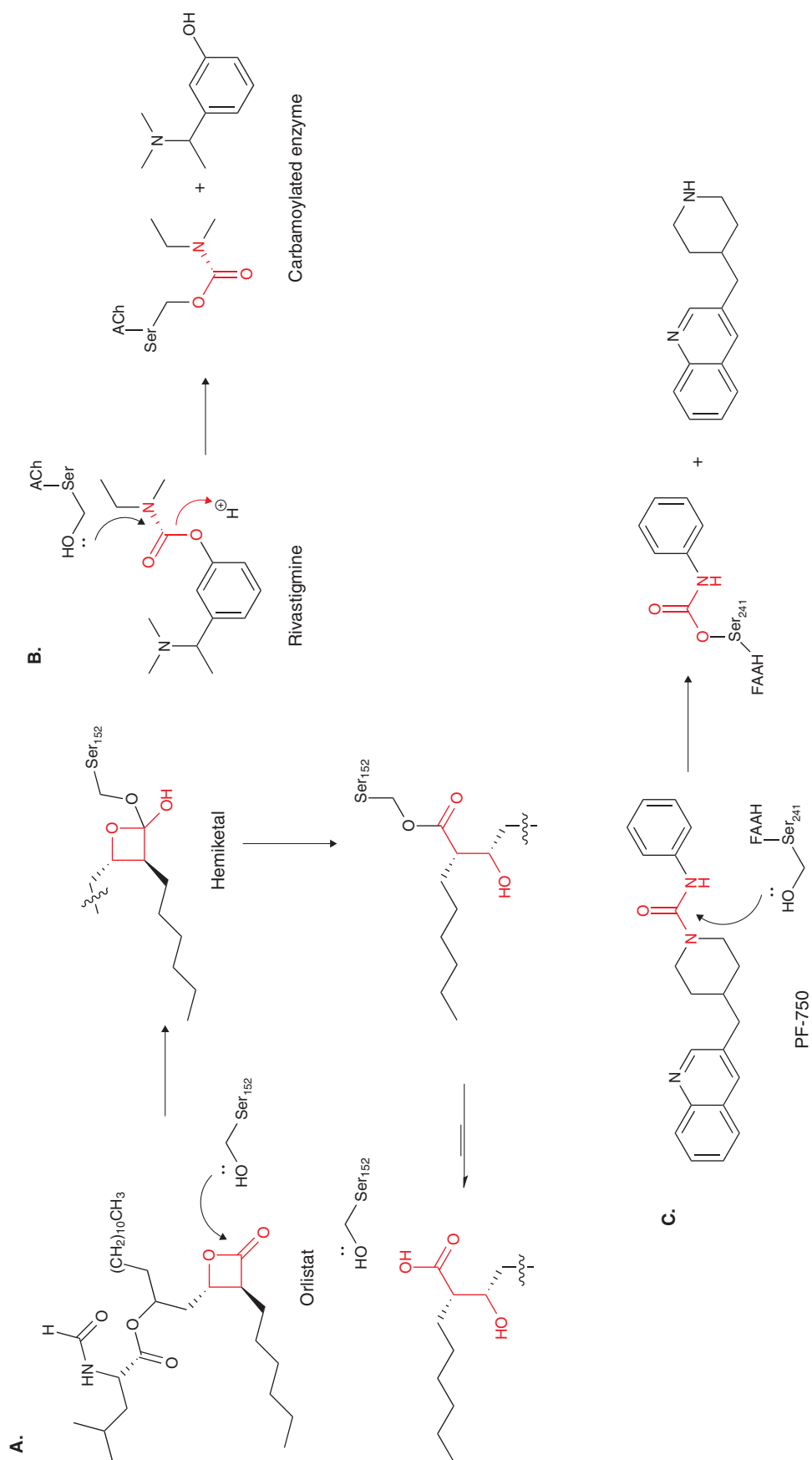


Figure 5. Acylation of serine residues by (A) orlistat, (B) rivastigmine, and (C) FAAH inhibitor (PF-750).

scaffold in the design of covalent drugs, particularly targeted toward serine hydrolases. Rivastigmine, which is used to treat Alzheimer's disease, is a slow-binding, reversible covalent inhibitor of the serine hydrolase, acetylcholinesterase. Co-crystallographic data of rivastigmine bound to the enzyme indicate the formation of the active site serine-carbamate adduct (Figure 5). The phenol-leaving group is retained in the "anionic site" of the enzyme in proximity to the site of reaction [37]. Recently, several groups have reported on the use of piperazine/piperidine aryl ureas as selective carbamoylating agents for fatty acid amide hydrolase (FAAH), a serine hydrolase [38-40]. FAAH is an integral membrane enzyme that degrades the fatty acid amide family of signaling lipids including the endocannabinoid anandamide; pharmacologic inactivation of FAAH in preclinical species leads to analgesic and anti-inflammatory effects [38,39]. A representative piperidine urea (PF-750) was shown to inhibit FAAH in a time-dependent manner by covalently modifying the active site serine through a carbamate linkage (Figure 5) [39,40]. Despite the covalent mechanism, PF-750 selectively inhibited FAAH relative to other mammalian serine hydrolases. A proposed mechanism that could potentially explain this exquisite selectivity is a specific binding-induced activation of the urea in the FAAH active site, which renders the reactivity of the urea similar to an amide [2].

3.2 Selection of electrophilic warheads for reversible, covalent inhibition

Reversible covalent modification has been recently considered to overcome potential toxicity concerns arising from irreversible covalent inhibition. Although tempered electrophiles (e.g., acrylamides) may react slowly with most cysteine residues in the active site of proteins, the probability of modifying a hyper-reactive cysteine (comprising up to ~10% of solvent-exposed cysteines) is generally unpredictable [41]. Reversible covalent drugs possess a tempered electrophile with very low reactivity toward cellular nucleophiles. Any adducts formed via off-target chemical reactivity, in theory, should be reversible in nature, and slowly revert back to the parent drug. Although esters and carbamates can form a reversible adduct (due to facile hydrolysis of the acyl moiety), optimal substituents include aldehydes, ketones, and nitriles. Aldehyde- and ketone-containing compounds generally bind to cysteine or serine residues in proteins resulting in a thiohemiacetal/thiohemiketal or hemiacetal/hemiketal bond formation (Figure 6). Some representative compounds containing aldehyde and/or ketone motifs (e.g., telaprevir and boseprevir in Figure 1) have been approved for clinical use. Telaprevir and boseprevir are NS3 serine protease inhibitors, which covalently interact with the active site serine to afford a reversible hemiketal intermediate [42,43].

Nitriles react with cysteine or serine residues to afford a reversible imidate or thioimide adducts (Pinner-type reaction) (Figure 6) [44]. Use of nitrile group as a warhead has gained considerable importance in covalent drug design,

since this functional group is chemically less reactive than aldehydes [45,46]. Nitrile-containing molecules have been known to inhibit cysteine proteases since 1960s when benzamidoacetonitrile was first shown to be an inhibitor of papain [47]. Three chemical classes of nitrile-containing inhibitors are known: i) heteroaromatic nitriles, ii) cyanamides, and iii) α -amidoacetonitriles (Figure 6) [48-52]. Although all three classes interact with proteins in a reversible manner, there are profound differences in the relative electrophilicities. Cyanamides and heteroaromatic nitriles are relatively more reactive than amidoacetonitriles, and are more promiscuous toward reaction with off-target nucleophiles [51,52]. For example, assessment of the electrophilicity (chemical reactivity) associated with the three functionalities following reaction with cysteine [53] resulted in 71% and 79% conversion of cyanamide and cyanopyrimidine derivatives to a cysteine adduct, whereas, a representative amidoacetonitrile analog only yielded 9% of the cysteine conjugate. Several amidoacetonitrile derivatives have been developed as reversible covalent modifiers of serine and/or cysteine proteases including dipetidyl peptidase IV (DPP IV) or cathepsin inhibitors for the treatment of type 2 diabetes and osteoporosis, respectively [46,53,54]. The cyanopyrrolidine derivative saxagliptin, a DPP IV inhibitor (Figure 6), was recently launched under the name of Onglyza [55], while the structurally related DPP IV inhibitor vildagliptin (Figure 6) was approved in Europe [56]. Similarly, a search for cathepsin K inhibitors led to discovery of odanacatib (Figure 6) as a reversible, covalent drug that is currently in Phase III clinical trials [52]. All of these agents covalently interact with an active site serine or cysteine residue resulting in a reversible imidate or thioimide adduct with a slow off rate for dissociation into parent compound and active enzyme (Figure 6) [57,58].

More recently, Taunton and his colleagues disclosed a strategy that utilizes electron-deficient olefins (including acrylamides) to reversibly react with noncatalytic cysteine residues in proteins [59]. Installation of a nitrile group increases the intrinsic reactivity of the olefin, but, paradoxically, eliminates the formation of irreversible adducts. Thus, addition of a nitrile group was shown to convert irreversible Michael acceptors (e.g., acrylate or acrylamide) into electrophiles that reacted with cysteine thiols under physiologic conditions in a manner that was energetically favorable yet rapidly reversible. Adding the nitrile group to the α -carbon of an acrylamide increases the susceptibility of the β -carbon to nucleophilic attack but also stabilizes the resultant carbanion. This leads to a kinetic regime in which thiol addition and elimination proceed on a subsecond-to-second time scale. Incorporation of the cyanoolefin motif into a noncovalent kinase-recognition scaffold produced slowly dissociating, covalent inhibitors of the p90 ribosomal protein S6 kinase (Figure 7). A cocrystal structure revealed specific noncovalent interactions that stabilize the complex by positioning the electrophilic carbon near the targeted cysteine. Disruption of these interactions by protein unfolding or proteolysis

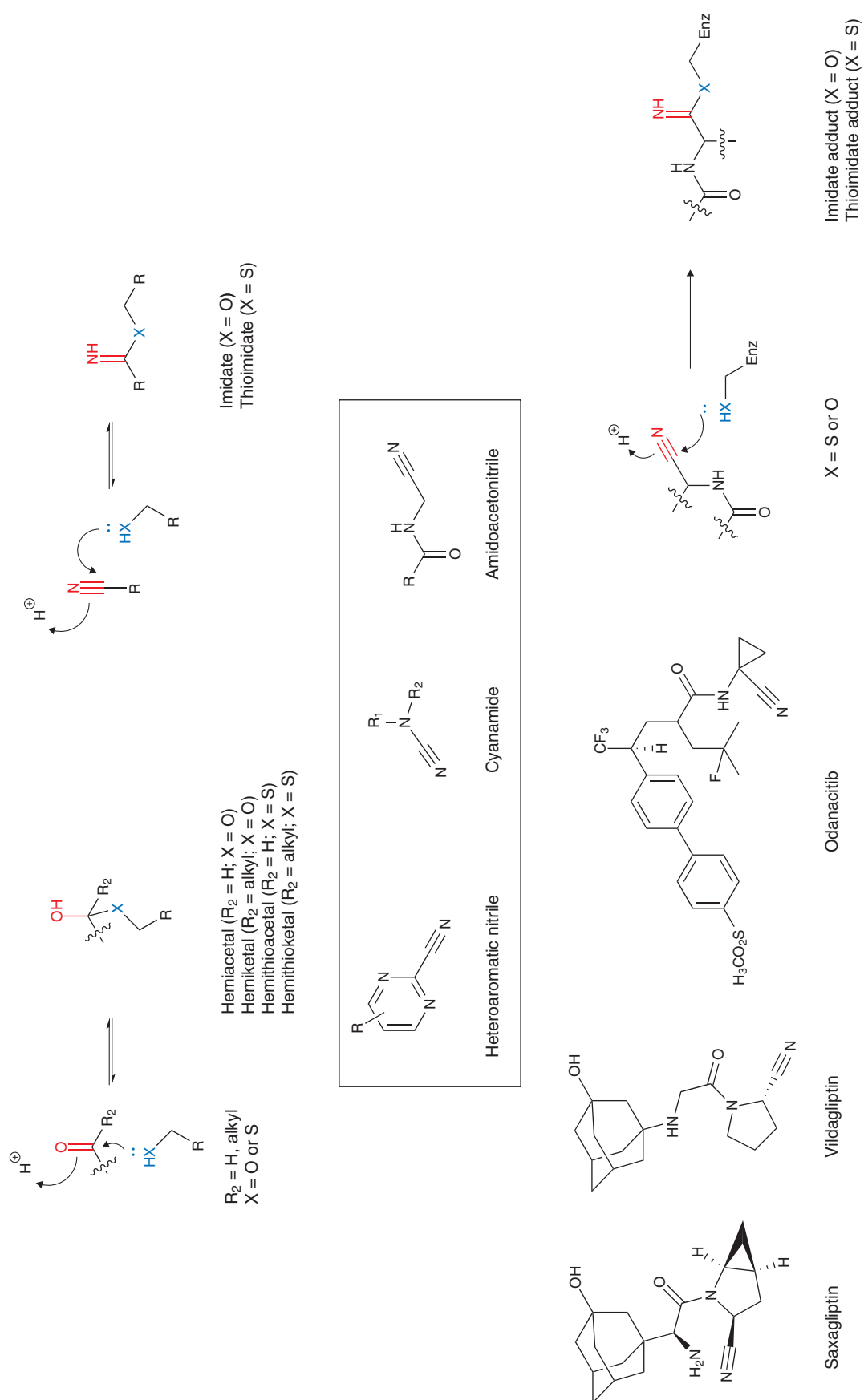


Figure 6. Reversible covalent modification by marketed drugs.

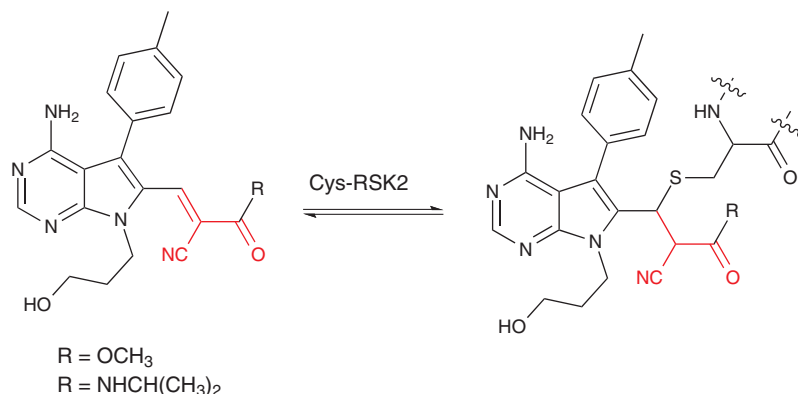


Figure 7. Cyanoolefins as reversible covalent inhibitors of p90 ribosomal protein S6 kinase.

promoted instantaneous cleavage of the covalent bond. The authors claim that the reversible covalent targeting strategy described herein can be applied to any kinase that has an exposed cysteine within striking distance of the active site, provided that the geometric requirements for covalent bond formation are satisfied with minimal strain.

4. Assessing the chemical (and off-target) reactivity of covalent drugs

Achieving a high degree of molecular selectivity with respect to covalent target engagement is a crucial endeavor in the design of covalent drugs. Safety concerns that must be mitigated include lack of specificity and the potential immunogenicity associated with non-specific covalent labeling. Both experimental and *in silico* methods have been described in the literature for assessing off-target reactivity of covalent inhibitors.

4.1 Experimental strategies to examine chemical/biochemical reactivity

The simplest method to examine the chemical reactivity of a covalent inhibitor involves the measurement of its reaction rate with a model nucleophile that acts as a protein surrogate. The model nucleophile can range from a small chemical entity such as *n*-butylamine (a lysine equivalent) or thiols such as GSH or *N*-acetylcysteine [23,24,60-65]. 4-(*p*-Nitrobenzyl)pyridine, which possesses nucleophilicity characteristics similar to those of DNA bases, has also been used to measure the alkylation potential of electrophiles [66,67]. The chemical reactions are initiated by addition of the covalent inhibitor (i.e., the electrophile) to a solution of the nucleophile in phosphate buffer (pH 7.4), and rates are estimated by monitoring the loss of the parent compound, or nucleophile by liquid chromatography tandem mass spectrometry (LC/MS-MS) and/or ultraviolet spectrophotometry. Alternatively, rates for covalent adduct formation can also be measured if an authentic standard of the adduct is available. These approaches provide relatively easy means of screening compounds to rank order their

reactivity potency as a indirect measure of non-specific covalent binding to macromolecules. The main source of error in these experiments is the degree of mismatch in the behavior of the nucleophile and a biologic system (such as proteins), since the nucleophilic entity cannot completely represent the behavior of a protein. For example, cysteines in a proteinaceous environment can have a significant different reactivity profile than a small molecule [68], which has raised doubts about the reliability of using thiol trapping agents such as GSH as a surrogate for protein targets.

In order to establish some threshold for acceptable *in vitro* chemical reactivity, and subsequent compound progression in preclinical discovery, it will be essential to compare reactivity rates (measured in terms of half-life) with reference agents (e.g., a marketed covalent drug with a good safety record). MacFaul and co-workers, in their risk assessment of a series of nitrile-containing covalent inhibitors, identified nilvadipine as a potential agent for benchmarking purposes (Figure 8) [69]. Nilvadipine is a calcium channel antagonist that has been on the market since 1991, and has demonstrated a good safety record over the years. The half-lives for conjugation of the cyano group in nilvadipine with cysteine and GSH is 2 and 40 h, respectively [69]. Based on the reactivity of nilvadipine, the authors proposed a target half-life of 40 h, or greater, as a potentially “safe” guideline for preclinical progression of nitriles. Huth *et al.* [70,71] also reported a high-throughput methodology involving a proteinaceous probe for measuring thiol reactivity. The method, referred to as ALARM NMR, is based on monitoring chemical shift or mass changes that occur in the human La antigen protein [72], upon cysteine modification by a covalent inhibitor. Recently, Chen *et al.* [73] have applied the ALARM NMR strategy toward the discovery of a irreversible hepatitis C virus (HCV) NS5b polymerase inhibitor (*p*-fluoronitrobenzyl derivative 3, Figure 8), which covalently modifies Cys366 in HCV polymerase. Lack of covalent interaction of 3 with reactive thiols in human La antigen suggested that the *p*-fluoro-nitro-substituted benzene moiety in 3 was not indiscriminately reactive toward off-target cysteines.

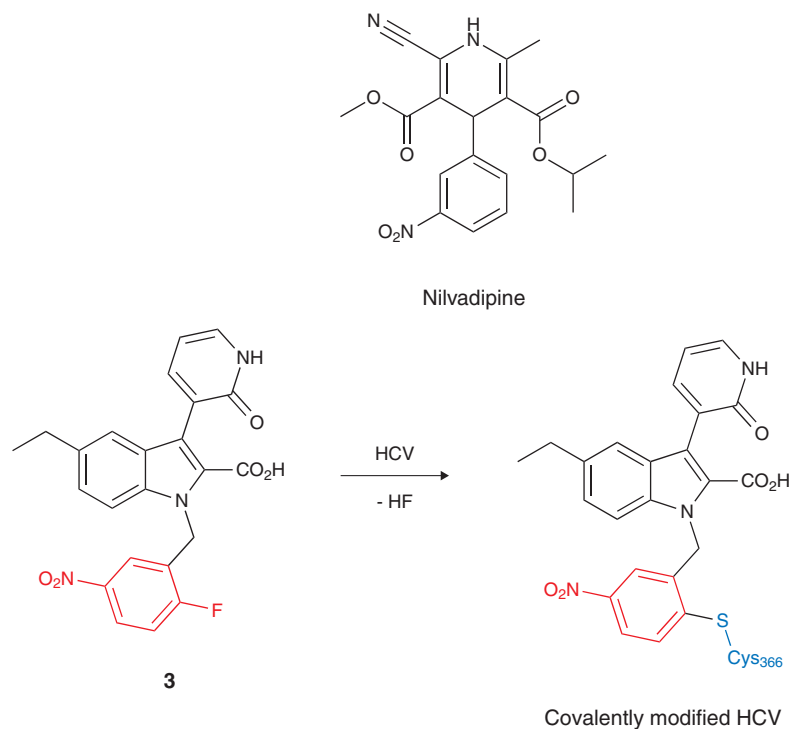


Figure 8. Structures of nilvadipine and the irreversible HCV NS5b polymerase inhibitor 3.

For more advanced candidates, the potential for performing covalent binding studies to assess the off-target reactivity of irreversible inhibitors may also be feasible. Covalent binding assays involving incubation of radiolabeled (^{14}C or ^3T) compound with hepatic tissue (e.g., liver microsomes or hepatocytes) are advantageous, since they allow a quantitative estimate of drug-protein adduct formed via off-target covalent labeling. Radiolabeling studies have demonstrated high specificity of covalent EGFR inhibitors in cellular lysates [74,75], and have been useful in demonstrating that neratinib (see Figure 1) forms a reversible covalent adduct with K190 of human serum albumin but not with mouse, rat, or rabbit serum albumin [76,77]. Recently, covalent binding studies have also been used to assess protein reactivity of nitrile-based inhibitors of cathepsin K [51,69]. Similar to the chemical/biochemical reactivity assays, defining an acceptable threshold of non-specific covalent binding for compound progression will require a side-by-side comparison with a control agent such as marketed covalent drug with demonstrated safety record. A primary drawback of covalent binding studies is the need for synthesis of radiolabeled material, which may not be conducive in early drug discovery for structure-activity relationship studies to minimize off-target covalent binding.

Apart from the traditional methods described above, proteomics-based approaches such as activity-based protein profiling (ABPP) have also been utilized to evaluate off-target reactivity of covalent drugs [2,38]. ABPP uses active site-directed covalent probes to examine the functional state

of enzymes in complex proteomes. The covalent probes contain an electrophile (attached to a linker or binding group) to covalently modify the active site of enzymes in proteomes, and a fluorescent reporter group (e.g., rhodamine or biotin) for detection and identification of protein targets. SDS-PAGE (in-gel-fluorescence) and/or mass spectrometric methods have been used to enable visualization of covalent labeling events from a complex proteome [78,79]. Selectivity of a covalent inhibitor against an enzyme family that is targeted by a particular activity-based probe (with a fluorescent reporter tag) can be examined by measuring the ability of an inhibitor to slow the rate of reaction of the enzyme with an activity-based probe [80,81]. Such assays can be performed in complex proteome mixtures, enabling the simultaneous evaluation of inhibitor potency and selectivity within a relevant native proteome. Recently, ABPP has also been integrated with click chemistry approaches wherein, replacing the bulky reporter groups with the smaller alkyne moiety enables the probe labeling step to occur *in vivo* in an intact living system [82,83]. Click chemistry can be applied to append an azide-functionalized reporter tag to the labeled proteins after cell lysis and homogenization (Figure 9). An important application of ABPP/click chemistry is the evaluation of target selectivity of covalent inhibitors in whole cells and animals. In many cases, an alkyne can be incorporated into a covalent inhibitor with minimal disruption to the cell permeability and binding interactions of the parent small molecule. The alkyne analog of the covalent inhibitor can be administered to mice;

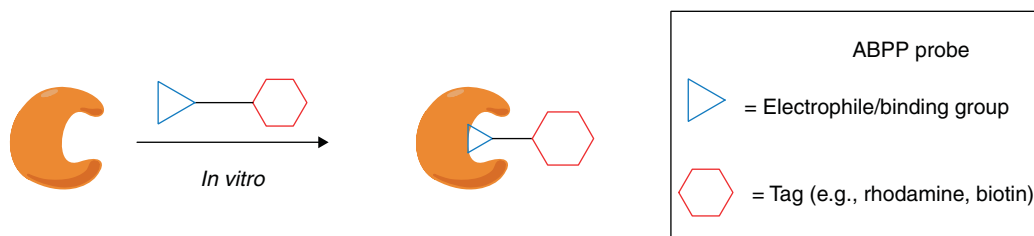
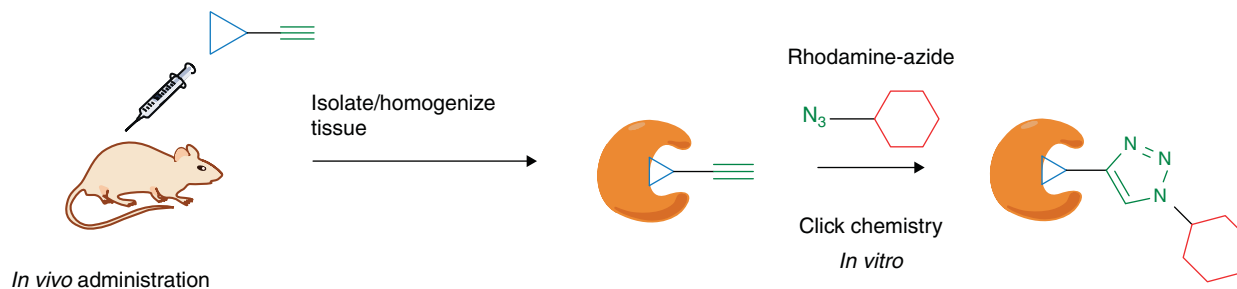
A. Activity-based protein profiling (ABPP)**B. Click chemistry ABPP**

Figure 9. Examining molecular selectivity of covalent inhibitors by ABPP and click chemistry.

the tissue of interest can be harvested and subjected to click chemistry conjugation with the desired reporter tag for identification of the target proteins. The application of ABPP/click chemistry was successfully used to probe the molecular selectivity of FAAH inhibitors resulting in the nomination of the clinical candidate PF-750 [38,81,84].

