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Señores

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**

**Dirección de Nuevas Creaciones**

E. S. D.



**Expediente:** **05-052.701**

**Titular:** **BAYER CROPSCIENCE S.A.**

**Asunto:** **CONTESTACIÓN A REQUERIMIENTO.**

**LUIS PATIÑO LEYVA,** mayor de edad, identificado y domiciliado como aparece al pie de mi firma, en mi calidad de apoderado de **BAYER CROPSCIENCE S.A.**, por medio del presente memorial acudo a la Dirección de Nuevas Creaciones de la Superintendencia de Industria y Comercio con el fin de **DAR RESPUESTA** oportuna al Oficio No. **9757**, de acuerdo como sigue:

**CLARIDAD Y SUFICIENCIA**

Con el fin de superar las objeciones que por claridad y suficiencia planteó su Despacho, presento nuevo pliego reivindicatorio compuesto por nueve (9) reivindicaciones, en el cual y siguiendo lo sugerido por el Señor Examinador, se han realizado las siguientes modificaciones. A saber:

i. La nueva reivindicación independiente 1 se ha modificado para definir los sustituyentes $R^1$, $R^2$, $R^3$, $R^4$, $R^5$, $R^6$, $R^{10}$, $R^{11}$, $R^{13}$, $R^{15}$, $R^{19}$ y W según las modalidades de la invención que se encuentran debidamente sustentadas en el capítulo descriptivo. En vista de lo anterior, las definiciones de $R^7$, $R^8$, $R^9$, $R^{12}$, $R^{14}$, $R16^6$, $R^{17}$, $R^{18}$, $R^{20}$, p y q han sido eliminadas. Adicionalmente "$S(O)_pR^{10}$" y "$S(O)_pR^{11}$" fueron reemplazados por "$SR^{10}$" y "$SR^{11}$".

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ii. Las reivindicaciones 5, 6 y 9 han sido eliminadas y las reivindicaciones restantes han sido reenumeradas y las dependencias ajustadas de conformidad.

iii. Las nuevas reivindicaciones dependientes 2 a 9 limitan las definiciones de los sustituyentes característicos de los compuestos de fórmula I, acorde con las modificaciones de la nueva reivindicación independiente 1.

En relación con el sustento de la actividad de los compuestos que se desea proteger, respetuosamente pongo de presente que la descripción tal como fue presentada brinda un soporte ejemplar para las reivindicaciones modificadas.

Dicha descripción revela cinco ejemplos biológicos diferentes (métodos A, B, C, D y E, páginas 72 a 75 y alrededor de 80 compuestos página75), en los cuales se evalúa la actividad parasiticida y plaguicida de los compuestos descritos en el nuevo pliego reivindicatorio. Por lo tanto, considero que las modificaciones a las reivindicaciones son una representación razonable de los compuestos reivindicados y atentamente solicito que las reivindicaciones que se presentan junto con este escrito sean aceptadas.

Finalmente, me permito anotar que las modificaciones realizadas al pliego reivindicatorio no amplían la materia objeto de protección, sino por el contrario limitan la misma, permitiendo una interpretación inequívoca del alcance de protección y su comparación con el estado del arte. En consecuencia, las mismas se encuentran en concordancia con los lineamientos de los artículos 28, 30 y 34 de la D.486.

Así las cosas, las modificaciones al nuevo pliego reivindicatorio dan por superadas las objeciones que por claridad y suficiencia planteó su Despacho, puesto que se están reivindicando compuestos, procesos y composiciones de conformidad con el artículo 14 de la D. 486.

Por consiguiente, atentamente solicito al señor Examinador acepte las modificaciones propuestas y proceda con el respectivo estudio de patentabilidad.

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**NIVEL INVENTIVO**

El Despacho considera que los documentos D1 y D2 afectan el nivel inventivo de los compuestos de la solicitud en estudio, porque se trata de compuestos análogos que poseen la misma actividad y los cuales son aplicables como pesticidas tanto de uso agrícola como veterinario, de manera que los compuestos objeto de la solicitud en estudio se pueden derivar del arte previo.

Establece el artículo 18:

> "Art. 18
> Se *considerará que una invención tiene nivel inventivo, 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* ".

A este respecto, el Manual Andino[1] en la sección 10.8. referente al nivel inventivo en áreas específicas de la tecnología, establece que en el área de la Química un compuesto o una composición química (preparación farmacéutica) tienen nivel inventivo si se cumple **alguno** de los dos requisitos siguientes :

> "- *el compuesto tenga una estructura inesperada (caso poco frecuente);*
> ***o***
> ***- presenta un efecto inesperado****, (es el caso mas frecuente sobre todo si el compuesto es similar a otros del estado de la técnica. El efecto inesperado puede ser completamente diferente de los descritos para los compuestos similares conocidos, o bien ser igual pero con una mejora en los resultados)" (Negrita fuera del texto).*

Continúa dicho Manual:

> *"¿Qué es necesario considerar para que un compuesto nuevo tengo nivel inventivo?*
> *Respuesta:*
> ***1. que el compuesto posea una estructura inesperada; o***

---

1 Manual Andino de Patentes. Pág.82.

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***2. que el compuesto exhiba un uso o efecto sorprendente***