4.2 *In silico*/computational strategies

In silico/computational techniques can provide alternate means for identification and rank ordering of electrophilic moieties in covalent inhibitors, especially where experimental measurements are not feasible or restricted due to biopharmaceutical constraints such as poor aqueous solubility. Computational methodology to calculate theoretical reactivities of structurally diverse nitriles was recently demonstrated by Oballa *et al.* [51] wherein, energy differences between the thioimide adduct, a precursor thiol nucleophile, and the parent nitriles were used to calculate reactivity indices. The calculated values were found to correlate well with the experimental reactivity data (using cysteine as a nucleophile). More recently, Schwöbel and co-workers have used quantum chemical transition state calculations to examine Michael addition of GSH to α,β -unsaturated carbonyl derivatives. The predicted kinetic rate constants (k_{GSH}) for reaction with GSH were found to be in good agreement with experimental k_{GSH} values [25,85]. In another approach, E_{LUMO} and E_{HOMO} values were computed for reactivities of α,β -unsaturated carbonyl electrophiles in relation to their biologic activities [86]. The study demonstrated that Michael acceptor reactivity correlated well with lower E_{LUMO} values, which is consistent with results obtained by O'Brien and co-workers [23,24].

5. Mitigating toxicity risks with covalent drug candidates

For the purposes of this discussion, it is important to first point out that highly electrophilic xenobiotics (e.g., alkyl and/or acyl halides) and/or reactive metabolites generated in the course of drug metabolism (e.g., quinones, arene oxides, etc.) will tend to exhibit significantly higher chemical reactivity than the weakly electrophilic (tempered) warheads that are incorporated in covalent drugs. Unlike covalent drugs that require a specific non-covalent interaction with their biochemical targets prior to covalent modification, highly reactive metabolites will exhibit a low degree of molecular selectivity, and will alkylate/acylate a broad range of proteins with which they come into contact. For instance, the quinone-imine species generated via the two-electron oxidation of acetaminophen by CYP isoforms is known to covalently adduct with over 20 hepatic proteins *in vivo* [87]. Consequently, it is tempting to argue that the likelihood of protein covalent modification leading to IADRs with a covalent drug is potentially lower than it is with a reactive electrophile that modifies biologic macromolecules on a relatively indiscriminate basis [1].

5.1 Mitigation of overt toxicity risks of covalent drugs

Discovery strategies to mitigate overt toxicity and/or DDI risks with covalent drugs will be generally similar to the ones adopted for non-covalent agents. For example, genotoxicity concerns arising from covalent interactions of the warhead with DNA can be addressed in the *Salmonella Ames*

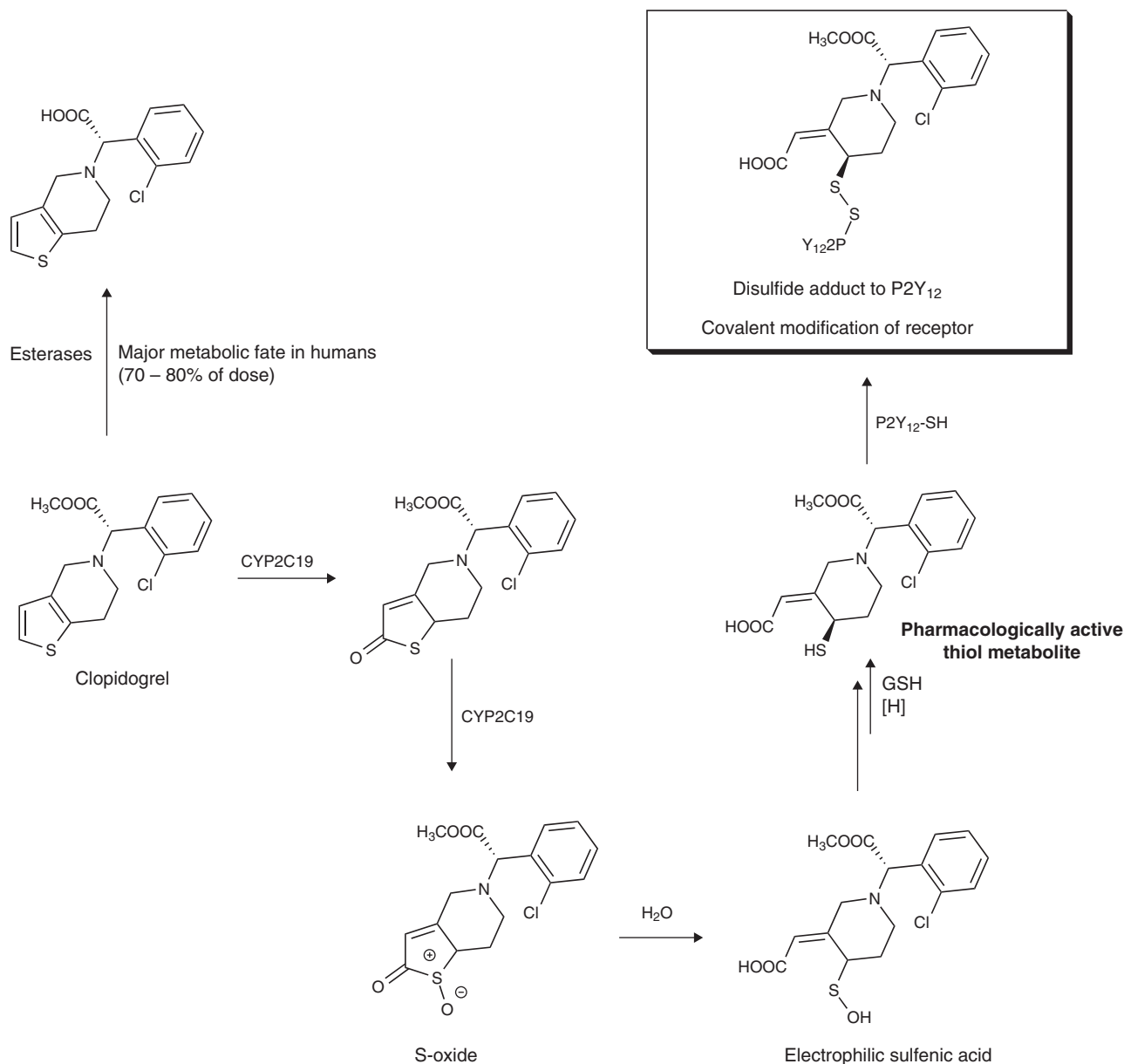


Figure 10. Metabolic activation of clopidogrel into a pharmacologically active reactive metabolite.

and *in vitro* micronucleus assays in the presence and absence of metabolic activation (ariclor-1254-induced rat liver S-9/ NADPH). Apart from the reactivity studies discussed in section 4, assessment of GSH reactivity toward covalent inhibitors in biologic systems (e.g., liver microsomes and liver cytosol from human and animals) that support glutathione transferase activity are essential from a toxicologic and pharmacokinetics perspective. In some scenarios (e.g., high daily dose covalent drugs), the GSH pool could be depleted in the process of metabolic detoxication (GSH conjugation to the warhead), and this can lead to hepatotoxicity in a manner similar to that noted with acetaminophen. Because liver injury caused by acetaminophen in humans can be replicated in

acute toxicity studies in animals [9], a logical next step would be the examination of GSH depletion and liver injury in pre-clinical species. *In vivo* short- and long-term target organ toxicity studies in preclinical species can be utilized to establish safety margins for clinical trials.

Clinical DDI risks due to reversible and time-dependent inhibition of major human CYP enzymes can be examined *in vitro* using human liver microsomes in the presence or absence of NADPH co-factor as exemplified with dacomitinib (see Figure 1), which causes potent inhibition of CYP2D6 activity ($IC_{50} = 60$ nM) resulting in clinical DDIs with the CYP2D6 substrate dextromethorphan [88]. Inhibition studies in the absence of NADPH are particularly

important since time- and concentration-dependent inhibition of CYP isoform(s) by a covalent inhibitor in the absence of co-factor can provide additional information pertaining to indiscriminate reaction with amino acid residue(s) on enzymes other than the pharmacologic target.

5.2 Mitigation of covert (idiosyncratic) toxicity

The concordance between drug toxicity in preclinical species and humans in relation to covert or idiosyncratic toxicity is very low [89], which increases the level of uncertainty around predicting IADR potential based on animal data. With respect to reactive metabolite involvement in IADRs, it is well recognized that many non-covalent drugs are bioactivated to electrophilic, reactive metabolites and/or covalently bind to macromolecules, but only a small fraction thereof cause toxicity *in vivo* [11]. Given the realm of uncertainty around IADR predictions, certain qualifying considerations have been proposed to support the clinical advancement of reactive metabolite-positive non-covalent drugs, and it is possible that the guidelines discussed below will also apply to covalent drug candidates.

5.2.1 Impact of daily dose

There is considerable evidence to suggest low daily dose as a key factor in mitigating IADR risks [11]. There do not appear to be examples of drugs that are dosed at < 20 mg/day that cause IADRs (whether or not these agents are prone to reactive metabolite formation). It is possible that the improved safety of low-dose drugs arises from a marked reduction in the total body burden to reactive metabolite exposure via efficient scavenging by GSH, and therefore unlikely to exceed the threshold needed for toxicity. The blockbuster cardiovascular drug and P2Y₁₂ purinoreceptor antagonist clopidogrel (Plavix®) is an example of a covalent drug that can be used as an illustration of this phenomenon. A prerequisite for the beneficial pharmacologic activity of clopidogrel is its conversion by CYP2C19 to a thiol metabolite (Figure 10), which inhibits platelet aggregation by covalently adducting to a cysteine residue in the P2Y₁₂ receptor in platelets [90-92]. Using human liver microsomes, Dansette *et al.* studied the pathway associated with the thiophene ring scission in clopidogrel, and intercepted a reactive sulfenic acid metabolite (see Figure 10), which can potentially react with protein nucleophiles [92]. Subsequent reduction of the electrophilic sulfenic acid intermediate by GSH was proposed to lead to the pharmacologically active thiol derivative. The bioactivation findings with clopidogrel pose a fundamental question from a structure-toxicity perspective: *Why is clopidogrel not associated with a high incidence of IADRs despite forming protein-reactive metabolite(s), and despite being administered at a relatively high daily dose of 75 mg?* A plausible reason is that the majority (> 70%) of the clopidogrel daily dose is rapidly hydrolyzed by human carboxylesterases to the inactive carboxylic acid metabolite (~ 80 – 85% of circulating metabolites) (see Figure 10) [93], which means that only a small percentage

of the parent drug (20 mg or less) is theoretically available for conversion to the active electrophilic species. Indeed, covalent binding to platelets accounts for only 2% of radiolabeled clopidogrel in human mass balance studies (Plavix package insert). While the feasibility of achieving a low daily dose covalent drug seems high (for the reasons stated in section 1), it is acknowledged that for certain drug classes (e.g., antibacterials, antivirals, etc.), this will be difficult to achieve.

5.2.2 Additional qualifying considerations for preclinical advancement of covalent drugs

Clinical indication is another qualifying consideration of significant importance. The level of risk that would be deemed acceptable for drug candidates intended to treat major unmet medical need and/or a life-threatening disease is likely to be significantly greater relative to treatment of chronic non-debilitating conditions where alternate treatment options are already available. This is clearly evident from Figure 1, where the majority of covalent drugs are being developed to treat rare, life-threatening cancers. An additional example of the benefit-risk argument is highlighted with chlorambucil, an aniline mustard derivative (Figure 11). Despite a black box warning (BBW) label for bone marrow toxicity/blood dyscrasias and mutagenic effects, chlorambucil is often used in the treatment of chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and Hodgkin's disease. The toxicity associated with chlorambucil (and related nitrogen mustards) is thought to arise via indiscriminate reactivity of the haloalkane motif with proteins and DNA (Figure 11). Certainly, GSH alkylation by chlorambucil (catalyzed by glutathione transferase) has been established, and is generally regarded as a detoxication pathway for most nitrogen mustards [94].

A second example is that of non-covalent tyrosine kinase inhibitors such as lapatinib, pazopanib and sunitinib (see Figure 11), which are also associated with a BBW label for hepatotoxicity, but are widely used in cancer treatment. While the mechanism by which lapatinib causes hepatotoxicity is unknown, a circumstantial link has been established with lapatinib metabolism to a reactive quinone-imine metabolite and its high daily dose of 1250 mg [11,95]. In clinical trials with pazopanib (daily dose ~ 800 mg), severe and fatal hepatotoxicity has been noted resulting in a BBW label. Similar to pazopanib, sunitinib is a multitargeted receptor tyrosine kinase inhibitor that has been approved for treatment of renal cell carcinoma as well as gastrointestinal stromal tumor. Although sunitinib qualifies as a relatively low dose drug (50 mg QD), results from preapproval clinical trials indicate an equivocal hepatic risk profile for sunitinib resulting in a BBW label. Despite this liability, sunitinib made the top 200 drug list based on total sales in 2009 [11].

Finally, selectivity with respect to target tissue distribution (locally acting drugs) can be potentially used to alleviate safety concerns. For example, the pharmacologic action of the anti-obesity drug orlistat is localized to the lumen with negligible exposure of the covalent drug in plasma. Likewise, in the

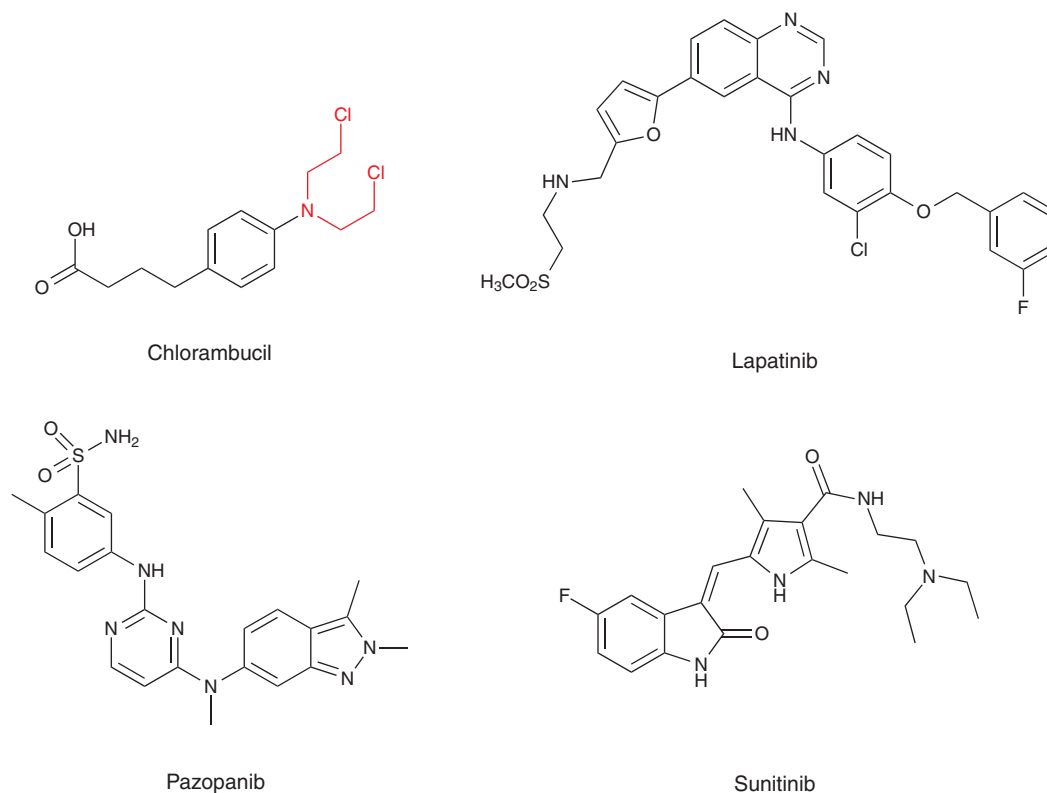


Figure 11. Structures of marketed covalent and non-covalent cancer drugs associated with a black box warning for toxicity.

case of the proton pump inhibitors such as omeprazole, mechanism-based unveiling of the electrophilic active metabolites (sulfenic acid/sulfenamide) is restricted to the acidic environment of the stomach [96], so systemic exposure to reactive electrophiles is unlikely to be achieved.

6. Expert opinion

Unlike highly reactive electrophilic xenobiotics that gave covalent drugs a bad name, today's covalent inhibitors utilize soft electrophiles as warheads that become locally reactive in the topology of an enzyme/receptor binding site. Paramount for success is the availability of detailed structural information on protein–ligand interaction, such as that derived from X-ray crystallography, to facilitate the refinements in inhibitor design (including the strategic placement of the warhead) as elegantly illustrated with EGFR inhibitors. Proteomic screening and chemical/biochemical reactivity measurements can provide systematic rank ordering of molecular selectivity and reactivity in the early discovery phase of covalent inhibitor programs, which can assist in candidate selection. However, the ability to prospectively predict immune-mediated IADR potential of covalent drugs using the output from these various endpoints is unknown. With non-covalent drugs, it is generally recognized that *in vitro* (or *in vivo*) protein reactivity of electrophilic species generated in the course of metabolism

is not a reliable predictor of IADR potential of the parent drugs. In the case of covalent drugs, opinions regarding an acceptable level of chemical reactivity/body burden for a *toxic* electrophile (e.g., alkylhalides that reacts with DNA, GSH, and/or proteins) versus a *non-toxic* electrophilic covalent drug have not been defined. This is clearly an area for future research among the drug metabolism and/or toxicology community. With the exception of β -lactam antibiotics and nitrogen mustards that are associated with a significant incidence of IADRs, there are no other covalent drugs (marketed or in advanced clinical trials) that have demonstrated unacceptable incidences of IADRs. The precise reason(s) for suspension of canertinib, the first covalent inhibitor of EGFR, from Phase II clinical trials may be due to its marginal efficacy and lack of differentiation from reversible EGFR inhibitors in the treatment of platinum-refractory or recurrent advanced-stage non-small-cell lung cancer [97]. The identification of the back-up agent dacomitinib (PF-00299804) with superior *in vivo* antitumor activity and PK properties may be an additional reason for canertinib termination [98]. Dacomitinib is currently in numerous Phase II and Phase III clinical trials in patients with advanced non-small-cell lung cancer [99]. In the case of canertinib, cutaneous lesions, which could be interpreted as a possible side effect of an immune-mediated IADR, have been reported in clinical trials. However, the skin lesions appear to be a consequence of

EGFR inhibition, and have been noted with reversible EGFR inhibitors [100].

Overall, increasing confidence in the use of proteomic, chemical, and biochemical reactivity screens in candidate identification will require a retrospective analysis with marketed drugs not associated with IADRs (e.g., orlistat), β -lactam antibacterials and nitrogen mustards with propensity to cause IADRs (e.g., amoxicillin and chlorambucil), and chemicals (e.g., alkylhalides) known to cause deleterious toxic effects via non-selective covalent binding. A systematic analysis is also needed with the rationally designed EGFR inhibitors (e.g., such as dacomitinib and neratinib). For instance, while mass balance studies on [^{14}C]-neratinib in humans indicate that elimination of neratinib-derived radioactivity is rapid and essentially

complete (~ 98.2%) after 216 h following oral administration of a single dose of 200 mg (unpublished observation), it is unclear how neratinib would fare in the *in vitro* proteomics and reactivity assays, especially since off-target covalent binding has been reported to human serum albumin [77]. In conclusion, ongoing clinical studies and post-marketing surveillance with an emerging cadre of highly selective covalent inhibitors will continue to offer insights into the therapeutic utility and possible IADR risks, which could dispute the historical prejudices against the development of irreversible inhibitors.

Declaration of interest

The authors are employed at Pfizer, Inc.

Bibliography

Papers of special note have been highlighted as either of interest (●) or of considerable interest (●●) to readers.

- Potashman MH, Duggan ME. Covalent modifiers: an orthogonal approach to drug design. *J Med Chem* 2009;52:1231-46
- Detailed review of the mechanisms of marketed covalent drugs.
- Johnson DS, Weerapana E, Cravatt BF. Strategies for discovering and derisking covalent, irreversible enzyme inhibitors. *Future Med Chem* 2010;2:949-64
- Insightful review of proteomics-based approaches to establish molecular selectivity of covalent inhibitors.
- Singh J, Petter RC, Baillie TA, Whitty A. The resurgence of covalent drugs. *Nat Rev Drug Discov* 2011;10:307-17
- Insightful review of the therapeutic potential of covalent drugs.
- Powers JC, Asgian JL, Ekici OD, James KE. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem Rev* 2002;102:4639-750
- Phillips EJ, Mallal SA. Pharmacogenetics of drug hypersensitivity. *Pharmacogenomics* 2010;11:973-87
- Shenton JM, Chen J, Uetrecht JP. Animal models of idiosyncratic drug reactions. *Chem Biol Interact* 2004;150:53-70
- Miller JA. Brief history of chemical carcinogenesis. *Cancer Lett* 1994;83:9-14
- Miller JA. The metabolism of xenobiotics to reactive electrophiles in chemical carcinogenesis and mutagenesis: a collaboration with Elizabeth Cavert Miller and our associates. *Drug Metab Rev* 1998;30:645-74
- An historical account of the groundbreaking work on aniline bioactivation by the Millers.
- Mitchell JR, Jollow DJ, Potter WZ, et al. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther* 1973;187:211-17
- Publication is part of a seminal series of articles describing the biochemical basis for acetaminophen liver injury.
- Dahlin DC, Miwa GT, Lu AY, Nelson SD. N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci USA* 1984;81:1327-31
- Identification of the reactive quinone-imine metabolite of acetaminophen.
- Stepan AF, Walker DP, Bauman J, et al. Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. *Chem Res Toxicol* 2011;24:1345-410
- Exhaustive analysis of structural alert, reactive metabolite and covalent binding trends as predictors of idiosyncratic adverse drug reactions.
- Graham SL, Shepard KL, Anderson PS, et al. Topically active carbonic anhydrase inhibitors. 2. Benzo[b] thiophenesulfonamide derivatives with ocular hypotensive activity. *J Med Chem* 1989;32:2548-54
- Woltersdorf OW Jr, Schwam H, Bicking JB, et al. Topically active carbonic anhydrase inhibitors. 1. O-acyl derivatives of 6-hydroxybenzothiazole-2-sulfonamide. *J Med Chem* 1989;32:2486-92
- Graham SL, Hoffman JM, Gautheron P, et al. Topically active carbonic anhydrase inhibitors. 3. Benzofuran- and indole-2-sulfonamides. *J Med Chem* 1990;33:749-54
- Robertson JG. Mechanistic basis of enzyme-targeted drugs. *Biochemistry* 2005;44:5561-71
- Van Der Ouderaa FJ, Buytenhek M, Nugteren DH, Van Dorp DA. Acetylation of prostaglandin endoperoxide synthetase with acetylsalicylic acid. *Eur J Biochem* 1980;109:1-8
- DeWitt DL, El-Harith EA, Kraemer SA, et al. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J Biol Chem* 1990;265:5192-8
- Amslinger S. The tunable functionality of alpha,beta-unsaturated carbonyl compounds enables their differential application in biological systems. *ChemMedChem* 2010;5:351-6
- Way JC. Covalent modification as a strategy to block protein-protein interactions with small-molecule drugs. *Curr Opin Chem Biol* 2000;4:40-6
- Jencks WP. Binding energy, specificity, and enzymic catalysis: the circe effect.

- Adv Enzymol Relat Areas Mol Biol 1975;43:219-410
21. LoPachin RM, Gavin T, DeCaprio A, Barber DS. Application of the hard and soft, acids and bases (HSAB) theory to toxicant – target interactions. *Chem Res Toxicol* 2011;25:239-351
 22. Pearson RG. Hard and soft acids and bases – the evolution of a chemical concept. *Coord Chem Rev* 1990;100:403-25
 23. Chan K, Poon R, O'Brien PJ. Application of structure-activity relationships to investigate the molecular mechanisms of hepatocyte toxicity and electrophilic reactivity of alpha, beta-unsaturated aldehydes. *J Appl Toxicol* 2008;28:1027-39
 24. Chan K, O'Brien PJ. Structure-activity relationships for hepatocyte toxicity and electrophilic reactivity of alpha, beta-unsaturated esters, acrylates and methacrylates. *J Appl Toxicol* 2008;28:1004-15
 25. Schwobel JAH, Wondrousch D, Koleva YK, et al. Prediction of Michael-type acceptor reactivity toward glutathione. *Chem Res Toxicol* 2010;23:1576-85
 26. Fry DW. Site-directed irreversible inhibitors of the erbB family of receptor tyrosine kinases as novel chemotherapeutic agents for cancer. *Anticancer Drug Des* 2000;15:3-16
 27. Cohen MS, Zhang C, Shokat KM, Taunton J. Structural bioinformatics-based design of selective, irreversible kinase inhibitors. *Science* 2005;308:1318-21
 28. Singh J, Petter RC, Kluge AF. Targeted covalent drugs of the kinase family. *Curr Opin Chem Biol* 2010;14:475-80
 29. Lu WP, Kincaid E, Sun Y, Bauer MD. Kinetics of beta-lactam interactions with penicillin-susceptible and-resistant penicillin-binding protein 2x proteins from *Streptococcus pneumoniae*. Involvement of acylation and deacylation in beta-lactam resistance. *J Biol Chem* 2001;276:31491-501
 30. Yocum RR, Rasmussen JR, Strominger JL. The mechanism of action of penicillin. Penicillin acylates the active site of *Bacillus stearothermophilus* D-alanine carboxypeptidase. *J Biol Chem* 1980;255:3977-86
 31. Zhao Z, Baldo BA, Rimmer J. Beta-Lactam allergenic determinants: fine structural recognition of a cross-reacting determinant on benzylpenicillin and cephalothin. *Clin Exp Allergy* 2002;32:1644-50
 32. Levine BB, Ovary Z. Studies on the mechanism of the formation of the penicillin antigen. III. The N-(D-alpha-benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G. *J Exp Med* 1961;114:875-904
 33. Uetrecht J. Immune-mediated adverse drug reactions. *Chem Res Toxicol* 2009;22:24-34
 34. Connor SC, Everett JR, Jennings KR, et al. High resolution 1H NMR spectroscopic studies of the metabolism and excretion of ampicillin in rats and amoxycillin in rats and man. *J Pharm Pharmacol* 1994;46:128-34
 35. Hadvary P, Sidler W, Meister W, et al. The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase. *J Biol Chem* 1991;266:2021-7
 36. Guercioli R. Mode of action of orlistat. *Int J Obes Relat Metab Disord* 1997;21(Suppl 3):S12-23
 37. Bar-On P, Millard CB, Harel M, et al. Kinetic and structural studies on the interaction of cholinesterases with the anti-Alzheimer drug rivastigmine. *Biochemistry* 2002;41:3555-64
 38. Ahn K, Johnson DS, Mileni M, et al. Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol* 2009;16:411-20
 39. Johnson DS, Ahn K, Kesten S, et al. Benzothioephene piperazine and piperidine urea inhibitors of fatty acid amide hydrolase (FAAH). *Bioorg Med Chem Lett* 2009;19:2865-9
 40. Mileni M, Johnson DS, Wang Z, et al. Structure-guided inhibitor design for human FAAH by interspecies active site conversion. *Proc Natl Acad Sci USA* 2008;105:12820-4
 41. Weerapana E, Wang C, Simon GM, et al. Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* 2010;468:790-5
 42. Lin C, Kwong AD, Perni RB. Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. *Infect Disord Drug Targets* 2006;6:3-16
 43. Kwo PY, Lawitz EJ, McCone J, et al. Efficacy of boceprevir, an NS3 protease inhibitor, in combination with peginterferon alfa-2b and ribavirin in treatment-naïve patients with genotype 1 hepatitis C infection (SPRINT-1): an open-label, randomised, multicentre phase 2 trial. *Lancet* 2010;376:705-16
 44. Dufour E, Storer AC, Menard R. Peptide aldehydes and nitriles as transition state analog inhibitors of cysteine proteases. *Biochemistry* 1995;34:9136-43
 45. Frizler M, Lohr F, Furtmann N, et al. structural optimization of azadipeptide nitriles strongly increases association rates and allows the development of selective cathepsin inhibitors. *J Med Chem* 2011;54:396-400
 46. Frizler M, Stirnberg M, Sisay MT, Gutschow M. Development of nitrile-based peptidic inhibitors of cysteine cathepsins. *Curr Top Med Chem* 2010;10:294-322
 47. Lucas EC, Williams A. The pH dependencies of individual rate constants in papain-catalyzed reactions. *Biochemistry* 1969;8:5125-35
 48. Deaton DN, Hassell AM, McFadyen RB, et al. Novel and potent cyclic cyanamide-based cathepsin K inhibitors. *Bioorg Med Chem Lett* 2005;15:1815-19
 49. Grabowski U, Chambers TJ, Shiroo M. Recent developments in cathepsin K inhibitor design. *Curr Opin Drug Discov Devel* 2005;8:619-30
 50. Altmann E, Cowan-Jacob SW, Missbach M. Novel purine nitrile derived inhibitors of the cysteine protease cathepsin K. *J Med Chem* 2004;47:5833-6
 51. Oballa RM, Truchon JF, Bayly CI, et al. A generally applicable method for assessing the electrophilicity and reactivity of diverse nitrile-containing compounds. *Bioorg Med Chem Lett* 2007;17:998-1002
 52. Gauthier JY, Chauret N, Cromlish W, et al. The discovery of odanacatib (MK-0822), a selective inhibitor of cathepsin K. *Bioorg Med Chem Lett* 2008;18:923-8

53. Black WC, Percival MD. The consequences of lysosomotropism on the design of selective cathepsin K inhibitors. *ChemBiochem* 2006;7:1525-35
54. Fleming FF, Yao L, Ravikumar PC, et al. Nitrile-containing pharmaceuticals: efficacious roles of the nitrile pharmacophore. *J Med Chem* 2010;53:7902-17
55. Dhillon S, Weber J. Saxagliptin. *Drugs* 2009;69:2103-14
56. Mathieu C, Degrande E. Vildagliptin: a new oral treatment for type 2 diabetes mellitus. *Vasc Health Risk Manag* 2008;4:1349-60
57. Li CS, Deschenes D, Desmarais S, et al. Identification of a potent and selective non-basic cathepsin K inhibitor. *Bioorg Med Chem Lett* 2006;16:1985-9
58. Metzler WJ, Yanchunas J, Weigelt C, et al. Involvement of DPP-IV catalytic residues in enzyme-saxagliptin complex formation. *Protein Sci* 2008;17:240-50
59. Serafimova IM, Pufall MA, Krishnan S, et al. Reversible targeting of noncatalytic cysteines with chemically tuned electrophiles. *Nat Chem Biol* 2012;8:471-476
60. Chan K, Jensen N, O'Brien PJ. Structure-activity relationships for thiol reactivity and rat or human hepatocyte toxicity induced by substituted p-benzoquinone compounds. *J Appl Toxicol* 2008;28:608-20
61. Kalgutkar AS, Mascitti V, Sharma R, et al. Intrinsic electrophilicity of a 4-substituted-5-cyano-6-(2-methylpyridin-3-yloxy)pyrimidine derivative: structural characterization of glutathione conjugates in vitro. *Chem Res Toxicol* 2011;24:269-78
62. Teffera Y, Colletti AE, Harmnag JC, et al. Chemical reactivity of methoxy 4-o-aryl quinolines: identification of glutathione displacement products in vitro and in vivo. *Chem Res Toxicol* 2008;21:2216-22
63. Litchfield J, Sharma R, Atkinson K, et al. Intrinsic electrophilicity of the 4-methylsulfonyl-2-pyridone scaffold in glucokinase activators: role of glutathione-S-transferases and in vivo quantitation of a glutathione conjugate in rats. *Bioorg Med Chem Lett* 2010;20:6262-7
64. Aptula AO, Patlewicz G, Roberts DW, Schultz TW. Non-enzymatic glutathione reactivity and in vitro toxicity: a non-animal approach to skin sensitization. *Toxicol In Vitro* 2006;20:239-47
65. Schultz TW, Yarbrough JW, Johnson EL. Structure-activity relationships for reactivity of carbonyl-containing compounds with glutathione. *SAR QSAR Environ Res* 2005;16:313-22
66. Cespedes-Camacho IF, Manso JA, Perez-Prior MT, et al. Reactivity of acrylamide as an alkylating agent: a kinetic approach. *J Phys Org Chem* 2010;23:171-5
67. Bardos TJ, Datta-Gupta N, Hebborn P, Triggler DJ. A study of comparative chemical and biological activities of alkylating agents. *J Med Chem* 1965;8:167-74
68. Wilson JM, Wu D, Motiu-DeGrood R, Hupe DJ. A spectrophotometric method for studying the rates of reaction of disulfides with protein thiol groups applied to bovine serum albumin. *J Am Chem Soc* 1980;102:359-63
69. MacFaul PA, Morley AD, Crawford JJ. A simple in vitro assay for assessing the reactivity of nitrile containing compounds. *Bioorg Med Chem Lett* 2009;19:1136-8
70. Huth JR, Mendoza R, Olejniczak ET, et al. ALARM NMR: a rapid and robust experimental method to detect reactive false positives in biochemical screens. *J Am Chem Soc* 2005;127:217-24
- **NMR studies to measure thiol reactivity of covalent inhibitor using human La antigen protein.**
71. Huth JR, Song D, Mendoza RR, et al. Toxicological evaluation of thiol-reactive compounds identified using a La assay to detect reactive molecules by nuclear magnetic resonance. *Chem Res Toxicol* 2007;20:1752-9
72. Jacks A, Babon J, Kelly G, et al. Structure of the C-terminal domain of human La protein reveals a novel RNA recognition motif coupled to a helical nuclear retention element. *Structure* 2003;11:833-43
73. Chen KX, Lesburg CA, Vibulbhan B, et al. A novel class of highly potent irreversible hepatitis C virus NS5B polymerase inhibitors. *J Med Chem* 2012;55:2089-101
74. Fry DW, Bridges AJ, Denny WA, et al. Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosine kinase inhibitor. *Proc Natl Acad Sci USA* 1998;95:12022-7
- **First example of targeted covalent inhibition of kinase with detailed in vitro and in vivo biochemical characterization.**
75. Torrance CJ, Jackson PE, Montgomery E, et al. Combinatorial chemoprevention of intestinal neoplasia. *Nat Med* 2000;6:1024-8
76. Chandrasekaran A, Shen L, Lockhead S, et al. Reversible covalent binding of neratinib to human serum albumin in vitro. *Drug Metab Lett* 2010;4:220-7
77. Wang J, Li-Chan XX, Atherton J, et al. Characterization of HKI-272 covalent binding to human serum albumin. *Drug Metab Dispos* 2010;38:1083-93
- **Non-specific covalent labeling of neratinib, an EGFR covalent inhibitor, to human serum albumin.**
78. Cravatt BF, Wright AT, Kozarich JW. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu Rev Biochem* 2008;77:383-414
79. Weerapana E, Speers AE, Cravatt BF. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP) – a general method for mapping sites of probe modification in proteomes. *Nat Protoc* 2007;2:1414-25
80. Leung D, Hardouin C, Boger DL, Cravatt BF. Discovering potent and selective reversible inhibitors of enzymes in complex proteomes. *Nat Biotechnol* 2003;21:687-91
81. Ahn K, Johnson DS, Fitzgerald LR, et al. Novel mechanistic class of fatty acid amide hydrolase inhibitors with remarkable selectivity. *Biochemistry* 2007;46:13019-30
82. Best MD. Click chemistry and bioorthogonal reactions: unprecedented selectivity in the labeling of biological molecules. *Biochemistry* 2009;48:6571-84
83. Speers AE, Cravatt BF. Profiling enzyme activities in vivo using click chemistry methods. *Chem Biol* 2004;11:535-46
- **Excellent review article on the basics of click chemistry/ABPP.**

84. Alexander JP, Cravatt BF. Mechanism of carbamate inactivation of FAAH: implications for the design of covalent inhibitors and in vivo functional probes for enzymes. *Chem Biol* 2005;12:1179-87
85. Schwobel JA, Madden JC, Cronin MT. Examination of Michael addition reactivity towards glutathione by transition-state calculations. *SAR QSAR Environ Res* 2010;21:693-710
86. Zoete V, Rougee M, Dinkova-Kostova AT, et al. Redox ranking of inducers of a cancer-protective enzyme via the energy of their highest occupied molecular orbital. *Free Radic Biol Med* 2004;36:1418-23
87. Park BK, Kitteringham NR, Maggs JL, et al. The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol* 2005;45:177-202
88. Bello CL, LaBadie RR, Ni G, et al. The effect of dacomitinib (PF-00299804) on CYP2D6 activity in healthy volunteers who are extensive or intermediate metabolizers. *Cancer Chemother Pharmacol* 2012;69:991-7
89. Olson H, Betton G, Robinson D, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol* 2000;32:56-67
90. Savi P, Pereillo JM, Uzabiaga MF, et al. Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* 2000;84:891-6
91. Pereillo JM, Maftouh M, Andrieu A, et al. Structure and stereochemistry of the active metabolite of clopidogrel. *Drug Metab Dispos* 2002;30:1288-95
92. Dansette PM, Libraire J, Bertho G, Mansuy D. Metabolic oxidative cleavage of thioesters: evidence for the formation of sulfenic acid intermediates in the bioactivation of the antithrombotic prodrugs ticlopidine and clopidogrel. *Chem Res Toxicol* 2009;22:369-73
93. Farid NA, Kurihara A, Wrighton SA. Metabolism and disposition of the thienopyridine antiplatelet drugs ticlopidine, clopidogrel, and prasugrel in humans. *J Clin Pharmacol* 2010;50:126-42
94. Ciaccio PJ, Tew KD, LaCreta FP. Enzymatic conjugation of chlorambucil with glutathione by human glutathione S-transferases and inhibition by ethacrynic acid. *Biochem Pharmacol* 1991;42:1504-7
95. Teng WC, Oh JW, New LS, et al. Mechanism-based inactivation of cytochrome P450 3A4 by lapatinib. *Mol Pharmacol* 2010;78:693-703
96. Shin JM, Cho YM, Sachs G. Chemistry of covalent inhibition of the gastric (H⁺ 3K⁺)-ATPase by proton pump inhibitors. *J Am Chem Soc* 2004;126:7800-11
97. Janne PA, von Pawel J, Cohen RB, et al. Multicenter, randomized, phase II trial of CI-1033, an irreversible pan-ERBB inhibitor, for previously treated advanced non-small-cell lung cancer. *J Clin Oncol* 2007;25:3936-44
98. Gonzales AJ, Hook KE, Althaus IW, et al. Antitumor activity and pharmacokinetic properties of PF-00299804, a second-generation irreversible pan-erbB receptor tyrosine kinase inhibitor. *Mol Cancer Ther* 2008;7:1880-9
99. Available from: http://www.pfizer.com/files/news/esmo/dacomitinib_pf_00299804_fact_sheet.pdf
100. Brown AP, Dunstan RW, Courtney CL, et al. Cutaneous lesions in the rat following administration of an irreversible inhibitor of erbB receptors, including the epidermal growth factor receptor. *Toxicol Pathol* 2008;36:410-19

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Irreversible Protein Kinase Inhibitors: Balancing the Benefits and Risks

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■ INTRODUCTION

In the relatively young but expanding field of irreversible kinase inhibitor drug discovery, there are two main developments that are of central importance. First, the patients have watched the first wave of low molecular weight protein kinase inhibitors becoming available to them over the past decade. These kinase inhibitors have been approved by the U.S. Food and Drug Administration (FDA) for therapeutic use in oncology indications and constitute an important addition to the arsenal of drugs to combat cancer (Table 1). Without exception, these marketed protein kinase inhibitors have been identified and developed using conventional approaches, i.e., reversible inhibitors that (partly) occlude the ATP pocket in the catalytic domain of the kinase. Although protein kinases are regarded as an attractive drug target family, it took (and still takes) huge efforts to master the human kinome, which comprises more than 500 protein kinases. The search for clinically applicable kinase inhibitors that target the highly conserved ATP pocket has been thwarted by a couple of well-known hurdles. Selectivity, cellular potency, and an increasingly crowded intellectual property arena are major points of attention.

The second important development is the renewed interest in covalent binding drugs (reviewed by Potashman and Duggan and by Singh et al).^{1,2} This recent revival results from a better understanding of the benefits of the covalent binding principle and the approval of effective and safe covalent drugs. Historically, drug discoverers have been taught to stay away from small molecular entities that harbor reactive electrophilic groups because these used to be equivalent to promiscuity.³ Promiscuous hits that relied on reactive groups were traditionally hard to optimize toward leads, since these were more than often interfering with the biochemical assay rather than truly modifying the activity of the target of interest.⁴ Even if the target modulation was real, indiscriminant reactivity was believed to trigger insurmountable toxic events that may surface in late stage clinical trials when larger patient populations are involved. As a consequence, “suicide inhibitors”, “warheads”, and covalent irreversible inhibitors developed a negative flavor over time and became almost synonymous with toxicity in some organizations. The skepticism toward irreversible drugs may evaporate as more examples of irreversible drugs progress clinically that demonstrate good efficacy and safety margins.

In a nutshell, the therapeutic applicability or the success of irreversible binding kinase inhibitors is dependent on whether or not the covalent bond can be confined solely to the protein kinase of interest. So this approach is in essence a story about two T's: treatment and toxicity. When relying on the covalent binding principle, it is important to discern adduct-based toxicity and adduct-based treatment, since the adducting

molecular entities in question obey overlapping fundamental rules in terms of reactivity. The only key difference is the nature and the function of the proteins that are covalently modified. Covalent kinase inhibitors with well-balanced recognition and reactivity should provide efficacy, selectivity, and ultimately the safety margins that are required for regulatory approval. If we strike the right balance, a third “T” will give enough comfort: therapeutic window.

This Perspective aims to give a comprehensive account from a medicinal chemist's point of view on the progress of irreversible kinase inhibitor drug discovery. The “state of the art” is reviewed by means of reported irreversible kinase inhibitors profiles and their chemical structures. The potential upsides and pitfalls that are associated with this concept are highlighted to provide a general understanding of the differences with respect to conventional drug discovery, as well as the future potential of this approach.

■ EFFECT OF RESIDENCE TIME ON THE THERAPEUTIC WINDOW

Residence Time and Efficacy. A kinase inhibitor will only be efficacious when it is modulating the action of the physiological kinase and can only do so when the inhibitor is bound. This general paradigm was effectively captured by Copeland and co-workers, who described the potential advantages of long residence time in terms of duration of action and target selectivity.⁵ The equilibrium dissociation constant (K_d) for the drug–target binary complex as determined in a closed (in vitro) system is a more accurate measure than the IC_{50} or K_i . Yet the equilibrium setting is different in vivo, since the drug concentrations are no longer constant, and the efficacy is dependent on the on-rate (k_{on}) and even more importantly the off-rate constant (k_{off}). The importance of the related occupancy time or half-life for the protein–drug complex was corroborated in the context of pharmacological agents for whom longer residence times seemed to be beneficial in terms of biological or clinical efficacy.^{6,7} In this respect, kinase inhibitors that exert covalent and irreversible binding can achieve the ultimate physiological goal: Efficacy is maintained until the target kinase is physically restored by the body to physiologically relevant levels (Figure 1).⁸ In other words, the pharmacodynamics will be a function of the de novo synthesis rate of the target protein rather than the trough level of the compound.

Unlike for kinase inhibitors with a reversible binding mode, we anticipate that not all ADME parameters need to be as efficiently optimized for covalent inhibitors. The general

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Table 1. FDA Approved Protein Kinase Inhibitors (as of March 2012)

generic (brand) name	year of approval	company	indication	target kinase
imatinib (Gleevec)	2001	Novartis	chronic myeloid leukemia (CML)	Abl, c-Kit, PDGFR α/β
gefitinib (Iressa)	2003	AstraZeneca	non-small-cell lung carcinoma (NSCLC)	EGFR
erlotinib (Tarceva)	2004	Genetech, OSI	NSCLC, pancreatic cancer	EGFR
sorafenib (Nexavar)	2005	Bayer, Onyx	hepatocellular carcinoma, renal cell carcinoma (RCC)	Raf, VEGFR2/3, c-Kit, PDGFR β
sunitinib (Sutent)	2006	Pfizer	gastrointestinal stromal tumor (GIST), RCC	c-Kit, VEGFR, PDGFR, FLT3
dasatinib (Sprycel)	2006	Bristol-Myers Squibb	CML	Abl, c-Kit, PDGFR, Src
nilotinib (Tasigna)	2007	Novartis	CML	Abl, c-Kit, PDGFR, Src, ephrin
lapatinib (Tykerb)	2007	GlaxoSmithKline	breast cancer	EGFR, ErbB2
pazopanib (Votrient)	2009	GlaxoSmithKline	RCC	VEGFR, PDGFR α/β , c-Kit
vandetanib (Caprelsa)	2011	AstraZeneca	thyroid cancer	VEGFR, EGFR, RET
vemurafenib (Zelboraf)	2011	Roche, Plexxicon	CML	Abl, c-Kit, PDGFR, Src, ephrin
crizotinib (Xalkori)	2011	Pfizer	NSCLC (ALK +ve)	ALK, MET
ruxolitinib (Jakafi)	2011	Incyte	myelofibrosis	JAK1/2
axitinib (Inlyta)	2012	Pfizer	RCC	VEGFR, PDGFR β , c-Kit

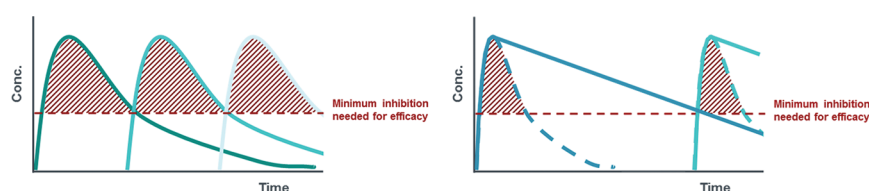


Figure 1. For conventional drugs, the pharmacodynamic effect is driven by the pharmacokinetics of the drug (left). For irreversible covalent drugs, the pharmacodynamic effect is driven by the turnover rate (de novo synthesis) of the protein target (right). The red hatched area is drug exposure not required for efficacy.

requirements for absorption and distribution characteristics remain relatively unchanged with respect to the conventional approach. It is still advantageous to have good oral availability and rapid distribution, but in the case of an irreversible kinase inhibitor it would be favorable to clear the drug from the body once the target kinase is maximally occupied. Thus, the *in vitro* and *in vivo* criteria for optimization cycles with respect to metabolism and excretion are very much different and should accentuate a swift clearance of the drug.

Residence Time and Toxicity. Drug-safety-related issues pose a serious problem to the pharmaceutical industry and have been a major contributing factor to attrition rates in the drug development trajectories.⁹ As discussed above, since no excessive circulating levels are required to maintain efficacy with irreversible binding drugs, rapid clearance should lead to a lower propensity for off-target related adverse effects. But this can only be achieved when off-target protein–drug interactions are short-lived because of the noncovalent nature of binding.

On the other hand, toxic events can also be associated with covalent binding, and this can be either on-target or off-target related. If the toxicity is a direct consequence of on-target modulation, then irreversible inhibition might exacerbate the adverse effect potential. In this case, the clinical benefit should outweigh the adverse event. Alternatively, prolonged residence times can occur if collateral targets are being adducted by the potential drug. In that case, a high degree of *in vivo* selectivity can only be accomplished when the off-target turnover rate is (much) faster than the on-target resynthesis rate.

For irreversible kinase inhibitors, the target selectivity thus should not only be discussed in terms of reversible binding to closely related kinases with respect to primary sequence homology. Especially kinases that (also) share the site of covalent modification are highly relevant to include in the selectivity panel. A good level of selectivity for those off-target

kinases is suggestive of a high degree of overall selectivity within the human kinome, as well as for other protein classes. Also, potential adverse effects due to covalent inhibition of specific off-target kinases can be designed out early in the optimization cycles to yield a better therapeutic window.

Much of the aversion to irreversible binders is predominantly the result of the negative experiences with reactive metabolites that could bind covalently to a variety of proteins.^{10,11} In such a case, the parent drugs are metabolically bioactivated to reactive species that potentially can bind covalently to all sorts of macromolecules. Next to the predictable intrinsic toxicity, this may lead to unpredictable idiosyncratic toxicity (IDT) in late stage clinical testing and beyond. In order to exclude or minimize the propensity of reactive metabolite formation, potential drugs are usually evaluated preclinically for (i) glutathione (GSH) adduct formation and depletion when incubated with recombinant enzymes or human liver tissue preparations and (ii) magnitude of covalent protein binding in liver microsomal fractions and in rodents. It is obvious that these models are highly relevant for the optimization of irreversible kinase inhibitors as well. The advantage with irreversible kinase inhibitors in this respect is that the parent molecules are the reactive species, which should have a better predictive power for the reactivity assays and body retention models involved.

Recently, the occurrence of IDT or hypersensitivity reaction has been linked by Nakayama et al. to the magnitude of covalent and irreversible binding to off-targets and the daily dose required in patients.¹² A classification system to assess the risk of IDT was proposed based on covalent binding to human hepatocytes as the best predictor, in combination with the daily dose.

Another potential event that has to be considered for covalent and irreversible kinase inhibitors is hapten formation,

i.e., triggering an immune response to the adducted protein.¹³ Experiences with reactive compounds and reactive metabolites indicate that additional danger signals (e.g., mitochondrial damage) are required next to the hapten mechanism to induce an immune response (for a review, see Zhang et al).¹⁴ Except for the β -lactam antibiotics, absence of reports of immune responses for recently marketed irreversible binding drugs does not support this concern as a general problem for covalent drugs.

Reactive Metabolite Considerations. Considerations that have been used historically within the pharmaceutical industry to assess the fate of conventional drugs with suspected reactive metabolites can provide guidance for irreversible binding kinase inhibitors as well.¹⁰ It is obvious that the “avoidance strategy”, i.e., complete elimination of reactive groups, cannot be applied on warhead-containing drugs, since the pharmacological effect is largely dependent on the reactive group. Perhaps it would be better to develop a reversible therapy for less severe disorders, especially if one can rationalize that reversible drugs should work equally well. Also, the anticipated human dose (for any drug) has to be considered, as the body burden has to be kept to a minimum (vide infra). In addition, a disease that needs acute dosing rather than chronic treatment might be more appropriate to engage with irreversible binding drugs. These and other questions need to be addressed before a covalent (kinase) inhibitor program can be progressed toward clinical development. Obviously, the key issue here is the benefit/risk ratio, and serious life threatening diseases may be particularly justified in this scenario.

One has to keep in mind though that most reactive metabolites are by no means tuned, and they can be uncontrollable like “unguided missiles”. In contrast, deliberately designed irreversible kinase inhibitors can be subjected to safety assays during optimization cycles to ensure selection of compounds that meet stringent safety prediction criteria. In some other instances, bioactivation is even required to generate the active drug, such as in the case of proton pump inhibitors like omeprazole and P2Y₁₂ receptor antagonist clopidogrel.¹ No matter the type of covalent approach involved, a decision tree that incorporates several safety assessment parameters will be required to mitigate the risks. In addition, the level of acceptance that has been set for the magnitude of covalent binding of reactive metabolites in vitro and in vivo also applies for covalent binding parent drugs.

■ THE CONCEPT APPLIED TO KINASES

The question is whether the members belonging to the superfamily of protein kinases are suitable targets at all for irreversible inhibition. First of all, the replenishment of the target kinase has to be sluggish enough to allow drug dosing once or twice a day. This has to be checked on a kinase-by-kinase basis. Second, unlike for instance the enzyme families of (cysteine) proteases or phosphatases, kinases lack a catalytically active amino acid residue that is key to the function of a particular enzyme by virtue of its overt nucleophilicity. The relatively unreactive cysteines, serines, threonines, and lysines in kinases may have to be exposed to excessive reactivity to ensure covalent bond formation. There are plenty of examples of overly reactive electrophiles that despite the best of intentions, in retrospect never had a chance to make it into a safe drug. Nevertheless, these prototypical molecules definitely marked the beginning of this emerging platform.

Non-ATP Competitiveness. There is one major upside that can specifically be exploited in the kinase field. Non-ATP competitive inhibitors offer distinct advantages to conventional ATP competitive binders, especially in terms of efficacy and selectivity.^{15,16} The highly conserved nature of the ATP binding pocket poses a challenge in the identification of selective kinase inhibitors, but more important is that high intracellular ATP concentrations affect the cellular potency of ATP competitive kinase inhibitors under physiological conditions.¹⁷ Besides allosteric binders of protein kinases, blockade of the ATP pocket with an irreversible inhibitor has emerged as an attractive and alternative strategy to achieve non-ATP competitive inhibition of kinase-mediated signaling.¹⁸ As a consequence, caution should be taken with the interpretation and/or comparison of the biochemical and functional IC₅₀ values, since irreversible inhibitors usually become more potent over time. The selection of the length of preincubation time will thus influence the observed value, unless maximal inhibition is achieved instantly. The inactivation of the kinase involves two steps: formation of a reversible complex based on affinity (K_i) followed by the covalent bond formation between the inhibitor and the kinase (k_{inact}).¹⁹ The first step depends on the ATP level used in the various assays reported. The second step is primarily influenced by the inherent reactivity of the electrophilic group, as well as the distance to the nucleophilic trap. The better the juxtaposition of the reacting partners, the better the inactivation step. As the kinase is effectively removed from the equilibrium with ATP and substrate, a shift in IC₅₀ is expected and, as demonstrated in Figure 2, the inhibitor shows an increasingly non-ATP competitive character as time progresses. Consequently, this would be beneficial for protein kinases with a low $K_{\text{m,ATP}}$ in particular.

Many research groups have already embarked intentionally, or unintentionally, on the search for irreversible kinase inhibitors (partly reviewed by Garuti et al).²⁰ In some instances, these inhibitors have become invaluable assets in the target

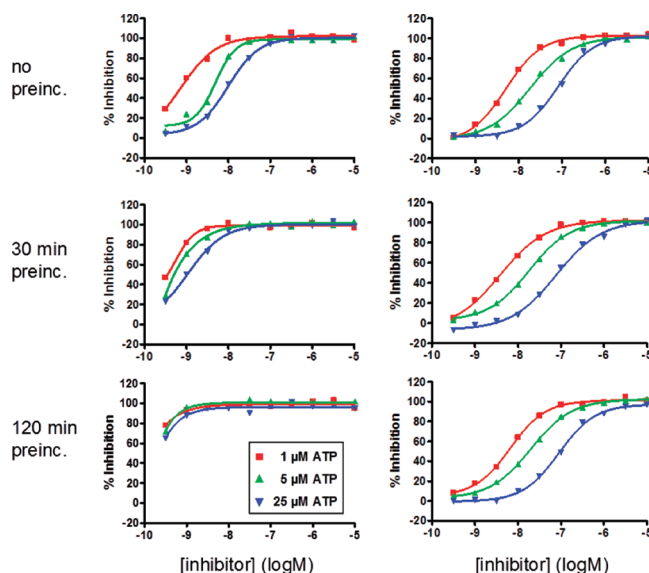


Figure 2. IC₅₀ determination depends on the ATP concentration and preincubation time for an irreversible kinase inhibitor (left), whereas an ATP competitive kinase inhibitor is only affected by ATP concentration (right). Data are from an unpublished kinase inhibitor study included for illustration purposes only.

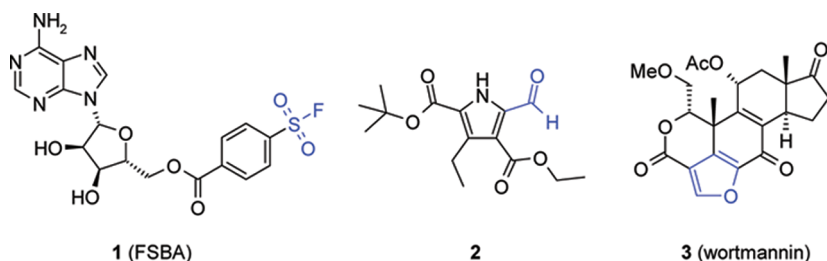


Figure 3. Examples of lysine trapping kinase inhibitors.

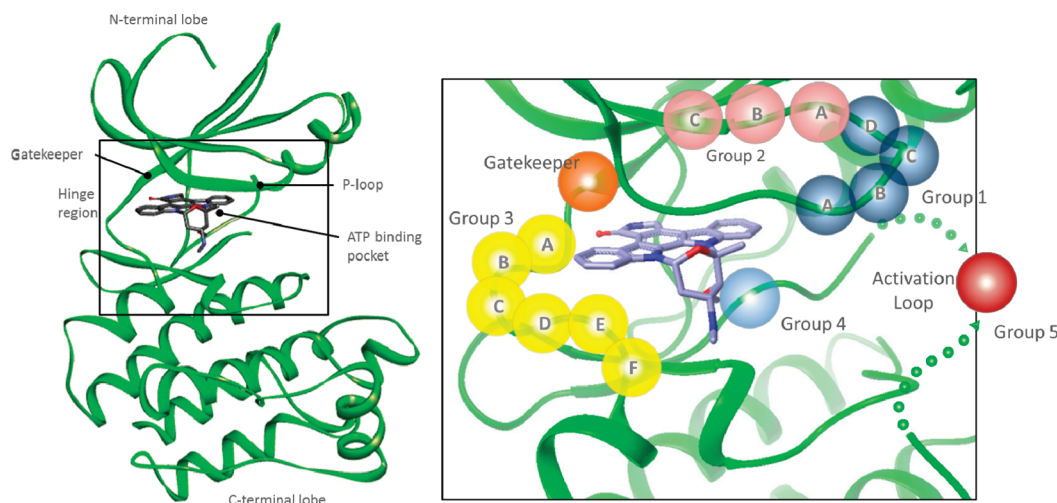


Figure 4. Schematic representation of available cysteines in or near the ATP binding pocket of kinases in the active conformation (based on the crystal structure of interleukin 2 tyrosine kinase, PDB code 1SM2). The spheres indicate the locations of cysteines that are in principle accessible for covalent modification: group 1, glycine-rich- or P-loop (dark blue); group 2, the roof of the ATP binding pocket (pink); group 3, hinge region and front pocket (yellow); group 4, DFG-motif neighboring cysteine (light blue). Group 5 (in dark red) denotes the cluster of cysteines that resides on the activation loop. The gatekeeper group is shown in orange. Adapted by permission from Macmillan Publishers Ltd.: *Nature Reviews Cancer* (<http://www.nature.com/nrc/index.html>), Copyright 2009.³⁸

validation stage. Others were deliberately designed to be the drugs of the future. The selection of examples that is reviewed in this Perspective covers both and in addition is primarily based on the diversity of cysteine locations within the known druggable binding regions of kinases. The electrophilic portions of these inhibitors (highlighted in blue in this Perspective) are either being trapped by the highly conserved lysine or cysteines at various positions of the kinases. To our knowledge, there are no reports to date that describe covalent kinase inhibitors that adduct other amino acid residues, such as serine or threonine.

Lysine-Trapping Covalent Kinase Inhibitors. Because of the well-conserved nature and its abundant presence, the lysine involved in the phosphate transfer machinery is not the most ideal candidate amino acid for trapping electrophilic inhibitors.²¹ This particular lysine resides in virtually every ATP-binding pocket of the known human kinases. Nevertheless, a couple of lysine trapping covalent inhibitors have appeared in literature. One of the first reported is ATP analogue 5'-fluorosulfonylbenzoyl adenosine (FSBA, **1**), a tool frequently used as an affinity label for kinases like EGFR,²² p38 γ ,²³ and brain PI4K (Figure 3).²⁴ As per example, Fox and co-workers used **1** for analysis of kinetic mechanisms and ATP-binding site reactivity toward p38 γ and found it to bind irreversibly to the unphosphorylated and active form.²³ Digest maps showed that Lys56 of p38 γ was selectively and covalently modified by **1**. The use of **1** has been reported in more than 200 publications, underscoring the usefulness of this particular chemical probe.

Bell and co-workers discovered a pyrrole-5-carboxaldehyde class of type 1 insulin-like growth factor receptor (IGF-1R) inhibitors with modest activity but good selectivity over a small panel of other kinases.²⁵ These aldehyde-based inhibitors were shown to bind in the ATP-binding pocket of IGF-1R in a covalent but reversible manner via imine formation between Lys1003 and the aldehyde functionality. Reduction of the imine adduct with sodium borohydride irreversibly inactivated the kinase, enabling a tryptic digest and X-ray crystallography examination. A representative example is inhibitor **2**, which demonstrated an IC_{50} of 0.49 μ M for IGF-1R.

A frequently studied lipid kinase inhibitor is represented by antiproliferative agent wortmannin (**3**, Figure 3). This PI3K inhibitor modifies the ATP pocket covalently and irreversibly via trapping of conserved Lys802 (p110 α numbering).²⁶ Analogues of **3** inhibit PI3K (reported IC_{50} values in the low nanomolar range) by virtue of an activated furan ring as a fairly unusual electrophile, which opens upon nucleophilic attack of the side chain of Lys802.²⁷ SAR studies on analogues of **3** demonstrated that omission (or modification) of this furan ring results in significant reduction in inhibitory activity. An X-ray crystallographic structure revealed the exact nature of the binding mode of **3**, and clear density was observed for the primary amine linking to the opened furane ring.²⁸ Later on, **3** was also shown to inhibit the protein kinase Plk1 (IC_{50} = 24 nM), shedding some doubt on the pharmacology that was previously attributed to PI3K inhibition.²⁹ Subsequently, it was

established that **3** also inhibits Plk3 by covalent modification of the conserved lysine in the ATP binding pocket.³⁰ In general, deliberately designed covalent drugs that target the invariant lysine in kinases are not to be expected. However, uniquely positioned lysines elsewhere in or near the ATP binding pocket may well be suitable for a covalent inhibitor approach.

Cysteine-Trapping Covalent Kinase Inhibitors. Unlike cysteines in kinases, cysteines involved in the catalytic machinery in, for instance, protein tyrosine phosphatases³¹ and cysteine proteases³² are part of very specific environments made up of neighboring amino acids and ternary architecture of these particular proteins. Part of the active site architecture is the position of these cysteines, residing frequently at the “bottom” of one or more α -helices. Backbone NH groups point downward generating a net positive charge that stabilizes the sulfhydryl anion and augments nucleophilicity.³³ The pK_a of a thiol in a typical cysteine residue is ~ 8.5 , whereas the presence of a polar or positively charged environment can decrease the pK_a up to 5 log units. In many protein families, cysteines contribute to maintaining ternary protein structures via disulfide bond formation. In the ATP-binding pocket of kinases, reduced cysteines generally do not seem overly nucleophilic and the side chain functionality may have a rudimentary role at most. There are, however, several recent reports that suggest that reaction of a cysteine thiol can regulate and control even protein kinase function, for instance, by means of redox signaling^{34,35} or S-alkylation with endogenous electrophiles.^{36,37} For this very same reason, reducing agents such as dithiothreitol (DTT) or dithioerythritol (DTE) are indispensable ingredients in biochemical assays for kinases, in order to keep the enzyme in an active and sensitive state. This also supports the notion that these cysteines are sensitive enough for electrophilic modification by designer drugs.

Recently, Zhang et al. have suggested a very useful classification system that originally binned the cysteines into four groups according to the relative positions in the ATP binding pocket (Figure 4).³⁸ It is justified to add at least two additional groups to this initial classification, involving the cysteines located at the gatekeeper position and a fifth group. Group 5 covers cysteines residing at various locations in the activation loop. Some cysteines are more unique than others, and there are at least 200 kinases that seem tractable for a covalent approach. Given the known conformational flexibility of kinases, this number may be even higher. Structural analyses of the different conformations, including the active kinase state and inactive “DFG-out” and “C-helix-out” conformations, unveil additional cysteines that could be targeted.³⁹ These cumulative analyses generate a fingerprint of the kinome active site toward cysteines, and the resulting “cystinome” is of great value for the design of covalent kinase inhibitors. There are now several examples of covalent kinase inhibitors that target this region, and in the following sections the several groups are reviewed. The order of appearance is dictated by the group numbering as indicated in Figure 4.

Group 1 Cysteines. So far, there is only one report that describes the identification of covalent kinase inhibitors targeting a cysteine on the lip of the glycine-rich loop (Figure 4). An irreversible group 1B inhibitor is represented by fibroblast growth factor receptor (FGFR) inhibitor **4** (FIIN-1, Figure 5), which was identified via a structure-based approach using available reversible FGFR inhibitors.⁴⁰ The FGFR family consists of four members, FGFR1–4 and plays an important role in tumor formation and progression as well as wound

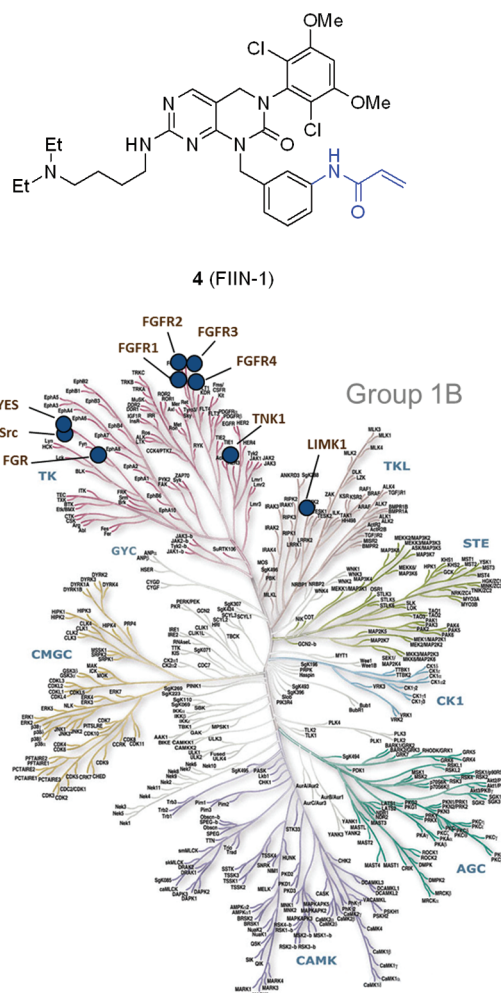


Figure 5. (Top) Chemical structure of FGFR inhibitor **4**. (Bottom) The human kinome and the group 1B cysteines. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, 298, 1912–1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).⁴²

healing.⁴¹ Compound **4** is a biochemically potent acrylamide-based inhibitor of FGFR1, FGFR2, and FGFR3 (IC_{50} values of 9.2, 6.2, and 11.9 nM, respectively) and is somewhat less potent on FGFR4 (IC_{50} = 189 nM), with decent selectivity over the other group 1B cysteine kinases, such as Src, YES, and TNK1 (Figure 5). The reversible propionyl analogue of **4** was 24- to 100-fold less potent in FGFR1 and FGFR3 harboring cells, respectively. With the aid of a biotinylated version of **4** (Figure 17), specific covalent wild type (WT) FGFR1 labeling was demonstrated in transfected HEK293 cells whereas the C486S mutant was barely modified. This is in line with the binding model that strongly suggests that **4** forms a covalent bond with FGFR1 via Cys486.

Group 2 Cysteines. Within this cluster, subgroup 2B comprises only 11 kinase members and has been the sole subject of proven covalent kinase targeting within the group 2 cysteines. Cohen et al. were the first to exploit this particular cysteine as a selectivity filter in addition to the threonine gatekeeper residue in p90 ribosomal protein S6 kinase (RSK).⁴³ The four closely related RSKs (RSK1–4) all have the P-loop cysteine, whereas RSK3 has the sterically demanding

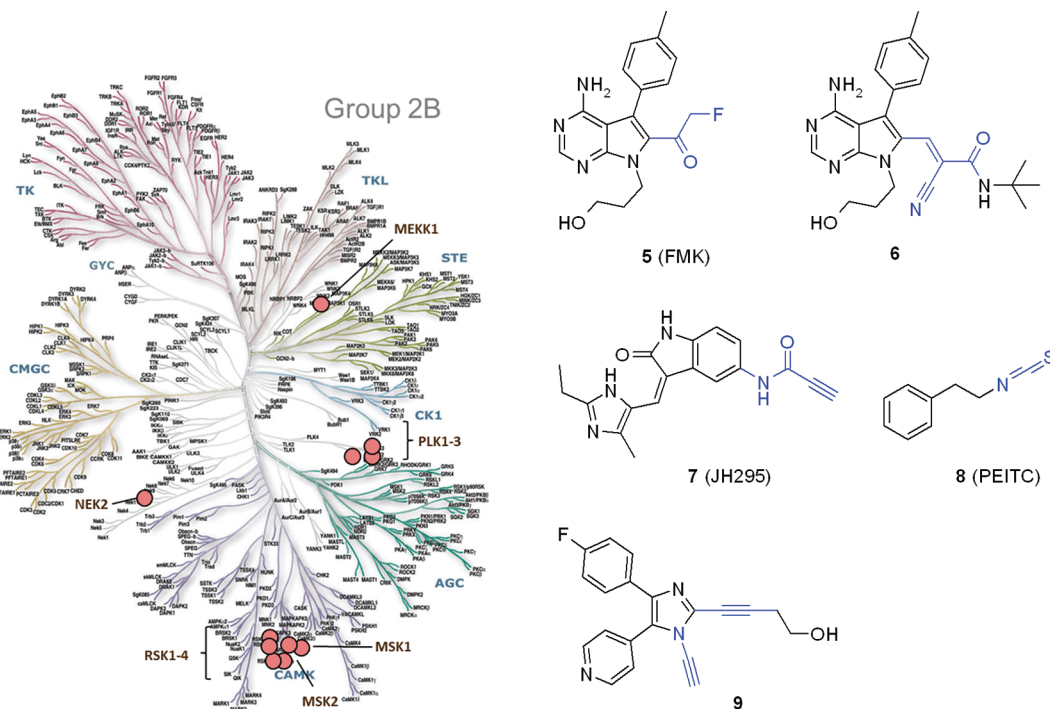


Figure 6. (Left) Human kinome and the group 2B cysteines. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, 298, 1912–1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).⁴² (Right) Chemical structures of group 2B inhibitor 5–8 and group 2C kinase inhibitor 9.

methionine as a gatekeeper as opposed to threonine in the other three RSK kinase members. This was sufficient to develop a fluoromethylketone (FMK) derivative **5** as a highly potent and selective RSK1 and RSK2 inhibitor, with an IC_{50} value against RSK2 of 15 nM (Figure 6). Selective covalent labeling of WT (but not the C436V mutant) RSK2 was demonstrated by FMK-biotin in transfected HEK293 cells. Also, Michael acceptors such as an acrylate or acrylonitrile were appended from the same pyrrolopyrimidine scaffold, yielding irreversible RSK2 inhibitors with modest biochemical IC_{50} values.⁴⁴ By contrast, the doubly activated electrophiles, as in cyanoacrylamide **6**, were 200-fold more potent (IC_{50} = 4 nM) in enzymatic assays than the monoactivated vinyl groups, yet in a fully reversible covalent binding mode. Owing to its greater reactivity, **6** demonstrated faster kinetics than **5** in cellular settings, but the duration of occupancy seemed to rely on protein resynthesis and was indistinguishable between the two inhibitors. Intriguingly, all RSK kinases also accommodate another cysteine which is located in the back of the ATP binding pocket, equivalent to Cys560 in RSK2 (group 4).⁴⁵ Potential trapping of derivatives of **5** by this cysteine was not investigated.

NEK2 is a centrosomal kinase that interferes with the spindle assembly checkpoint, affecting mitosis. It harbors a cysteine (Cys22) in an identical position as the RSK family of kinases.⁴⁶ Henise and Taunton developed a reasonably potent (IC_{50} = 770 nM) and cell permeable irreversible NEK2 inhibitor (**7**) based on an oxindole scaffold using a propynamide as the warhead. Selective covalent NEK2 inhibitors, such as **7**, are important validation assets to investigate the consequences of intracellular inactivation of NEK2 on regulating the proliferation of tumor cells overexpressing this kinase.

The same specific cysteine on the P-loop of MEKK1 was previously identified as a glutathionylation site following

oxidative stress.⁴⁷ Bioactive nutrients harbor electrophilic isothiocyanates that intervene with stress-signaling kinase pathways. Reported activities include cell cycle effects and protection of experimental carcinogenesis in animal models. The SAPK/JNK signaling pathway was investigated by means of phenylethyl isothiocyanate (PEITC) **8**, which was shown to inhibit WT MEKK1 (group 2B) but failed to intervene with the C1238V mutant in both biochemical and cellular assays.⁴⁸ It was the second covalent kinase inhibitor reported for this subgroup, and although far from being druglike, it demonstrates the potential for covalent kinase inhibitors as research tools in the exploratory biology of kinase signaling pathways.

One group 2C suspect has been reported, which is a p38 α inhibitor with moderate biochemical potency (IC_{50} = 200 nM). Dialkynylimidazole derivative **9** did covalently modify p38 α following prolonged incubation and as determined by ESI-MS.⁴⁹ The authors propose trapping via an intricate cyclization and rearrangement mechanism involving both ethynyl groups of **9**, but the exact mode of action and site of covalent binding were not confirmed. Cys39 could be adducted given its position in the roof of the ATP pocket of p38 α and the known binding mode of the imidazole class of p38 inhibitors.⁵⁰ However, the cysteine sulfhydryl group is directed away from the pocket itself, and as a consequence, this cysteine is unlikely to be involved in adduct formation.

Group 3 Cysteines. The 3F cluster of the group 3 cysteine-containing kinases has received by far the most attention because of the clinical potential of kinase inhibitors belonging to this subgroup.¹⁸ In addition, the relatively high nucleophilicity with respect to the other cysteines facilitates the reaction with all sorts of electrophilic agents. The pK_a of the sulfhydryl group of this particular cysteine is most likely lower than average because of the position at the end of a C-lobe α -helix (Figure 4). The net positive charge through the backbone NH

groups stabilizes the thiolate anion, thus making it more amenable for electrophile trapping. Depending on the sequence alignment used, there are 10 kinases with a cysteine on this position (Figure 7). The TEC family (Bmx, Btk, Itk, TEC, and Txk) and ErbB family (EGFR, ErbB2, and ErbB4) constitute the majority of this subgroup and in addition comprise Blk and JAK3.

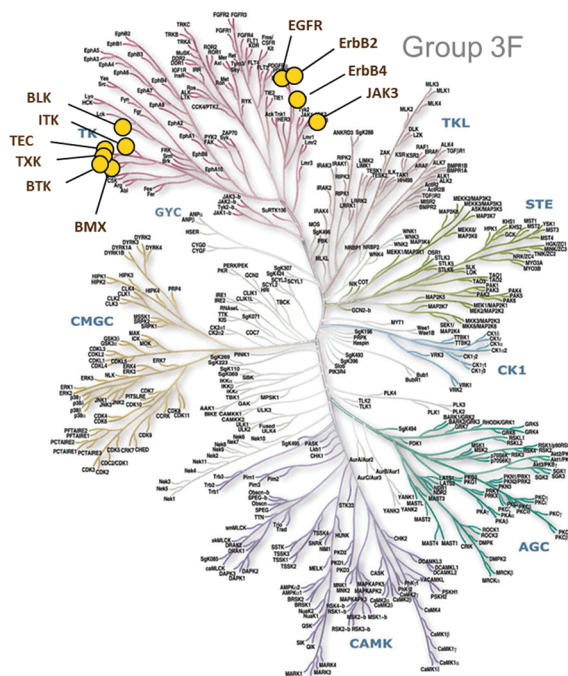


Figure 7. Human kinome and the group 3F kinases. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912–1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).⁴²

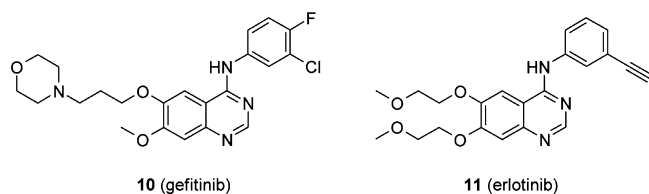


Figure 8. Reversible ATP competitive EGFR inhibitors gefitinib and erlotinib.

The ErbB family kinases were the first ones to be deliberately scrutinized via a targeted irreversible covalent approach. Reversible small molecule kinase inhibitors gefitinib (**10**) and erlotinib (**11**) have shown excellent clinical benefit, particularly in the treatment of non-small-cell lung carcinomas (NSCLC). The chemical structures of these reversible kinase inhibitors are presented in Figure 8. Unfortunately, the ability of these ATP competitive EGFR inhibitors to treat NSCLC patients effectively is short-lived partly because of acquired drug resistant mutations in EGFR which concerns roughly 50% of the treated population.^{51,52} One of these mutations involves the threonine gatekeeper, which is converted to a methionine (T790M). This prevents reversible inhibitors from binding at higher ATP concentrations.⁵³ An additional postulated

explanation is the increased internalization of EGFR as observed in drug resistant tumor cell lines.⁵⁴ Both resistance mechanisms may efficiently be circumvented by irreversible kinase inhibitors and have spurred the search for irreversible (pan) ErbB inhibiting drug candidates.

In 1997, it was demonstrated by Singh and colleagues of the Parke-Davis group that EGFR and ErbB2 could be covalently modified by an adenosine thiol analogue (**12**, Figure 9).⁵⁵ Inactivation of EGFR was shown to be time-dependent, a hallmark for covalent binding, and the binding model suggested the proximity of the 2'-thiol group of **12** to Cys797 in EGFR and to Cys805 in ErbB2. Around the same time, the highly potent and selective **13** (PD-168393) was shown to inactivate EGFR and ErbB2 in an irreversible manner by means of an acrylamide (Figure 9).⁵⁶ While the subnanomolar potency of **13** for EGFR ($IC_{50} = 0.7$ nM) was indistinguishable from the reversible propionyl counterpart, a range of data (MS, site-directed mutagenesis, ¹⁴C-labeling) demonstrated unequivocally the alkylation of Cys797. This translated into a significant advantage in a xenograft mouse model, since the duration of action had become a function of the protein turnover rate (de novo synthesis) rather than the pharmacokinetic profile of the inhibitor. Although **13** should be regarded as the prototype 4-anilinoquinazoline-based irreversible EGFR inhibitor, this particular compound did not make it into the clinic. A plethora of newer ErbB inhibitors have been identified that may display advantageous properties. One line of research focused on replacement of the acrylamide function by alternative and less reactive electrophiles, since this warhead was initially considered as too aggressive.

A nearly identical and highly potent analogue discovered by Wyeth researchers is **14** (CL-387,785, $IC_{50} = 0.37$ nM), which harbors a 2-butyramide as the Michael acceptor (Figure 10).⁵⁷ It showed potent inhibition of EGF-induced EGFR autophosphorylation in A431 cells ($IC_{50} \approx 5$ nM) and blocked tumor growth in a xenograft mouse model when given orally (80 mg kg⁻¹ day⁻¹, q.d.). This compound, also known as EKI-785, was the first irreversible EGFR inhibitor considered for further clinical development but was abandoned later because of poor bioavailability.⁵⁸ A major improvement was observed when introducing a dialkylamino group as in compound **15**. This electrophile serves both as an internal base to accelerate the reaction with the cysteine and as a water-solubilizing group.

Carmi et al. explored a variety of warheads characterized by different reaction mechanisms with respect to nucleophiles.⁵⁹ The electrophiles studied included epoxides, phenoxymethylamides, carbamates, nitriles, and (benz)isothiazolinones. Epoxide derivative **16** was the EGFR inhibitor that stood out in terms of biochemical potency ($IC_{50} = 1.2$ nM) and inhibition of EGFR autophosphorylation in cells with IC_{50} values of 7 nM (immediately following 1 h of incubation with EGF) and 44 nM (8 h after removal of inhibitor from culture medium, following 1 h of incubation with inhibitor), respectively. The hysteresis in efficacy observed is in line with covalent binding, resulting in prolonged inhibition only reversed following de novo synthesis of EGFR.

A less obvious and less reactive electrophile is the acetylene moiety on an electron-deficient thienopyrimidine scaffold as in **17** (Figure 9).⁶⁰ Covalent adduct formation of this type of pan-ErbB kinase inhibitors was demonstrated by means of X-ray crystallography and mass spectrometry. Covalent modification of EGFR with **17** was much slower compared to **22** (CI-1033, Figure 11),⁶¹ while the enzymatic IC_{50} values for EGFR, ErbB2,

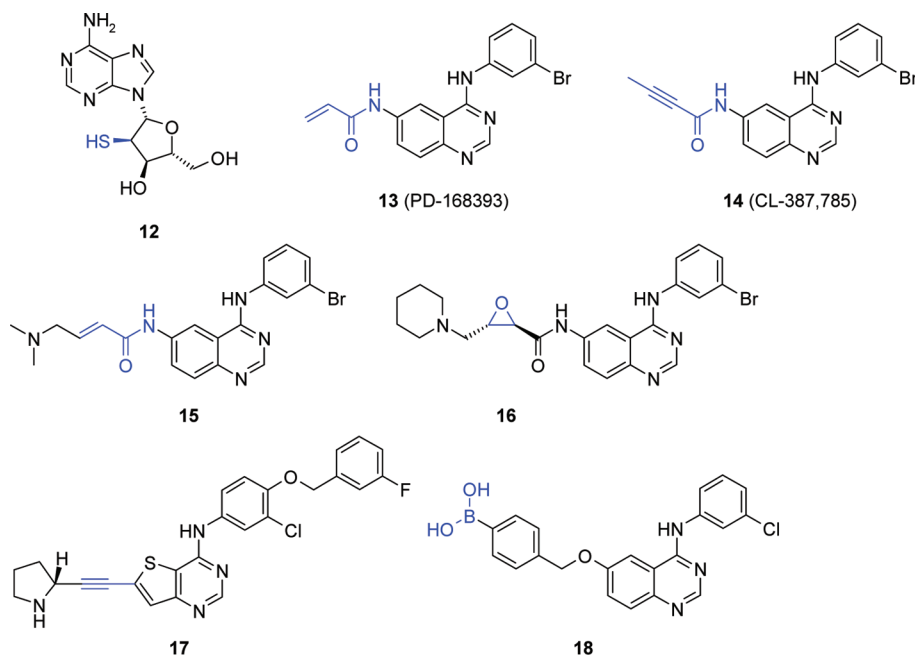


Figure 9. Group 3F irreversible ErbB family kinase inhibitors 12–18.

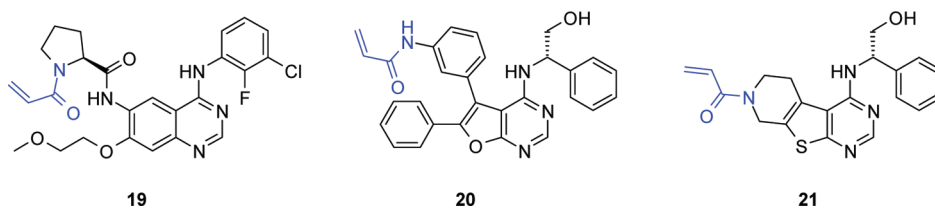


Figure 10. Group 3F irreversible ErbB family kinase inhibitors 19–21.

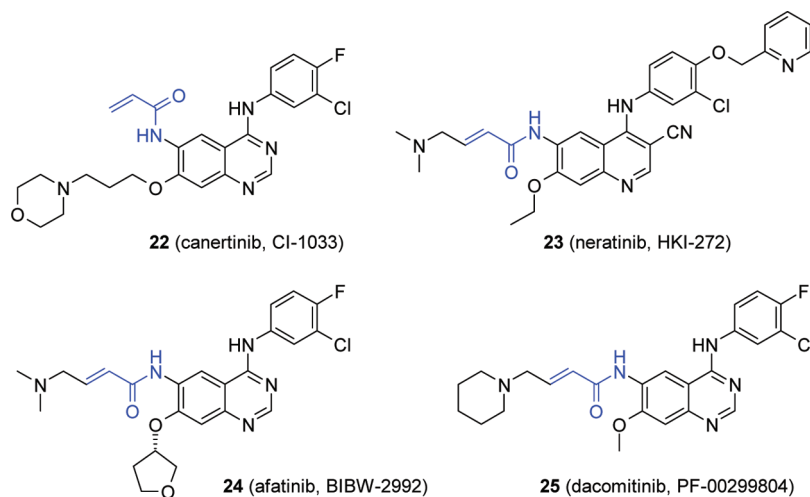


Figure 11. Irreversible pan-ErbB kinase inhibitors in clinical development.

and ErbB4 ranged from 7 to 66 nM. At doses of 10, 30, and 100 mg/kg (b.i.d.), 17 displayed a dose-dependent inhibition of tumor regression in a BT474 xenograft mouse model.

Although not extremely potent, boron-conjugated 4-anilinoquinazolines were also shown to inhibit EGFR with submicromolar IC_{50} values.⁶² Unlike the other boronic acid derivatives reported in this paper, compound 18 demonstrated irreversible inhibition, which was attributed to a covalent bond to Asp800 instead of Cys797. Unfortunately, no empirical proof

was provided to strengthen this hypothesis. Additional warheads that have been employed in the search for ErbB family kinase inhibitors have recently been extensively reviewed.⁶³

Another line of focus is optimization of the nature and size of the linker between the 4-anilinoquinazoline scaffold and the warhead, with the aim to fine-tune warhead orientation. Cha et al. partially locked the orientation of the acrylamide with a conformationally rigid L-proline, arriving at 19 as a potent and

Table 2. Late Stage Clinical Development Irreversible ErbB Kinase Inhibitors

originator	compd	enzyme IC ₅₀ (nM)			stage of development ^a	ref
		EGFR	ErbB2	ErbB4		
ParkeDavis ^b	22, canertinib	1.5	5	10	Ph 2, discontinued	61
Wyeth ^b	23, neratinib	92	59		Ph 3 (breast cancer)	74
Boehringer Ingelheim	24, afatinib	0.5	14		Ph 3 (NSCLC, breast neoplasms, squamous cell carcinoma head and neck)	71
Pfizer	25, dacomitinib	6	46	74	Ph 3 (NSCLC)	72

^aSource: Thomson Reuters and ref 67. ^bNow Pfizer.

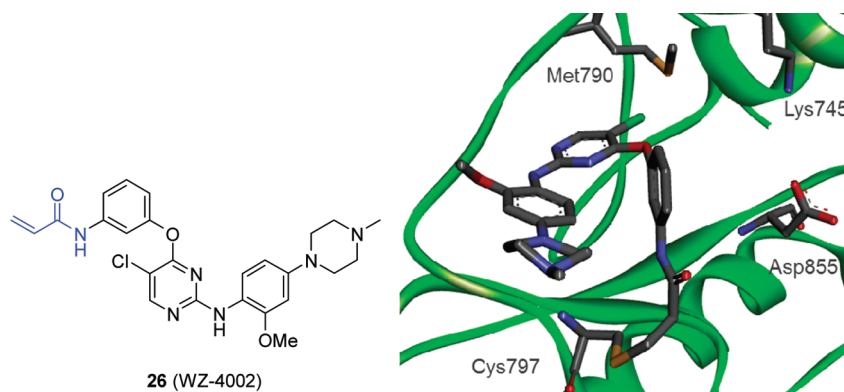


Figure 12. Crystal structure of EGFR T790M covalently bound to 26 (3IKA.pdb; P-loop omitted for clarity).

selective dual inhibitor of EGFR and ErbB2 with IC₅₀ values of 13 and 42 nM, respectively (Figure 10).⁶⁴ Its favorable cellular potency (IC₅₀ = 8 nM) and pharmacokinetic profile translated to robust reductions of tumor size in the A431 xenograft mouse model at 3 mg kg⁻¹ day⁻¹ (po).

The use of completely new scaffolds also has led to the discovery of irreversible ErbB family kinase inhibitors, as was demonstrated by Coumar et al., who identified furanopyrimidine derivative 20.⁶⁵ Docking studies of its des-acrylamido hit (IC₅₀ = 223 nM on EGFR) suggested the optimal position of the warhead. This led to a 30-fold improvement of the biochemical potency to 7 nM for 20, along with potent antiproliferative activity in the HCC827 lung cancer cell line. The same group took a hybrid approach with another micromolar tetrahydrobenzothienopyrimidine hit, eventually resulting in 21.⁶⁶ This EGFR inhibitor showed an IC₅₀ of 8 nM biochemically, 3 nM toward inhibition of EGFR autophosphorylation in the HCC827 cell line, and again regression of tumor size in vivo in mice following 5–15 mg kg⁻¹ day⁻¹ iv dosing.

Since the first reports of covalent irreversible ErbB family kinase inhibitors in the late 1990s, a number of these inhibitors have progressed to late stage clinical development.⁶⁷ The irreversible inhibitors fall into two related structural categories: 4-anilinoquinazolines and the reminiscent 4-anilino-3-cyanoquinolines. The chemical structures of the most prominent compounds are depicted in Figure 11, and data associated with these compounds are shown in Table 2. So far, these candidates seem to live up to the potential upsides, as prolonged inhibition of both the WT enzymes and primary resistance mutants has been demonstrated preclinically. However, it remains to be established whether the effectiveness observed for all of these agents in vitro against, for instance, the EGFR T790M mutants persists at concentrations realistic for successful clinical use.⁶⁸ Dose-limiting toxicity for neratinib (23, HKI-272) may have prevented progression toward NSCLC as a primary indication⁶⁹ but is currently in phase 3 clinical trials for ErbB2-positive breast cancer.⁷⁰ The most advanced clinical trials for

afatinib (24, BIBW-2992) concern several cancer indications, as a single agent or in combination therapy.^{67,71} A phase 3 clinical trial of dacomitinib (25, PF-00299804) is ongoing for NSCLC, and the study is expected to be completed in 2013.^{72,73} Interestingly, all research groups independently of each other selected candidates that are decorated with a dialkylamino substituted crotonamide warhead. Although ω -alkyl substitution of the acrylamide normally is detrimental to reactivity, introduction of the amine function offsets this loss by deprotonation of the sulfhydryl group (vide infra). In addition, solubility is favorably impacted, and the volume of distribution is high because of the lower intracellular pH, presumably stimulating uptake in the target tissues.

With the observed on-target related adverse effects (skin rash and diarrhea) for EGFR inhibitors, development of EGFR mutant selective inhibitors for relapsed NSCLC due to mutated EGFR could show great promise. These mutant-selective agents may be as efficacious but would be better tolerated when WT EGFR is not affected. As many of the irreversible ErbB family kinase inhibitors reported herein have dual activity toward both the EGFR wild-type and T790M or T790M/L858R mutants, truly effective inhibition of the latter two are usually hampered by a large back-pocket substituent that clashes with the sterically demanding gatekeeper methionine. The first mutant selective EGFR inhibitors that are based on entirely new chemical scaffolds have recently appeared in the literature.⁷⁵ To this end, covalent pyrimidine inhibitors were identified by screening, and 26 (WZ-4002) was extracted as the best inhibitor in terms of affinity with a K_d of 0.13 μ M for EGFR L858R/T90M and about 100-fold less for WT EGFR (Figure 12). The overall selectivity provides a good starting point for further optimization, but there is clearly room for improvement.⁷⁶ Nevertheless, 26 is less potent in vitro and in vivo against WT EGFR than irreversible quin(az)oline-based inhibitors and 30- to 100-fold more potent against EGFR T790M. 26 is efficacious in murine models of T790M-containing cancers, as was shown by reduced tumor volumes

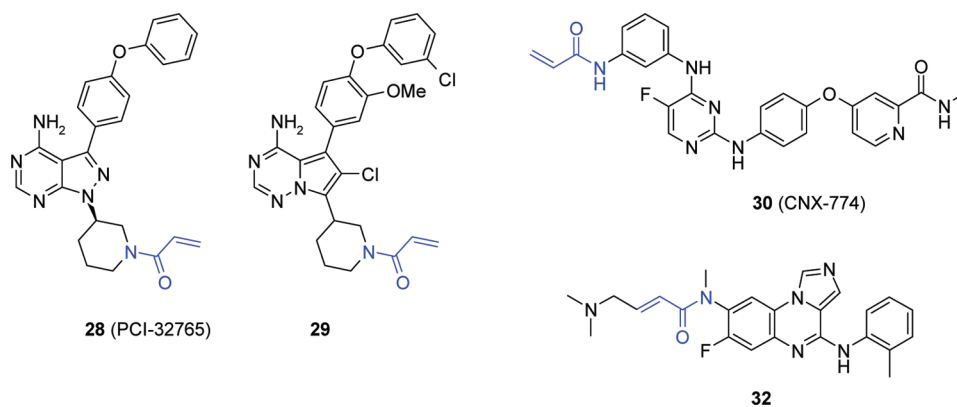


Figure 13. Chemical structures of group 3F irreversible Btk inhibitors 28–32.

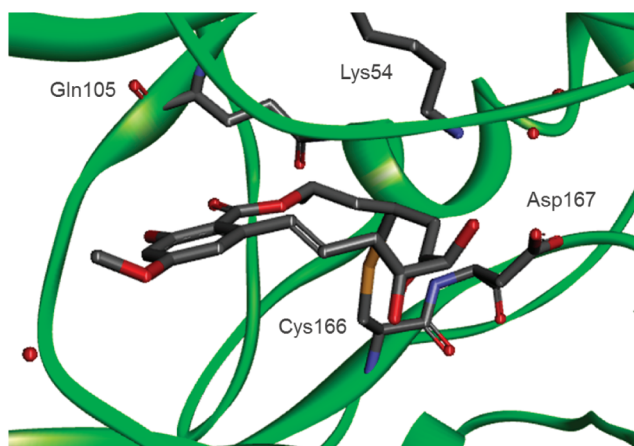
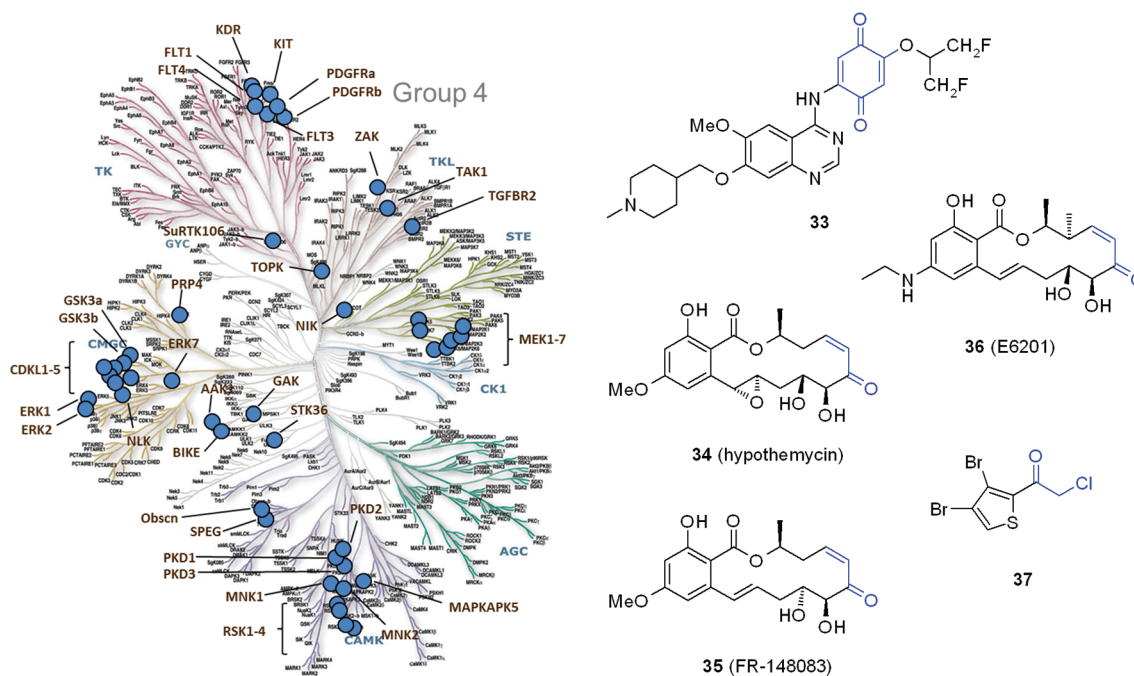


Figure 14. (Top-Left) Human kinome and the group 4 kinases. Adapted illustration reproduced with permission from AAAS (Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, 298, 1912–1934) and courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com).⁴² (Top-Right) Chemical structures of selected irreversible group 4 kinase inhibitors 33–37. (Bottom) Compound 35 bound in the active site of Erk2 (2E14.pdb).

following a daily oral dose of 25 mg/kg.⁷⁵ Next to the binding mode, covalent binding to the Cys797 of EGFR T790M was confirmed via X-ray crystallography. Elucidation of the structure also revealed that **26** binds the active conformation of the kinase, as opposed to **23**, which binds the inactive form. One other mutant-selective inhibitor (**27**, CO-1686) has been described in a joint effort by Avila Therapeutics and Clovis Oncology, but this structure has not been disclosed.⁷⁷ **27** inhibits cell proliferation of cell lines expressing mutant EGFR T790M and EGFR signaling in, for instance, L858R/T790M expressing H1975 cells with an IC_{50} of 62 nM while sparing WT EGFR. Dose-dependent tumor regression in the H1975 and HCC827 xenograft models was achieved with complete inhibition at the highest dose (100 mg kg⁻¹ day⁻¹, po), and **27** has recently been progressed to the clinic for the NSCLC indication. These mutant-selective irreversible inhibitors can be considered the third generation EGFR inhibitors, after the first (reversible, ATP competitive) and second (irreversible) generation WT EGFR inhibitors.

The second kinase of the subgroup 3F that received considerable interest is Bruton's tyrosine kinase (Btk). Btk is a key kinase in the signaling pathway activated via the B-cell receptor (BCR), regulating B-cell proliferation and activation. Inhibition of Btk shows great potential in the treatment of B-cell malignancies and autoimmune diseases. Celera researchers were the first to report on irreversible (Btk) inhibitors, including ibrutinib (**28**, PCI-32765, Figure 13) that was subsequently taken to clinical development by Pharmacyclics.⁷⁸ Compound **28** is a potent and fairly selective Btk inhibitor with an IC_{50} of 0.5 nM by virtue of covalent binding to Cys481. It was efficacious in collagen-induced arthritis (CIA) and lupus models in mice and in spontaneous canine B-cell lymphomas.⁷⁹ Phase 2 clinical studies in non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) have been concluded successfully for this Btk inhibitor, showing an excellent response rate in CLL patients and in subtypes of NHL.⁸⁰ The reminiscent covalent Btk inhibitor **29** was disclosed recently, displaying an IC_{50} of 2 nM for Btk.⁸¹

CNX-774 (**30**) from Avila Therapeutics also targets Cys481 and is a potent Btk inhibitor ($IC_{50} < 1$ nM) of yet another chemotype.⁸² Btk activity in Ramos cells was strongly inhibited with an IC_{50} of 1–10 nM, and **30** has demonstrated efficacy in a CIA mouse model at 60 mg kg⁻¹ day⁻¹. However, the in vivo target occupancy reached only ~70% as determined via a chemical probe method. Avila Therapeutics continued activities on a covalent Btk inhibitor, resulting in the development of the more potent compound **31** (AVL-292, structure not disclosed).⁸³ **31** potently inhibits Btk in biochemical ($IC_{50} < 0.5$ nM) and cellular assays ($EC_{50} = 1$ –10 nM), including α -IgM stimulation of B-cell proliferation and B-cell activation. The overall favorable efficacy and selectivity profile warranted clinical development, and early observations from a phase 1b clinical study suggest that **31** is safe and well tolerated in NHL and CLL patients.⁸⁴

Pfizer has reported on imidazoquinoxaline **32** and derivatives acting as irreversible Btk inhibitors, emphasizing its interest in this area.⁸⁵ Compound **32** combined good biochemical Btk potency ($IC_{50} = 1.93$ nM; no preincubation) with selectivity over the Src family kinase members albeit not over ErbB family member EGFR. The nanomolar inhibition of B-cell proliferation ($IC_{50} = 3.41$ nM) in combination with favorable pharmacokinetic properties led to dose-dependent efficacy in

the mouse CIA model following 0.3–10 mg/kg via oral route dosing.

Group 4 Cysteines. Although this cluster is confined to a single cysteine adjacent to the DFG-motif and near the bottom of the ATP binding pocket, nearly 10% of all known kinases (48) have this particular cysteine at this very position (Figure 14). Intuitively one would guess that in this case selectivity is a major issue to resolve. However, the fact that the kinases involved are clustered in eight or nine evolutionary families may clearly present options for additional selectivity filters.

After the success of bringing the irreversible EGFR/ERbB2 inhibitors to the clinic, Wissner et al. also explored the 4-anilinoquinazoline scaffold for covalent binding with Cys1045 in KDR (VEGFR-2).⁸⁶ In their binding model, the aniline portion was located near the cysteine, and for this reason it was replaced by a reactive quinone group. Inhibitors were screened for KDR inhibition with and without GSH to ascertain a sulfhydryl reaction confined to the kinase of interest and at high and low ATP concentrations to confirm the non-ATP competitive nature. Compound **33** emerged as an attractive irreversible inhibitor with an IC_{50} of 5 nM along with good selectivity and in vivo efficacy in a mouse xenograft model (Figure 14). Although only a small panel of deselection kinases was used, other group 4 member kinases, such as GSK and MEK1, were reportedly not inhibited. Even though caution should be taken (many more group 4 kinases were not included), this suggests that it is feasible to induce selectivity even within this large cluster of kinases, as long as the chemical scaffold is appropriately chosen.

Resorcylic acid lactones (RAL) that contain a *cis*-enone Michael acceptor were shown to be irreversible inhibitors of most of the group 4 kinases. Hypothemycin (**34**) turned out to be rather unselective within this cluster, inhibiting 18 out of 19 tested kinases containing this conserved cysteine.⁸⁷ A tryptic digest confirmed covalent binding to Erk2 but did not yet dismiss the other electrophilic functionality, the epoxide, as the reacting warhead. To this end, the crystal structure of the Erk2–hypothemycin complex provided proof that a covalent bond with Cys166 of Erk2 was formed with the *cis*-enone moiety.⁸⁸ Around the same time, another group reported on a small set of hypothemycin analogues, exemplified by **35** (FR-148083).⁸⁹ In analogy with **34**, X-ray crystallography showed the covalent interaction of **35** with Erk2 (Figure 14). Next to Erk2 ($IC_{50} = 80$ nM), the MAP2K kinases MEK1 ($IC_{50} = 6$ nM) and MKK7 (MEK7; $IC_{50} = 300$ nM) were substantially inhibited, whereas MKK4 (MEK4) showed $IC_{50} > 3$ μ M. No other kinases of this cluster were included in the selectivity profiling. The related analogue **36** (E6201) was evaluated for its anti-inflammatory and antihyperproliferative potential, reportedly owing to the inhibition of MEK1 ($IC_{50} = 5.2$ nM) as well as several other MAP2K kinases and the upstream group 2B kinase MEKK1 ($IC_{50} = 31$ nM).⁹⁰ Reduction of the *cis*-enone double bond was detrimental to cellular activity, and introduction of the γ -methyl was necessary to enhance metabolic stability. In animal models of dermatitis, compound **36** was active following topical administration⁹¹ and is currently in clinical trials as a topical formulation for psoriasis.⁶⁷ *cis*-Enone harboring RAL derivatives remain an intriguing class of irreversible kinase inhibitors that in some instances have shown very potent in vivo effects in preclinical oncology models,⁹² but highly selective inhibitors are usually not obtained. Other very similar RAL analogues appeared almost a decade earlier as fairly potent pan-MEK or TAK-1 inhibitors, which is not surprising

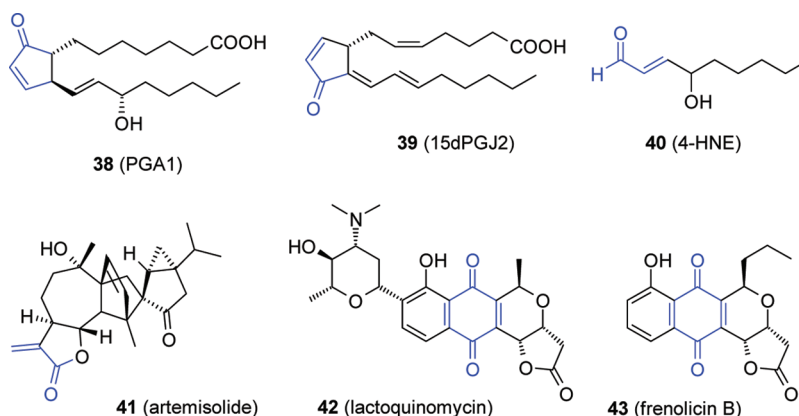


Figure 15. Irreversible group 5 kinase inhibitors 38–43.

given their incorporated α,β -unsaturated ketone moiety and the fact that the kinase involved belongs to group 4.⁹³ Nevertheless, the potential for irreversible binding was not interrogated at the time.

The latest addition of irreversible kinase inhibitors in this cluster of kinases are thienyl halomethyl ketones.⁹⁴ Thiophene derivative 37 displayed a biochemical IC_{50} of 0.5 μM for GSK3 β , representing the first reported irreversible inhibitors for this kinase to date. Although moderately potent and far from being druglike, these agents can be considered as fragments that can be optimized for affinity toward GSK3 α and/or GSK3 β .

Group 5 Cysteines. The location of this cluster is outside the ATP binding pocket and on the activation loop (regulatory or T-loop) of various kinases (Figure 4). It is not within the scope of this Perspective to present a thorough analysis of all potential cysteines via alignment of the kinase sequences. The flexible nature of the activation loops with respect to its length, but also the potential position of the cysteines, hampers a proper analysis. Therefore, it is practical to put all these cysteines in one group. In addition, because of flexibility of the activation loop, it is difficult to target these cysteines deliberately via a structure-based drug design approach, since 3D information for this region is scarce. Density with sufficient resolution is sometimes observed for activation loops but often not visible in crystal structures of kinases. On the other hand, it will probably be only a matter of time before the research community will identify and disclose meaningful ways of visualizing this structural “blind spot” for the majority of the kinases.

Group 5 is an interesting cluster because Mother Nature already seems to incorporate activation loop cysteines in her kinase signaling pathways. For instance, some of the effects of naturally occurring ligands have been tied to allosteric covalent modification of activation loop cysteines. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IKK α/β , and they do so by covalent binding through a cysteine at position 179 within the activation loop.⁹⁵ Several lines of evidence were generated. First, the inhibitory effect was completely insensitive to high concentrations (1 mM) of ATP in a biochemical setting, which emphasized the non-ATP competitiveness, albeit not the site of covalent binding per se. Interestingly, both IKKs also have a cysteine located in the ATP binding pocket belonging to group 3B.³⁸ Second, comparing inhibitory responses of both PGA1 (38) and 15dPGJ2 (39) on WT IKK β and the C179A mutant in vitro confirmed the probable site of binding (Figure 15). The WT kinase was fully

inhibited, whereas the mutant was not, yet both isoforms were equally responsive to the same stimuli. In line with these findings, the saturated analogous compounds cyclopentanone and cyclopentene derivatives failed to intervene with NF- κB activity.⁹⁶ The micromolar concentrations of cyclopentenone derivatives needed for inhibition of IKK β have been detected at the site of inflammation, and so it can be inferred that inhibition of the NF- κB transcriptional activity is at least partly attributable to this covalent binding mechanism. Another endogenous degradation product, 4-hydroxy-2-nonenal (4-HNE, 40), was also shown to block the NF- κB pathway.⁹⁷ In yet another study, artemisolid (41) was presented as an inhibitor of IKK β by means of Cys179 targeting.⁹⁸ The common denominator in all these agents is the α,β -unsaturated ketone functionality, which can act as a Michael acceptor. Interestingly, the kinase LKB1 is a kinase with a nucleophilic Cys210 on the activation loop that aligns nicely with Cys179 in IKK α/β and is also prone to inhibition by some of these reactive lipids, such as 38 and 40.⁹⁹

The kinase Akt1 accommodates two cysteines on the activation loop (Cys296 and Cys310) and is sensitive to inhibition by pyranonaphthoquinone derivatives, such as lactoquinomycin (42, IC_{50} = 149 nM) and frenolicin B (43, IC_{50} = 313 nM).¹⁰⁰ Because of the redox sensitive nature of the cysteines involved, the authors speculated righteously that the cysteines could be covalently adducted by these pyranonaphthoquinone-containing inhibitors. First, preincubation with cysteine led to ~60-fold loss of potency for the inhibitors, and second, both the C296A and C310A mutants were less sensitive to pyranonaphthoquinone derivatives. In contrast, staurosporine inhibited the WT and mutant kinases equally well.

Although the activation loop has not yet been thoroughly addressed, from these collective data it can be inferred that covalent trapping in this region shows potential for the future. Especially when the ATP binding pocket is highly conserved within a kinase family, inactivation via covalent targeting specific activation loop cysteines may be an attractive alternative.

Miscellaneous Cysteines. Beyond the kinase regions discussed so far, a first example of trapping a cysteine located on the catalytic loop has also been reported. While analyzing additional cysteines that are in the vicinity of the ATP binding pocket, taking into account the inactive “DFG-out” and “C-helix-out” conformations of protein kinases, the Winssinger group came across a cysteine located at the beginning of the

catalytic loop of c-KIT and PDGFR.³⁹ BCR-Abl inhibitor imatinib (**44**), which also blocks c-KIT and PDGFR via a type II allosteric binding conformation, served as the chemical starting point (Figure 16). The cysteine involved (Cys788 and

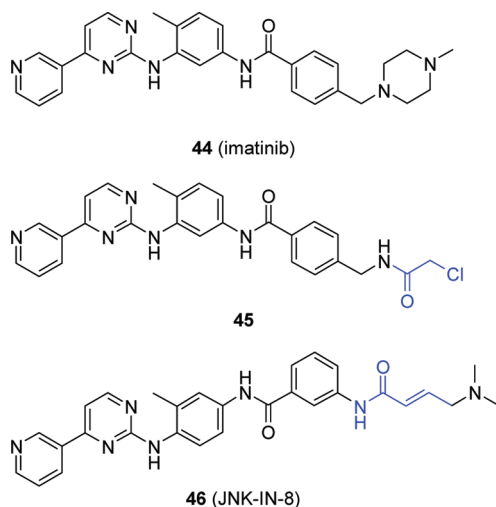


Figure 16. Transformation of imatinib into an irreversible c-KIT/PDGFR inhibitor **45** and JNK inhibitor **46**.

Cys814 in c-KIT and PDGFR α , respectively) was targeted by exchanging the *N*-methylpiperazine moiety for electrophilic groups. The resulting chloroacetamide derivative **45** was subsequently shown to adduct c-KIT and PDGFR covalently on the envisaged cysteine as shown in MS/MS experiments. The biochemical activity for the two kinases did not improve compared to **44**, but some advantage in terms of biochemical selectivity was achieved. Also, **45** lost some affinity for BCR-Abl, the prime target for **44**, which has a phenylalanine in the corresponding cysteine position. A serendipitous finding by Gray and co-workers with near identical compounds yielded potent and selective covalent inhibitors of JNK1, JNK2, and JNK3.¹⁰¹ While working on covalent inhibitors for c-KIT, they observed significant inhibition of JNK. In particular, incorporation of 1,4-dianilino and 1,3-benzamide motifs, such as in **46**, yielded efficient and specific electrophilic inhibitors. The phenylaminopyrimidine analogues trapped Cys116 in JNK1 and JNK2, and equivalent Cys154 in JNK3, via a type I binding conformation, as was demonstrated via cocrystal structures with JNK3. The fairly unique cysteine in JNK1, JNK2, and JNK3 resides just outside the ATP pocket on the C-terminal domain α -helix near the group 3F position (Figure 4). With enzymatic low nanomolar IC₅₀ values for JNK1 (4.7 nM), JNK2 (18.7 nM), and JNK3 (0.98 nM) and selective cellular inhibition of c-Jun phosphorylation, **46** is among the JNK inhibitors that are well suited to further study of JNK-mediated intracellular signaling.

CHEMICAL REPORTER PROBES FOR PROTEIN KINASES

Highly efficacious and selective kinase inhibitors are extremely useful in *in vitro* or *in vivo* validation settings, especially when the kinase involved is blocked irreversibly. First, the inhibition observed is very often time-dependent, which is one of the hallmarks of covalent and irreversible binding. This helps to delineate the pathway involved, and the markers investigated are easier to tie to the kinase under scrutiny. Second and

perhaps more importantly, appropriate decoration of a selective inhibitor with a linker-substituted label renders a very powerful chemical probe (also termed activity-based probe) to study intracellular phenomena (comparative application).¹⁰² Also, such a selective chemical probe can aid in the determination of target occupancy of the (pre)clinical candidate irreversible inhibitors (competitive application).

Several compelling examples have been reported for kinases underlining the strength of this platform, and many irreversible kinase inhibitors reviewed herein have been successfully transformed into such useful chemical probes. The irreversible inhibitors can be biotinylated to study *in vitro* or *in vivo* selectivity via (strept)avidin enrichment or (once the functional selectivity has been established) appended with a fluorescent tag to support visualization of intracellular processes (Figure 17, see insert).

One of the challenges for designing appropriate probes is the site of attachment of the linker on the core scaffold. Ideally, this position does not interfere with the potency that was obtained with the original parent inhibitor. To this end, Blair and co-workers decided to functionalize the quinazoline C7 position of **13** while searching for a selective EGFR affinity probe.¹⁰³ Both the PEG linker and the NBD fluorophore are solvent exposed, and the chance of disrupting the potency is minimal. The resulting fluorescent probe (**47**) was used in this case used to quantitate the relationship between EGFR stimulation and downstream kinases in living cells.

For some of the validation work with respect to covalent FGFR inhibition, FIIN-1-biotin (**48**) was utilized (*vide infra*).⁴⁰ In the design, the authors were allowed to elongate the acrylamide of parent **4** with a dialkylaminomethylene moiety, since the resulting reactivity is normally in the range of the unsubstituted warhead (Figure 17). Also in this case, the cellular EC₅₀ was comparable to that of parent inhibitor **4**.

In some instances, the derived chemical probes fail to present the necessary properties like permeability. For this reason, a biotinylated FMK derivative was inappropriate for targeting RSK1 and RSK2 in intact cells, and also FMK-BODIPY (**49**) showed modest cellular activity (Figure 17).⁴³ This was very elegantly solved by Cohen et al., who incorporated the clickable propargyl linker allowing for postlabeling intracellular conjugation with an azide-labeled biotin or reporter molecule (biotin-azide or TAMRA-azide).¹⁰⁴ On this improved propargyl amine version of FMK (FMK-PA, **50**), the vector of the tethered reporter tag is extending from pyrrolopyridine portion into the ribose pocket and toward the solvent. The application of this highly versatile *in situ* click conjugation concept contributes significantly to the power of irreversible kinase inhibition with respect to validation of specific target kinases in signaling pathways.

To specifically profile members of the PI3-kinase family, several activity-based probes have been developed based on wortmannin as a scaffold.¹⁰⁵ As per example, a wortmannin-BODIPY probe (structure not shown) turned out to be cell permeable allowing for labeling of proteins in intact living cells.¹⁰⁶ The additional finding by Liu et al. that **3** also targets the conserved lysine of protein kinases Plk1 and Plk3 via covalent binding can in part be attributed to the application of TAMRA-based probe of **3** (**51**, AX-7503) as shown in Figure 17.^{29,30}

The same group reported the utility of a selective PKC α/β probe via a slightly unorthodox design angle.¹⁰⁷ It was different, because a cysteine located just outside the ATP pocket of

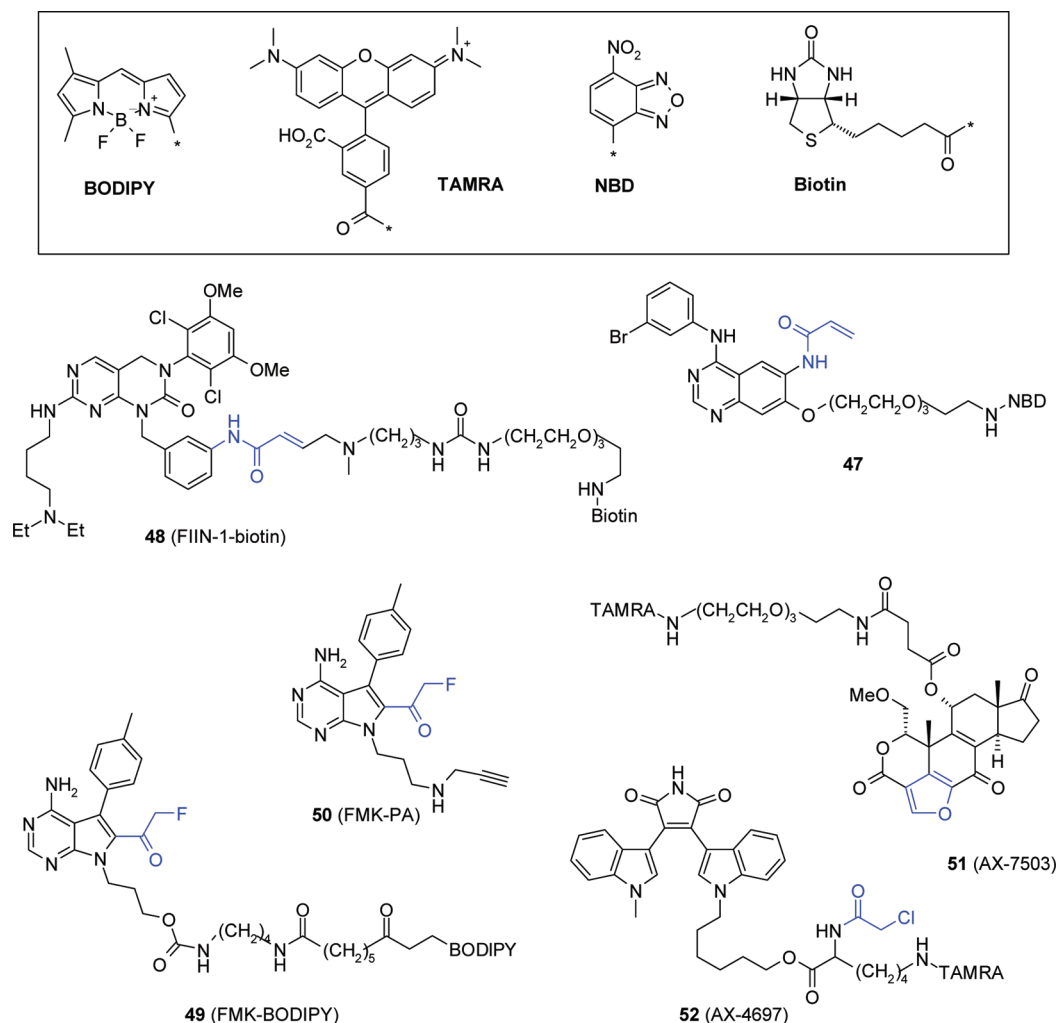


Figure 17. Examples of chemical probes based on irreversible protein kinase inhibitors. TAMRA = tetramethylrhodamine. NBD = nitrobenzoxadiazole. BODIPY = boron dipyrromethene.

PKC α (C619) and PKC β (C622) was targeted for covalent trapping. On the well-known PKC inhibitor bisindolylmaleimide core, a chloroacetamide warhead was incorporated on the TAMRA-tagged linker reaching out to the cysteine mentioned. The resulting exo-affinity probe **52** (AX-4697) provided a sensitive and permeable tool for further examination of the consequences of pharmacological intervention in PKC-mediated biology.

The determination of target occupancy has been an integral part of the (pre)clinical development of irreversible Btk inhibitors **31** and **28** (Figure 13). Honigberg et al. created the cell-permeable, fluorescently labeled derivative PCI-33380 (**53**, Figure 18) of their clinical candidate.⁷⁹ The level of Btk target occupancy by **28** was determined using **53**, and this occupancy was shown to tightly correlate with the inhibition of BCR signaling and in vivo efficacy in mice and dogs. In the clinic, the same fluorescent probe was used to correlate target occupancy and pharmacodynamics effects in cancer patients.⁸⁰ To this end, Avila Therapeutics assessed ex vivo Btk occupancy with a biotinylated covalent probe and quantitated by ELISA.⁸⁴

These probes provide excellent biomarkers, allowing efficacy and target occupancy to be linked in with the analysis of pharmacodynamics. Another upside for the utilization of probes is that it aids the selection of the highest relevant dose in the patient. In oncology clinical trials, patients are treated with

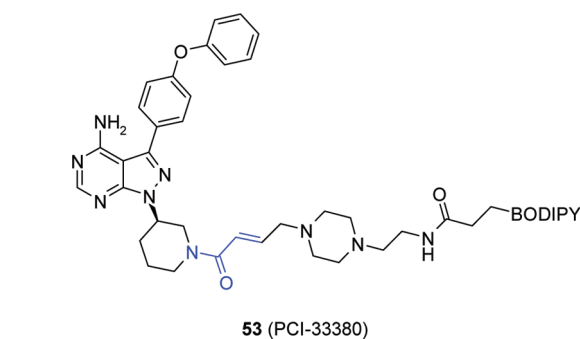


Figure 18. Chemical structure of the BODIPY-derived Btk chemical probe **53**.

increasing doses of the drug until dose limiting toxicity is observed. For a covalent drug, a modest excess of exposure with respect to the dose where full target occupancy is observed should be more than sufficient.

Clearly, one should be able to design and synthesize suitable probes for diagnosis and target occupancy purposes with modest efforts. Covalent kinase inhibitor probes should constitute an integral part of kinase research, especially when the identification and development of irreversible inhibitors come into play.

CHEMICAL GENETIC ENGINEERING

The physical evidence of irreversible inhibition, and the site of modification on the WT kinase, is frequently corroborated with genetically engineered kinase mutants. To be specific, the naturally occurring nucleophilic amino acid residue is replaced by a nonreactive isoster that is not interfering with the functional activity of the kinase. Hence, the relative contribution of covalent binding to the WT kinase inhibition can be studied. Some research groups have exploited this genetic technique by reversing this concept and introduced a nucleophilic anchor point for irreversible binding that was previously not in the active site. Mutation of strategically positioned key residues into a cysteine creates an engineered pocket that allows unparalleled specificity, especially in combination with additional selectivity filters (often a mutated gatekeeper residue). SAR that has previously been developed for WT kinases, bearing a cysteine in the same position, can now be efficiently exploited for the mutant kinase via selective irreversible inhibition. The groups of Shokat and Rauh have been at the forefront of this so-called chemical genetic engineering platform. In one case, c-Src was subjected to two point mutations in the ATP binding pocket, gatekeeper T338G and S345C at the group 3F position.¹⁰³ The latter position is homologous to Cys797 in EGFR, and c-Src-dm has become extremely sensitive to irreversible prototype EGFR inhibitor **13** (Figure 9) and analogues. The mutant IC₅₀ values ranged from 1 to 250 nM for these analogues, as opposed to >27 μ M for WT c-Src.

Similarly, two selectivity filters were applied on fission yeast aurora (Ark1) in order to tailor the kinase for covalent trapping by these quinazoline derivatives.¹⁰⁸ The first mutant filter at the gatekeeper position, L166A, allowed better accommodation of the aniline portion of analogues of **13**, and the second E173C mutation provided the anchor point for covalent modification. The electrophilic inhibitors efficiently inhibited the newly engineered mutant Ark1 in the low nanomolar range, and both selectivity filters were necessary because neither the WT kinase nor the mono E173C mutant was inhibited biochemically (IC₅₀ > 20 μ M). Subsequently, "analogue-sensitive" Ark1-mediated signaling in recombinant cells was efficiently and irreversibly blocked by the propynamide derivative of **13** while no detectable toxicity was observed.

This covalent complementarity approach was taken one step further by mutating the gatekeeper residue into a unique cysteine.¹⁰⁹ This cysteine has not been previously targeted for covalent binding. Only two human protein kinases (MOK and SgK494) have this endogenous cysteine gatekeeper residue which corresponds with the orange sphere in Figure 4. Thus, cysteine gatekeeper engineering in theory allows electrophilic inhibitor discovery for most kinases, as long as the mutant kinase activity is not impaired. Again, the concept was validated employing c-Src T338C in combination with warhead substituted 3-benzylpyrazolopyrimidines, such as **54**, and 4-anilinoquinazolines (Figure 19). Compound **54** showed a time-dependent inhibition of T338C c-Src and displayed an IC₅₀ of 114 nM (versus >5 μ M for WT c-Src). MOK inhibition was hardly impacted by **54**, and a few off-target effects were noted when screening against a panel of 307 kinases. A couple of TEC family kinases were hit more than 50% by **54** at 1 μ M but not when the fluoromethyl ketone moiety was employed as the electrophile. A crystal structure of **54** in complex with T338C c-Src was solved, which nicely demonstrated the formation of a

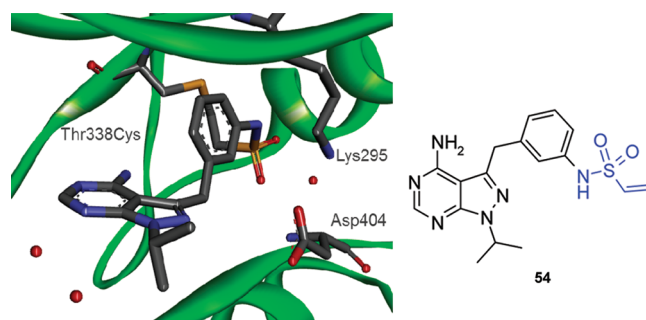


Figure 19. Pyrazolopyrimidine derivative **54** in covalent complex with c-Src T338C (3SVV.pdb; P-loop omitted for clarity).

covalent bond between the vinylsulfonamide warhead and the gatekeeper cysteine residue.

KINASE KINETICS AND STRUCTURE–REACTIVITY RELATIONSHIPS (SRR)

It is easy to get enchanted with extraordinary potencies and superb selectivity. However, embarking on a project with the aim to identify covalent inhibitors that should serve patients warrants an adapted flowchart for selecting candidate compounds with the required druglike properties. It is of paramount importance to incorporate specific off-target assays that are central in the flowchart (vide infra). These can be both computational and empirical of nature. Many of those assays, such as homologue cross-screening and monitoring of glutathione depletion, are already part of the conventional drug discovery toolbox. More importantly, addressing the kinetics of irreversible kinase inhibition and generation of robust structure–reactivity relationships are the real keys to success in the discovery of safe irreversible drugs. The activity of the ultimate irreversible kinase inhibitor should be an optimal mix of reversible binding affinity (SAR) and warhead reactivity (SRR). If the efficacy of the inhibitor depends solely on reactivity, the overall selectivity and consequently the safety profile will undoubtedly be compromised. When the reactivity is too low, the inhibitor will behave like a conventional reversible inhibitor and become more dependent on the clearance of the compound from the circulation again.

A suitable and straightforward assay system for the rate of kinase silencing has been reported by Rauh and co-workers. The kinetics of kinase inhibition were determined by monitoring changes in the intrinsic fluorescence signal upon covalent bond formation between electrophilic inhibitors and the S345C c-Src and the clinically relevant EGFR mutants.¹¹⁰ Also, electrophilicity calculations by means of enthalpy changes (ΔH) were employed in analogy with previously published procedures for nitrile warhead containing compounds.¹¹¹ The potencies of irreversible inhibitors were extrapolated from the initial reaction velocities found in the linear range of the curves. With this technique, the influence of the altered gatekeeper residue size on the initial reactivity rate of the irreversible inhibitors could be directly monitored. A pragmatic way of ranking irreversible kinase inhibitors based on the intrinsic electrophilic strength has been described by Wyeth researchers, who rank-ordered the explored warheads based on simple glutathione adduct formation in a THF–water–methanol solution.⁵⁸ Others have incubated irreversible kinase inhibitor leads with glutathione or whole blood and monitored the stability over time.⁸²

■ OUTLOOK

For many kinases, a crucial regulatory role has been associated with a variety of malignancies, making this protein family an attractive drug target family. The challenge for this target family has been the identification and development of potent inhibitors with sufficient selectivity to generate efficacious drugs with a good safety profile. For a number of reasons, the kinase field in particular seems well equipped for the covalent inhibitor concept with clear potential advantages versus reversible inhibitors. The compelling upsides have been reviewed in this Perspective. First, with high intracellular ATP levels, non-ATP competitiveness is a major upside. Therefore, irreversible protein kinase inhibitors could outperform reversible drugs based on superior efficacy and functional selectivity. Indeed, the loss in cellular potency for biochemically potent reversible inhibitors is in many cases not observed for an irreversible inhibitor. Second, a substantial part of the human kinome harbors a specific cysteine in or near the ATP binding pocket that may be used to achieve both potency and selectivity. Third, the rate of turnover for many kinases, required for return of function of the target that has been effectively knocked down via irreversible kinase inhibition, renders this protein family suitable for less frequent dosing regimens. In addition, lower overall exposure is to be expected, since the pharmacodynamics effects are dependent on *de novo* synthesis of the target protein, rather than higher dosing required to maintain certain circulating levels for the drug to retain efficacy over time. This lower body burden is beneficial in circumventing off-target related adverse effects. Fourth, protein kinase inhibitors introduced to the market to date have without exception been approved for oncology indications, and it is in this area that irreversible kinase drugs might gain easier acceptance. Drug-induced resistance in many cancers remains a serious problem, and also here, irreversible binding kinase inhibitors could make a difference. It has been proposed that this mutational pressure even might exist for cysteines, which may render the irreversible kinase inhibitors currently in clinical development for oncology indications increasingly ineffective over time.⁶⁹ Future follow-up in these patients is required to substantiate the occurrence and prevalence of these inactivating mutations.

Last but not least, irreversible kinase inhibitors as such are invaluable compounds as research tools in the validation of kinase signaling pathways or can with relative ease be appended with reporter tags. This may significantly speed the overall drug discovery process, as the biological validation of the target can be tackled early on and the availability of biomarker target occupancy probes facilitates dose-finding in clinical development.

Monitoring SRR is key in order to progress the most suitable covalent inhibitor candidates through the pipeline, since off-target toxicity related to the electrophile remains a substantial threat. The future will tell whether irreversible binding kinase inhibitors will live up to the expectations that have been generated by the research community; however, the progress that has been made in the past 2 decades has been impressive. The most advanced irreversible kinase inhibitors are in phase 3 clinical trials. Recently, two major deals have been struck within the pharmaceutical industry. Johnson & Johnson purchased phase 2 irreversible Btk inhibitor 28 from Pharmacyclics. Avila Therapeutics, a biotechnology company relying on the covalent binding platform, was recently acquired by Celgene. These

developments seem exemplary for the general growing confidence in irreversible kinase inhibitors as effective and safe treatments. If irreversible kinase inhibition is shown to be sufficiently safe in cancer indications, chronic treatment for other disorders may also become accepted. Covalent drugs are already widely applied in indications outside the oncology arena. It is our belief that the covalent binding principle will gain common ground as we get more accustomed to the upsides while still keeping a critical eye on the potential risks. Those covalent drugs that have the appropriate benefit/risk balance are “bound to succeed”.

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Notes

The authors declare no competing financial interest.

Biographies

Tjeerd Barf obtained his Ph.D. in Medicinal Chemistry from the University of Groningen, The Netherlands, under the supervision of Prof. Håkan Wikström in 1996. He joined Pharmacia & Upjohn in Uppsala, Sweden, in 1997 and continued his employment in the spin-off company Biovitrum (2000). He has led medicinal chemistry programs concerning metabolic diseases and assumed overall program leadership later on. In 2005, he moved to Legacy Organon (Oss, The Netherlands), and as a Group Leader, his responsibilities encompassed heading immunology lead finding projects. After the acquisition by Schering-Plough and the merger with Merck, he led a global cross-site lead optimization effort for an autoimmune disease. In 2011, he cofounded Covalution Pharma and presently holds the position of Head of Chemistry within that biotechnology company.

Allard Kaptein received his Ph.D. in 1993 in Biopharmaceutical Sciences at Gaubius Institute TNO/University of Leiden, The Netherlands. After 2 years as a Postdoctoral Fellow at Glaxo in Les Ulis, France, he joined the GlaxoWellcome in Stevenage, U.K. In 2000 he joined Organon in Oss, The Netherlands. From 2004 to 2008 he was head of the immunology lead finding program activities, and after 2009 headed a lead optimization program at Schering-Plough and Merck following the takeover of Organon. In July 2011 he founded, together with Tjeerd Barf, the R&D startup Covalution Pharma focusing on covalent kinase inhibitors in oncology and autoimmune diseases. His main areas of expertise include small molecule drug discovery on different target families, translational sciences, and cross-disciplinary knowledge bridging biology and chemistry in research.

■ ABBREVIATIONS USED

(Bcr)-Abl, (breakpoint cluster region)-Abelson kinase; ALK, anaplastic lymphoma kinase; Akt1 (PKB), Ak mouse strain thymoma; Ark1, Aurora kinase; BCR, B-cell receptor; Blk, B-lymphocyte kinase; Bmx, bone marrow tyrosine kinase gene in chromosome X; Btk, Bruton's tyrosine kinase; CIA, collagen-induced arthritis; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; ErbB, erythroblastosis oncogene B; EGFR (ErbB1), epidermal growth factor receptor; Erk2, extracellular signal-regulated kinase-2; FGFR, fibroblast growth factor receptor; FLT3, *fms*-like tyrosine kinase-3; GIST, gastrointestinal stromal tumor; GSK, glycogen synthase kinase; IDT, idiosyncratic toxicity; IGF-1R, insulin-like growth factor receptor; IKK α/β , inhibitor of kappa-B kinase subunit α/β ; Itk, IL2-inducible T-cell kinase; JAK, Janus kinase; JNK (SAPK), c-Jun N-terminal kinase; c-Kit, steel growth factor receptor;

LKB1, liver kinase B1; MEK (or MKK or MAP2K), mitogen-activated protein kinase kinase; MEKK1 (MAP3K), mitogen-activated protein kinase kinase kinase 1; MET, hepatocyte growth factor receptor; MOK, MAPK/MAK/MRK overlapping kinase; NEK2 (NIMA), never in mitosis gene a related kinase 2; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung carcinoma; PDGFR, platelet derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; PKC, protein kinase C; Plk, polo-like kinase; Raf, rapidly accelerated fibrosarcoma; RCC, renal cell carcinoma; RET, rearranged during transfection; RSK, p90 ribosomal protein S6 kinase; SgK494, Sugan kinase 494; c-Src, Rous sarcoma oncogene cellular homologue; SRR, structure-reactivity relationship; TAK, TGF- β activated kinase; TEC, tyrosine kinase expressed in hepatocellular carcinoma; TNK1, tyrosine kinase nonreceptor 1; Txk, T and X cell expressed kinase; VEGFR, vascular endothelial growth factor receptor; WT, wild type; YES, Yamaguchi sarcoma viral oncogene homologue

REFERENCES

- (1) Potashman, M. H.; Duggan, M. E. Covalent modifiers: an orthogonal approach to drug design. *J. Med. Chem.* **2009**, *52*, 1231–1246.
- (2) Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A. The resurgence of covalent drugs. *Nat. Rev. Drug Discovery* **2011**, *10*, 307–317.
- (3) Baell, J. B.; Holloway, G. A. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* **2010**, *53*, 2719–2740.
- (4) Huth, J. R.; Mendoza, R.; Olejniczak, E. T.; Johnson, R. W.; Cothron, D. A.; Liu, Y.; Lerner, C. G.; Chen, J.; Hajduk, P. J. ALARM NMR: a rapid and robust experimental method to detect reactive false positives in biochemical screens. *J. Am. Chem. Soc.* **2005**, *127*, 217–224.
- (5) Copeland, R. A.; Pompliano, D. L.; Meek, T. D. Drug–target residence time and its implications for lead optimization. *Nat. Rev. Drug Discovery* **2006**, *5*, 730–739.
- (6) Tummino, P. J.; Copeland, R. A. Residence time of receptor–ligand complexes and its effect on biological function. *Biochemistry* **2008**, *47*, 5481–5492.
- (7) Smith, A. J. T.; Zhang, X.; Leach, A. G.; Houk, K. N. Beyond picomolar affinities: quantitative aspects of noncovalent and covalent binding of drugs to proteins. *J. Med. Chem.* **2009**, *52*, 225–233.
- (8) Lewandowicz, A.; Tyler, P. C.; Evans, G. B.; Furneaux, R. H.; Schramm, V. L. Achieving the ultimate physiological goal in transition state analogue inhibitors for purine nucleoside phosphorylase. *J. Biol. Chem.* **2003**, *278*, 31465–31468.
- (9) Kola, I.; Landis, J. Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discovery* **2004**, *3*, 711–715.
- (10) Park, B. K.; Boobis, A.; Clarke, S.; Goldring, C. E. P.; Jones, D.; Kenna, J. G.; Lambert, C.; Lavery, H. G.; Naisbitt, D. J.; Nelson, S.; Nicoll-Griffith, D. A.; Obach, R. S.; Routledge, P.; Smith, D. A.; Tweedie, D. J.; Vermeulen, N.; Williams, D. P.; Wilson, I. D.; Baillie, T. A. Managing the challenge of chemically reactive metabolites in drug development. *Nat. Rev. Drug Discovery* **2011**, *10*, 292–306.
- (11) Guengerich, F. P.; MacDonald, J. S. Applying mechanisms of chemical toxicity to predict drug safety. *Chem. Res. Toxicol.* **2007**, *20*, 344–369.
- (12) Nakayama, S.; Atsumi, R.; Takakusa, H.; Kobayashi, Y.; Kurihara, A.; Nagai, Y.; Nakai, D.; Okazaki, O. A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug. Metab. Dispos.* **2009**, *37*, 1970–1977.
- (13) Erve, J. C. L. Chemical toxicology: reactive intermediate and their role in pharmacology and toxicology. *Expert Opin. Drug Metab. Toxicol.* **2006**, *2*, 923–946.
- (14) Zhang, X.; Liu, F.; Chen, X.; Zhu, X.; Uetrecht, J. Involvement of the immune system in idiosyncratic drug reactions. *Drug. Metab. Pharmacokinet.* **2011**, *26*, 47–59.
- (15) Garuti, L.; Roberti, M.; Bottegoni, G. Non-ATP competitive protein kinase inhibitors. *Curr. Med. Chem.* **2010**, *17*, 2804–2821.
- (16) Qin, J.; Dhondi, P.; Huang, X.; Aslanian, R.; Fossetta, J.; Tian, F.; Lundell, D.; Palani, A. Discovery of a potent dihydrooxadiazole series of non-ATP competitive MK2 (MAPKAPK2) inhibitors. *ACS Med. Chem. Lett.* **2012**, *3*, 100–105.
- (17) Knight, Z. A.; Shokat, K. M. Features of selective kinase inhibitors. *Chem. Biol.* **2005**, *12*, 621–637.
- (18) Singh, J.; Petter, R. D.; Kluge, A. F. Targeted covalent drugs of the kinase family. *Curr. Opin. Chem. Biol.* **2010**, *14*, 475–480.
- (19) Krippendorf, B.-F.; Neuhaus, R.; Lienau, P.; Reichel, A.; Huisinga, W. Mechanism-based inhibition: deriving K_i and k_{inact} directly from time-dependent IC_{50} values. *J. Biomol. Screening* **2009**, *14*, 913–923.
- (20) Garuti, L.; Roberti, M.; Bottegoni, G. Irreversible protein kinase inhibitors. *Curr. Med. Chem.* **2011**, *18*, 2981–2994.
- (21) Carrera, A. C.; Alexandrov, K.; Roberts, T. M. The conserved lysine of the catalytic domain of protein kinases is actively involved in the phosphotransfer reaction and not required for anchoring ATP. *Proc. Nat. Acad. Sci. U.S.A.* **1993**, *90*, 442–446.
- (22) (a) Buhrow, S. A.; Cohen, S.; Staros, J. V. Affinity labeling of the protein kinase associated with the epidermal growth factor receptor in membrane vesicles from A431 cells. *J. Biol. Chem.* **1982**, *257*, 4019–4020. (b) Scoggins, R. M.; Summerfield, A. E.; Stein, R. A.; Guyer, C. A.; Staros, J. V. 5'-(*p*-Fluorosulfonylbenzoyl)-2'(or 3')-(methylanthraniloyl)adenosine, fluorescent affinity labels for adenine nucleotide binding sites: Interaction with the kinase active site of the receptor for epidermal growth factor. *Biochemistry* **1996**, *35*, 9197–9203.
- (23) Fox, T.; Fitzgibbon, M. J.; Fleming, M. A.; Hsiao, H.-M.; Brummel, C. L.; Su, M. S.-S. Kinetic mechanism and ATP-binding site reactivity of p38 γ MAP kinase. *FEBS Lett.* **1999**, *461*, 323–328.
- (24) Vereb, G.; Balla, A.; Gergely, P.; Wymann, M. P.; Gulkan, H.; Suer, S.; Heilmeyer, L. M. G. The ATP-binding site of brain phosphatidylinositol 4-kinase PI4K230 as revealed by 5'-*p*-fluorosulfonylbenzoyladenine. *Int. J. Biochem. Cell Biol.* **2001**, *33*, 249–259.
- (25) Bell, I. M.; Stirdivant, S. M.; Ahern, J.; Culbertson, C.; Darke, P. L.; Dinsmore, C. J.; Drakas, R. A.; Gallicchio, S. N.; Graham, S. L.; Heimbrook, D. C.; Hall, D. L.; Hua, J.; Kett, N. R.; Kim, A. S.; Kornienko, M.; Kuo, L. C.; Munshi, S. K.; Quigley, A. G.; Reid, J. C.; Trotter, B. W.; Waxmann, L. H.; Williams, T. M.; Zartman, C. B. Biochemical and structural characterization of a novel class of inhibitors of the type 1 insulin-like growth factor and insulin receptor kinases. *Biochemistry* **2005**, *44*, 9430–9440.
- (26) (a) Powis, G.; Bonjouklian, R.; Berggren, M. M.; Gallegos, A.; Abraham, R.; Ashendel, C.; Zalkow, L.; Matter, W. F.; Dodge, J.; Grindey, G.; Vlahos, C. J. Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res.* **1994**, *54*, 2419–2423. (b) Wymann, M. P.; Bulgarelli-Leva, G.; Zvelebil, M. J.; Pirolo, M. L.; Van-haesebroeck, B.; Waterfield, M. D.; Panayotou, G. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* **1996**, *16*, 1722–1733.
- (27) Norman, B. H.; Shih, C.; Toth, J. E.; Ray, J. E.; Dodge, J. A.; Johnson, D. W.; Rutherford, P. G.; Schultz, R. M.; Worzalla, J. F.; Vlahos, C. J. Studies on the mechanism of phosphatidylinositol 3-kinase inhibition by wortmannin and related analogs. *J. Med. Chem.* **1996**, *39*, 1106–1111.
- (28) Walker, E. H.; Pacold, M. E.; Perisic, O.; Stephens, L.; Hawkins, P. T.; Wymann, M. P.; Williams, R. Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporin. *Mol. Cell* **2000**, *6*, 909–919.
- (29) Liu, Y.; Shreder, K. R.; Gai, W.; Corral, S.; Ferris, D. K.; Rosenblum, J. S. Wortmannin, a widely used phosphoinositide 3-kinase inhibitor, also potently inhibits mammalian Polo-like kinase. *Chem. Biol.* **2005**, *12*, 99–107.

- (30) Liu, Y.; Jiang, N.; Wu, J.; Dai, W.; Rosenblum, J. S. Polo-like kinases inhibited by wortmannin. *J. Biol. Chem.* **2007**, *282*, 2505–2511.
- (31) Barr, A. J.; Ugochukwu, E.; Lee, W. H.; King, O. N. F.; Filippakopoulos, P.; Alfano, I.; Savitsky, P.; Burgess-Brown, N. A.; Müller, S.; Knapp, S. Large-scale structural analysis of the classical human protein tyrosine phosphatome. *Cell* **2009**, *136*, 352–363.
- (32) Roberts, R. Lysosomal cysteine proteases: structure, function and inhibition of cathepsins. *Drug News Perspect.* **2005**, *18*, 605–614.
- (33) Kortemme, T.; Creighton, T. E. Ionisation of cysteine residues at the termini of model alpha-helical peptides. Relevance to unusual thiol pK_a values in proteins of the thioredoxin family. *J. Mol. Biol.* **1995**, *253*, 799–812.
- (34) Paulsen, C. E.; Carroll, K. S. Orchestrating redox signaling networks through regulatory cysteine switches. *ACS Chem. Biol.* **2009**, *5*, 47–62.
- (35) Paulsen, C. E.; Truong, T. H.; Garcia, F. J.; Homann, A.; Gupta, V.; Leonard, S. E.; Carroll, K. S. Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. *Nat. Chem. Biol.* **2012**, *8*, 57–64.
- (36) Leonberg, A. K.; Chai, Y.-C. The functional role of cysteine residues for c-Abl kinase activity. *Mol. Cell. Biochem.* **2007**, *304*, 207–212.
- (37) Satoh, T.; Lipton, S. A. Redox regulation of neuronal survival mediated by electrophilic compounds. *Trends Neurosci.* **2006**, *30*, 37–45.
- (38) Zhang, J.; Yang, P. L.; Gray, N. S. Targeting cancer with small molecule kinase inhibitors. *Nat. Rev. Cancer* **2009**, *9*, 28–39.
- (39) Leproult, E.; Barluenga, S.; Moras, D.; Wurtz, J.-M.; Winssinger, N. Cysteine mapping in conformationally distinct kinase nucleotide binding sites: application to the design of selective covalent inhibitors. *J. Med. Chem.* **2011**, *54*, 1347–1355.
- (40) Zhou, W.; Hur, W.; McDermott, U.; Dutt, A.; Xian, W.; Ficarro, S. B.; Zhang, J.; Sharma, S. V.; Brugge, J.; Meyerson, M.; Settleman, J.; Gray, N. A. A structure-guided approach to creating covalent FGFR inhibitors. *Chem. Biol.* **2010**, *17*, 285–295.
- (41) Powers, C. J.; McLeskey, S. W.; Wellstein, A. Fibroblast growth factors, their receptors and signaling. *Endocr.-Relat. Cancer* **2000**, *7*, 165–197.
- (42) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912–1934.
- (43) Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. Structural bioinformatics-based design of selective, irreversible kinase inhibitors. *Science* **2005**, *308*, 1318–1321.
- (44) Serafimova, I. M.; Pufall, M. A.; Krishnan, S.; Duda, K.; Cohen, M. S.; Maglathlin, R. L.; McFarland, J. M.; Miller, R. M.; Frödin, M.; Taunton, J. Reversible targeting of noncatalytic cysteines with chemically tuned electrophiles. *Nat. Chem. Biol.* **2012**, *8*, 471–476.
- (45) Malakhova, M.; Tereshko, V.; Lee, S.-Y.; Yao, K.; Cho, Y.-Y.; Bode, A.; Dong, Z. Structural basis for activation of the autoinhibitory C-terminal kinase domain of p90 RSK2. *Nat. Struct. Mol. Biol.* **2008**, *15*, 112–113.
- (46) Henise, J. C.; Taunton, J. Irreversible Nek2 kinase inhibitors with cellular activity. *J. Med. Chem.* **2011**, *54*, 4133–4146.
- (47) Cross, J. V.; Templeton, D. J. Oxidative stress inhibits MEKK1 by site-specific glutathionylation in the ATP-binding domain. *Biochem. J.* **2004**, *381*, 675–683.
- (48) Cross, J. V.; Foss, F. W.; Rady, J. M.; Macdonald, T. L.; Templeton, D. J. The isothiocyanate class of bioactive nutrients covalently inhibit the MEKK1 protein kinase. *BMC Cancer* **2007**, *7*, 183.
- (49) Li, J.; Kaoud, T. S.; Laroche, C.; Dalby, K. N.; Kerwin, S. M. Synthesis and biological evaluation of p38α kinase-targeting dialkynylimidazoles. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6293–6297.
- (50) Fitzgerald, C. E.; et al. Structural basis for p38α MAP kinase quinazolinone and pyridol-pyrimidine inhibitor specificity. *Nat. Struct. Biol.* **2003**, *10*, 764–769.
- (51) Pao, W.; Miller, V. A.; Politi, K. A.; Riely, G. J.; Somwar, R.; Zakowski, M. F.; Kris, M.; Varmus, H. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med.* **2005**, *2*, 1–11.
- (52) Kobayashi, S.; Boggon, T. J.; Dayaram, T.; Jänne, P. A.; Kocher, O.; Meyerson, M.; Johnson, B. E.; Eck, M. J.; Tenen, D. G.; Halmos, B. EGFR mutation and resistance on non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* **2005**, *352*, 786–792.
- (53) Yun, C.-H.; Mengwasser, K. E.; Toms, A. V.; Woo, M. S.; Greulich, H.; Wong, K.-K.; Meyerson, M.; Eck, M. J. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2070–2075.
- (54) Kwak, E. L.; Sordella, R.; Bell, D. W.; Godin-Heymann, N.; Okimoto, R. A.; Brannigan, B. W.; Harris, P. L.; Driscoll, D. R.; Fidias, P.; Lynch, T. J.; Rabindran, S. K.; McGinnis, J. P.; Wissner, A.; Sharma, S. V.; Isselbacher, K. J.; Settleman, J.; Haber, D. A. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7665–7670.
- (55) Singh, J.; Dobrusin, E. M.; Fry, D. W.; Haske, T.; Whitty, A.; McNamara, D. J. Structure-based design of a potent, selective and irreversible inhibitor of the catalytic domain of the erbB receptor subfamily of protein tyrosine kinases. *J. Med. Chem.* **1997**, *40*, 1130–1135.
- (56) Fry, D. W.; Bridges, A. J.; Denny, W. A.; Doherty, A.; Greis, K. D.; Hicks, J. L.; Hook, K. E.; Keller, P. R.; Leopold, W. R.; Loo, J. A.; McNamara, D. J.; Nelson, J. M.; Sherwood, V.; Smail, J. B.; Trumpp-Kallmeyer, S. T.; Dobrusin, E. M. Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosin kinase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12022–12027.
- (57) Discafani, C. M.; Carroll, M. L.; Floyd, M. B.; Hollander, I. J.; Husain, Z.; Johnson, B. D.; Kitchen, D.; May, M. K.; Malo, M. S.; Minnick, A. A.; Nilakantan, R.; Shen, R.; Wang, Y.-F.; Wissner, A.; Greenberger, L. M. Irreversible inhibition of epidermal growth factor receptor tyrosine kinase with in vivo activity by N-[4-[3-bromophenyl]amino]-6-quinazolinyl]-2-butynamide (CL-387,875). *Biochem. Pharmacol.* **1999**, *57*, 917–925.
- (58) Wissner, A.; Mansour, T. S. The development of HKI-272 and related compounds for the treatment of cancer. *Arch. Pharm. Chem. Life Sci.* **2008**, *341*, 465–477.
- (59) Carmi, C.; Cavazzoni, A.; Vezzosi, S.; Bordin, F.; Vacondio, F.; Silva, C.; Rivara, S.; Lodola, A.; Alfieri, R. R.; La Monica, S.; Galetti, M.; Ardizzoni, A.; Petronin, P. G.; Mor, M. Novel irreversible epidermal growth factor receptor inhibitors by chemical modulation of the cysteine-trap portion. *J. Med. Chem.* **2010**, *53*, 2038–2050.
- (60) Wood, E. R.; Shewchuk, L. M.; Byron Ellis, B.; Brignola, P.; Brashears, R. L.; Caferro, T. R.; Dickerson, S. H.; Dickson, H. D.; Donaldson, K. H.; Gaul, M.; Griffin, R. J.; Hassell, A. M.; Keith, B.; Mullin, R.; Petrov, K. G.; Reno, M. J.; Rusnak, D. W.; Tadepalli, S. M.; Ulrich, J. C.; Wagner, C. D.; Vanderwall, D. E.; Waterson, A. G.; Williams, J. D.; White, W. L.; Uehling, D. E. 6-Ethynylthieno[3,2-d] and 6-ethynylthieno[2,3-d]pyrimidin-4-anilines as tunable covalent modifiers of ErbB kinases. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 2772–2778.
- (61) Brigdes, A. J. Chemical inhibitors of protein kinases. *Chem. Rev.* **2000**, *101*, 2541–2571.
- (62) Ban, H. S.; Usui, T.; Nabeyama, W.; Morita, H.; Fukuzawa, K.; Nakamura, H. Discovery of boron-conjugated 4-anilinoquinazoline as a prolonged inhibitor of EGFR tyrosine kinase. *Org. Biomol. Chem.* **2009**, *7*, 4415–4427.
- (63) Carmi, C.; Lodola, A.; Rivara, S.; Vacondio, F.; Cavazzoni, A.; Alfieri, R. R.; Ardizzoni, A.; Petronini, P. G.; Mor, M. Epidermal growth factor receptor irreversible inhibitors: chemical exploration of the cysteine-trap portion. *Mini-Rev. Med. Chem.* **2011**, *11*, 1019–1030.
- (64) Cha, M. Y.; Lee, K.-O.; Kim, J. W.; Lee, C. G.; Song, J. Y.; Kim, Y. H.; Lee, G. S.; Park, S. B.; Kim, M. S. Discovery of a novel Her-1/Her-2 dual tyrosine kinase inhibitor for the treatment of Her-1 selective inhibitor-resistant non-small cell lung cancer. *J. Med. Chem.* **2010**, *52*, 6880–6888.
- (65) Coumar, M. S.; Chu, C.-Y.; Lin, C.-W.; Shiao, H.-Y.; Ho, Y.-L.; Reddy, R.; Lin, W.-H.; Chen, C.-H.; Peng, J.-S.; Lien, T.-W.; Huang,

- C.-T.; Fang, M.-Y.; Wu, S.-H.; Wu, J.-S.; Chittimalla, S. K.; Song, J.-S.; Hsu, J. T.-A.; Wu, S.-Y.; Liao, C.-C.; Chao, Y.-S.; Hsieh, H.-P. Fast-forwarding hit to lead: Aurora and epidermal growth factor receptor kinase inhibitor lead identification. *J. Med. Chem.* **2010**, *53*, 4980–4988.
- (66) Wu, C.-H.; Coumar, M. S.; Chu, C.-Y.; Lin, W.-H.; Chen, Y.-R.; Chen, C.-T.; Shiao, H.-Y.; Rafi, S.; Wang, S.-Y.; Hsu, H.; Chen, C.-H.; Chang, C.-Y.; Chang, T.-Y.; Lien, T.-W.; Fang, M.-Y.; Yeh, K.-C.; Chen, C.-P.; Yeh, T.-K.; Hsieh, S.-H.; Hsu, J. T.-A.; Liao, C.-C.; Chao, Y.-S.; Hsieh, H.-P. Design and synthesis of tetrahydropyridothieno-[2,3-*d*]pyrimidine scaffold based epidermal growth factor receptor (EGFR) kinase inhibitors: the role of side chain chirality and Michael acceptor group for maximal potency. *J. Med. Chem.* **2010**, *53*, 7316–7326.
- (67) Clinical Trials. <http://clinicaltrials.gov/>.
- (68) Sos, M. L.; Rode, H. B.; Heynck, S.; Peifer, M.; Fischer, F.; Klüter, S.; Pawar, V. G.; Reuter, C.; Heuckmann, J. M.; Weiss, J.; Ruddigkeit, L.; Rabiller, M.; Koker, M.; Simar, J. R.; Getlik, M.; Yuza, Y.; Chen, T.-H.; Greulich, H.; Thomas, R. K.; Rauh, D. Chemo-genomic profiling provides insights into the limited activity of irreversible EGFR inhibitors in tumor cells expressing the T790M EGFR resistance mutation. *Cancer Res.* **2010**, *70*, 868–874.
- (69) Godin-Heymann, N.; Ulkus, L.; Brannigan, B. W.; McDermott, U.; Lamb, J.; Maheswaran, S.; Settleman, J.; Haber, D. A. The T790M “gatekeeper” mutation in EGFR mediates resistance to low concentrations of an irreversible EGFR inhibitor. *Mol. Cancer Ther.* **2008**, *7*, 874–879.
- (70) Ocana, A.; Amir, E. Irreversible pan-ErbB tyrosine kinase inhibitors and breast cancer: current status and future directions. *Cancer Treat. Rev.* **2009**, *35*, 685–691.
- (71) Eskens, F. A. L. M.; Mom, C. H.; Planting, A. S. T.; Gietema, J. A.; Amelsberg, A.; Huisman, H.; Doorn, L.; van, Burger, H.; Stopfer, P.; Verweij, J.; de Vries, E. G. A phase I dose escalation study of BIBW 2992, an irreversible dual inhibitor of epidermal growth factor receptor 1 (EGFR) and 2 (HER2) tyrosine kinase in a 2-week on, 2-week off schedule in patients with advanced solid tumours. *Br. J. Cancer* **2008**, *98*, 80–85.
- (72) Engelman, J. A.; Zejnullahu, K.; Gale, C.-M.; Lifshits, E.; Gonzales, A. J.; Shimamura, T.; Zhao, F.; Vincent, P. W.; Naumov, G. N.; Bradner, J. E.; Althaus, I. W.; Gandhi, L.; Shapiro, G. I.; Nelson, J. M.; Heymach, J. V.; Meyerson, M.; Wong, K.-K.; Jänne, P. A. PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. *Cancer Res.* **2007**, *67*, 11924–11932.
- (73) Gonzales, A. J.; Hook, K. E.; Althaus, I. W.; Ellis, P. A.; Trachet, E.; Delaney, A. M.; Harvey, P. J.; Ellis, T. A.; Amato, D. M.; Nelson, J. M.; Fry, D. W.; Zhu, T.; Loi, C.-M.; Fakhoury, S. A.; Schlosser, K. M.; Sexton, K. E.; Winters, R. T.; Reed, J. E.; Bridges, A. J.; Lettiere, D. J.; Baker, D. A.; Yang, J.; Lee, H. T.; Tecle, H.; Vincent, P. W. Antitumor activity and pharmacokinetic properties of PF-00299804, a second-generation irreversible pan-erbB receptor tyrosine kinase inhibitor. *Mol. Cancer Ther.* **2008**, *7*, 1880–1889.
- (74) Tsou, H.-R.; Overbeek-Klumpers, E. G.; Hallett, W. A.; Reich, M. F.; Floyd, M. B.; Johnson, B. D.; Michalak, R. S.; Nilakantan, R.; Discafani, C.; Golas, J.; Rabindran, S. K.; Shen, R.; Shi, X.; Want, Y.-F.; Upeslakis, J.; Wissner, A. Optimization of 6,7-disubstituted-4-(arylamino)quinoline-3-carbonitriles as orally active, irreversible inhibitors of human epidermal growth factor receptor-2 kinase activity. *J. Med. Chem.* **2005**, *48*, 1107–1131.
- (75) Zhou, W.; Ercan, D.; Chen, L.; Yun, C.-H.; Li, D.; Capelletti, M.; Cortot, A. B.; Chirieac, L.; Lacob, R. E.; Padera, R.; Engen, J. R.; Wong, K.-K.; Eck, M. J.; Gray, N. S.; Jänne, P. A. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nat. Lett.* **2009**, *462*, 1070–1074.
- (76) Zhou, W.; Ercan, D.; Jänne, P. A.; Gray, N. S. Discovery of selective irreversible inhibitors for EGFR-T790M. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 638–643.
- (77) Walter, A. O.; Tjin, R.; Haringsma, H.; Lin, K. K.; Dubrovskiy, A.; Lee, K.; St. Martin, T.; Karp, R.; Zhu, Z.; Niu, D.; Nacht, M.; Petter, R. C.; Westlin, W. F.; Singh, J.; Raponi, M.; Allen, A. CO-1886, an Orally Available, Mutant-Selective Inhibitor of the Epidermal Growth Factor Receptor (EGFR), Causes Tumor Shrinkage in Non-Small Cell Lung Cancer (NSCLC) with T790M Resistance Mutations. Presented at the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, San Francisco, CA, November 12–16, 2011.
- (78) Pan, Z.; Scheerens, H.; Li, S.-J.; Schultz, B. E.; Sprengeler, P. A.; Burrill, L. C.; Mendonca, R. V.; Sweeney, M. D.; Scott, K. C. K.; Grothaus, P. G.; Jeffery, D. A.; Spoerke, J. M.; Honigberg, L. A.; Young, P. R.; Dalrymple, S. A.; Palmer, J. T. Discovery of selective irreversible inhibitors for Bruton's tyrosine kinase. *ChemMedChem* **2007**, *2*, 58–61.
- (79) Honigberg, L. A.; Smith, A. M.; Sirisawad, M.; Verner, E.; Loury, D.; Chang, B.; Li, S.; Pan, Z.; Thamm, D. H.; Miller, R. A.; Buggy, J. J. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc. Nat. Acad. Sci. U.S.A.* **2010**, *107*, 13075–13080.
- (80) Advani, R.; Sharman, J.; Smith, S.; Boyd, T.; Grant, B.; Kolibaba, K.; Furman, R.; Buggy, J.; Loury, D.; Hedrick, E.; Izumi, R.; Hamdy, A.; Fowler, N. The Btk Inhibitor PCI-32765 Is Highly Active and Well Tolerated in Patients with Relapsed/Refractory B Cell Malignancies: Final Results from a Phase I Study. Presented at the 11th International Conference on Malignant Lymphoma (ICML), Lugano, Switzerland, June 15–18, 2011.
- (81) Moriarty, K. J.; Konteatis, Z.; Moffett, K.; Lee, Y.; Chao, W. Int. Pat. Appl. WO 2010/126960 A1, 2010.
- (82) Labenski, M.; Chaturvedi, P.; Evans, E.; Mazdiasni, H.; Sheets, M.; Aslanian, S.; Niu, D.; Nacht, M.; Singh, J.; Westlin, W. In Vitro Reactivity Assessment of Covalent Drugs Targeting Bruton's Tyrosine Kinase. Presented at the 17th North America Regional Meeting of International Society for the Study of Xenobiotics, Atlanta, GA, October 16–20, 2011; Poster P211.
- (83) Petter, R. Targeted Covalent Kinase Inhibitors: AVL 292 as a Potent, Irreversible and Selective Inhibitor of Btk. Presented at the 5th Anglo-Swedish Medicinal Chemistry Symposium (ASMC), Åre, Sweden, March 20–23, 2011.
- (84) Evans, E.; Tester, R.; Aslanian, S.; Mazdiasni, H.; Ponader, S.; Tesar, B.; Chaturvedi, P.; Nacht, M.; Stiede, K.; Witowski, S.; Lounsbury, H.; Silver, B.; Burger, J.; Brown, J. R.; Mahadevan, D.; Sharman, J.; Harb, W.; Petter, R.; Singh, J.; Westlin, W. Clinical Development of AVL-292: A Potent, Selective Covalent Btk Inhibitor for the Treatment of B Cell Malignancies. Presented at the ASH Annual Meeting, San Diego, CA, December 10–13, 2011.
- (85) Kim, K.-H.; Maderna, A.; Schnute, M. E.; Hegen, M.; Mohan, S.; Miyashiro, J.; Lin, L.; Li, E.; Keegan, S.; Lussier, J.; Wrocklage, C.; Nickerson-Nutter, C. L.; Wittwer, A. J.; Soutter, H.; Caspers, N.; Han, S.; Kurumbail, R.; Dunussi-Joannopoulos, K.; Douhan, J.; Wissner, A. Imidazo[1,5-*a*]quinoxalines as irreversible BTK inhibitors for the treatment of rheumatoid arthritis. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6258–6263.
- (86) Wissner, A.; Floyd, M. B.; Johnson, B. D.; Fraser, H.; Ingalls, C.; Nittoli, T.; Dushin, R. G.; Discafani, C.; Nilakantan, R.; Marini, J.; Ravi, M.; Cheung, K.; Tan, X.; Musto, S.; Annable, T.; Siegel, M. M.; Loganzo, F. 2-(Quinazolin-4-ylamino)-[1,4]benzoquinones as covalent-binding, irreversible inhibitors of the kinase domain of vascular endothelial growth factor receptor-2. *J. Med. Chem.* **2005**, *48*, 7560–7581.
- (87) Schirmer, A.; Kennedy, J.; Murli, S.; Reid, R.; Santi, D. V. Targeted covalent inactivation of protein kinases by resorcylic acid lactone polyketides. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 4234–4239.
- (88) Rastelli, G.; Rosenfeld, R.; Reid, R.; Santi, D. V. Molecular modeling and crystal structure of ERK2-hypothemycin complexes. *J. Struct. Biol.* **2008**, *164*, 18–23.
- (89) Otori, M.; Kioshita, T.; Yoshimura, S.; Warizaya, M.; Nakajima, H.; Miyake, H. Role of a cysteine residue in the active site of ERK and the MAPKK family. *Biochem. Biophys. Res. Commun.* **2007**, *353*, 633–637.

- (90) Goto, M.; Chow, J.; Muramoto, K.; Chiba, K.-I.; Yamamoto, S.; Fujita, M.; Obaishi, H.; Tai, K.; Mizui, Y.; Tanaka, L.; Young, D.; Yang, H.; Wang, Y. J.; Shirota, H.; Gusovsky, F. E6201 [(3S,4R,5Z,8S,9S,11E)-14-(ethylamino)-8,9,16-trihydroxy-3,4-dimethyl-3,4,9,19-tetrahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione], a novel kinase inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)-1 and MEK kinase-1: in vitro characterization of its anti-inflammatory and antihyperproliferative activities. *J. Pharmacol. Exp. Ther.* **2009**, *331*, 485–495.
- (91) Muramoto, K.; Goto, M.; Inoue, Y.; Ishii, N.; Chiba, K.-I.; Kuboi, Y.; Omae, T.; Wang, Y. J.; Gusovsky, F.; Shirota, H. E6201, a novel kinase inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1: in vivo effects on cutaneous inflammatory responses by topical administration. *J. Pharmacol. Exp. Ther.* **2010**, *335*, 23–31.
- (92) Barluenga, S.; Jogireddy, R.; Koripelly, G. K.; Winssinger, N. In vivo efficacy of natural product-inspired irreversible kinase inhibitors. *ChemBioChem* **2010**, *11*, 1692–1699.
- (93) Reviewed in the following: Gaestel, M.; Mengel, A.; Bothe, U.; Asadullah, K. Protein kinases as small molecule inhibitor targets in inflammation. *Curr. Med. Chem.* **2007**, *14*, 2214–2234.
- (94) Perez, D. I.; Conde, S.; Perez, C.; Gil, C.; Simon, D.; Wandosell, F.; Moreno, F. J.; Gelpi, J. L.; Luque, F. J.; Martinez, A. Thiénylhalomethylketones: irreversible glycogen synthase kinase 3 inhibitors as useful pharmacological tools. *Bioorg. Med. Chem.* **2009**, *17*, 6914–6925.
- (95) Rossi, A.; Kapahi, P.; Natoli, G.; Takahashi, T.; Chen, Y.; Karin, M.; Santoro, M. G. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of I κ B kinase. *Nature* **2000**, *403*, 103–108.
- (96) Straus, D. S.; Pascual, G.; Li, M.; Welch, J. S.; Ricote, M.; Hsiang, C.-H.; Sengchanthalangsy, L. L.; Ghosh, G.; Glass, C. K. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ inhibits multiple steps in the NF- κ B signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 4844–4849.
- (97) Ji, C.; Kozak, K. R.; Marnett, L. J. I κ B kinase, a molecular target for inhibition by 4-hydroxy-2-nonenal. *J. Biol. Chem.* **2001**, *276*, 18223–18228.
- (98) Kim, B. H.; Lee, J.-Y.; Seo, J. H.; Lee, H. Y.; Ryu, S. Y.; Ahn, B. W.; Lee, C.-K.; Hwang, B. Y.; Han, S.-B.; Kim, Y. Artemisolide is a typical inhibitor of I κ B kinase β targeting cysteine-179 residue and down-regulates NF- κ B-dependent TNF- α expression in LPS-activated macrophages. *Biochem. Biophys. Res. Commun.* **2007**, *361*, 593–598.
- (99) Wagner, T. M.; Mullally, J. E.; Fitzpatrick, F. A. Reactive lipid species from cyclooxygenase-2 inactivate tumor suppressor LKB1/STK11. *J. Biol. Chem.* **2006**, *281*, 2598–2604.
- (100) Toral-Barza, L.; Zhang, W.-G.; Huang, X.; McDonald, L. A.; Salaski, E. J.; Barbieri, L. R.; Ding, W.-D.; Krishnamurthy, G.; Hu, Y. B.; Lucas, J.; Bernan, V. S.; Cai, P.; Levin, J. I.; Mansour, T. S.; Gibbons, J. J.; Abraham, R. T.; Yu, K. Discovery of lactoquinomycin and related pyranonaphthoquinones as potent and allosteric inhibitors of AKT/PKB: mechanistic involvement of AKT catalytic activation loop cysteines. *Mol. Cancer Ther.* **2007**, *6*, 3028–3038.
- (101) Zhang, T.; Inesta-Vaquera, F.; Niepel, M.; Zhang, J.; Ficarro, S. B.; Machleidt, T.; Xie, T.; Marto, J. A.; Kim, N.; Sim, T.; Laughlin, J. D.; Park, H.; LoGrasso, P. V.; Patricelli, M.; Nomanbhoy, T. K.; Sorger, P. K.; Alessi, D. R.; Gray, N. S. Discovery of potent and selective covalent inhibitors of JNK. *Chem. Biol.* **2012**, *19*, 140–154.
- (102) Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem.* **2008**, *77*, 383–414.
- (103) Blair, J. A.; Rauh, D.; Kung, C.; Yun, C.-H.; Fan, Q. W.; Rode, H.; Zhang, C.; Eck, M. J.; Weiss, W. A.; Shokat, K. V. Structure-guided development of affinity probes for tyrosine kinases using chemical genetics. *Nat. Chem. Biol.* **2007**, *3*, 229–238.
- (104) Cohen, M. S.; Hadjivassiliou, H.; Taunton, J. A clickable inhibitor reveals context-dependent autoactivation of p90 RSK. *Nat. Chem. Biol.* **2007**, *3*, 156–160.
- (105) Yuan, H.; Luo, J.; Field, S.; Weissleder, R.; Cantley, L.; Josephson, L. Synthesis and activity of C11-modified wortmannin probes for PI3 kinase. *Bioconjugate Chem.* **2005**, *16*, 669–675.
- (106) Yee, M.-C.; Fas, S. C.; Stohlmeyer, M. M.; Wandless, T. J.; Cimprich, K. A. A cell-permeable, activity-based probe for protein and lipid kinases. *J. Biol. Chem.* **2005**, *280*, 29053–29059.
- (107) Liu, Y.; Wu, J.; Weissig, H.; Betancort, J. M.; Gai, W. Z.; Leventhal, P. S.; Patricelli, M. P.; Samii, B.; Szardenings, A. K.; Shreder, K. R.; Kozarich, J. W. Design and synthesis of AX4697, a bisindolylmaleimide exo-affinity probe that labels protein kinase C α and β . *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5955–5958.
- (108) Koch, A.; Rode, H. R.; Richters, A.; Rauh, D.; Hauf, S. A chemical genetic approach for covalent inhibition of analogue-sensitive aurora kinase. *ACS Chem. Biol.* **2012**, *7*, 723–731.
- (109) Garske, A. L.; Peters, U.; Cortesi, A. T.; Perez, J. L.; Shokat, K. M. Chemical genetic strategy for targeting protein kinases based on covalent complementarity. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 15046–15062.
- (110) Klüter, S.; Simard, J. R.; Rode, H. R.; Grütter, C.; Pawar, V.; Raaijmakers, H. A.; Barf, T. A.; Rabiller, M.; Otterlo, van, W. A. L.; Rauh, D. Characterization of irreversible kinase inhibitors by directly detecting covalent bond formation: a tool for dissecting kinase drug resistance. *ChemBioChem* **2010**, *11*, 2557–2566.
- (111) Oballa, R. M.; Truchon, J. F.; Bayly, C. I.; Chauret, N.; Day, S.; Crane, S.; Berthelette, C. A generally applicable method for assessing the electrophilicity and reactivity of diverse nitrile-containing compounds. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 998–1002.