*b. 1 Estructura inesperada*

*Es un caso poco frecuente. La estructura del nuevo compuesto no hubiera podido ser deducida por el técnico medio en la materia. El examinador no necesita examinar si este compuesto posee o no un uso o efecto sorprendente puesto que la mera estructura ya le confiere nivel inventivo*

*b.2 Efecto o uso no esperado*

*b.2.1 La existencia de un efecto o uso sorprendente es la manera más común de establecer altura inventiva para compuestos nuevos, particularmente cuando tienen estructuras muy próximas a las del estado de la técnica.*

*b.2.2* ***Tipos de efecto sorprendente: (i) completamente diferente de los usos o efectos conocidos de compuestos descritos en el estado de la técnica; y (ii) una mejora sustancial de un efecto de las misma índole que exhiba un compuesto conocido del estado de la técnica más próximo.***

*b.2.3 Uso o efecto no descrito previamente: un uso o efecto se considera sorprende cuando para los compuestos descritos en el estado de la técnica no haya sido descrito ningún uso o efecto y éste no pueda ser derivable del conocimiento general."*[2] Negrita fuera del texto.

Por lo tanto, a partir de los lineamientos del artículo 18 y los conceptos expuestos por el Manual Andino procedo a demostrar que la invención de mi representada no se derivaría de manera obvia y evidente de D1 y D2, por cuanto las características técnicas divulgadas en dichos documentos no enseñan ni sugieren la invención reivindicada por mi representada, la cual tiene efecto técnico sorprendente.

D1 revela de forma general amplia un compuesto 1-fenilopirazol como se muestra a continuación, en donde Z en la posición - 5 del anillo de pirazol

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[2] Ibídem. Pág 83

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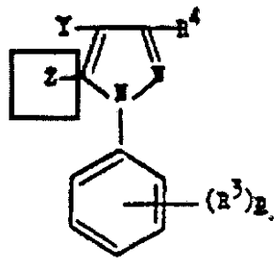
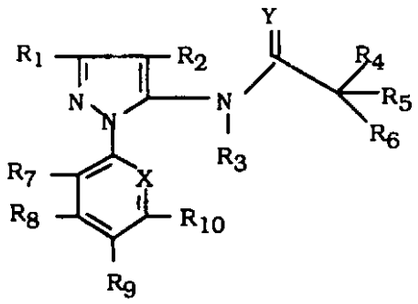
puede ser alquilosulfenilamino. En particular, en D1 se revelan acilaminopirazoles y sulfenilaminopirazoles.



D2 por su parte describe de forma general compuestos de pirazol sustituidos y métodos de utilización para el control de parásitos tales como helmintos y nematodos en animales. Específicamente, D2 describe compuestos 1-arilo-5-(sustituido con alquilo(tio)amido)pirazol que se muestran a continuación (véase también la página 2 de D2).



(I)

A pesar del extremadamente amplio rango y enorme número de posibles compuestos revelados, tanto en D1 y D2, dichos documentos no sugieren ni revelan los compuestos de la invención reivindicada por mi representada. **En particular, D1 y D2 no enseñan ni sugieren las sustituciones de sulfonilamido en la posición - 5 del anillo de pirazol que caracteriza a los compuestos de la presente invención**. En consecuencia, los efectos que tendrán los compuestos en su papel como agentes pesticidas no serán los mismos, y probablemente ni siquiera serán parecidos. Dada la falta de predictibilidad en las técnicas de la química, una persona que tenga habilidades normales en la técnica no necesariamente utilizaría D1 o D2 como motivación para llegar a la presente invención.

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En este sentido, respetuosamente pongo de presente que un experto en la materia basado en sus conocimientos puede afirmar que la modificación de un compuesto farmacéutico activo conocido, tiene uno de cuatro resultados en términos de actividad. A saber, (i) el compuesto modificado puede no tener actividad o tener una actividad diferente; (ii) el compuesto modificado puede tener un nivel de actividad, reducido pero todavía detectable; (iii) el compuesto modificado puede tener el mismo nivel de actividad; y (iv) el compuesto modificado puede tener un nivel de actividad incrementado.

Por lo tanto, sin un entendimiento muy detallado de la relación estructura-actividad de una clase particular de compuestos, no es posible predecir cuál de los cuatro descritos será el resultado que se observará para cualquier compuesto particular probado – sin importar la cercanía estructural de dicho compuesto con relación al compuesto activo conocido.

En el presente caso, se observa que un entendimiento detallado de la relación estructura-actividad de los compuestos reivindicados no pudo haber sido derivado simplemente del estudio de D1 y D2. Por consiguiente, una persona versada en la materia, partiendo de las anterioridades citadas no habría esperado que la diferencia en la sustitución de sulfonilamido en la posición - 5 del anillo de pirazol diera lugar a compuestos con actividad parasiticida y plaguicida mejorada, como los que se reivindican en la presente invención.

En este respecto, pongo en evidencia que a partir de las pruebas aportadas se observa claramente que los compuestos reivindicados son parasiticidas y plaguicidas efectivos en concentraciones tan bajas como a 5 ppm. (Método A, página 72 de la descripción).

Efectos diferentes de compuestos que son similares en su estructura es un hecho bien conocido. Por ejemplo, Kubinyi describe ligeras modificaciones estructurales de análogos que pueden destruir la actividad biológica (*véase Anexo 2)*. En efecto, Kubinyi describe compuestos similares que son inhibidores de termolisina que tienen capacidades bastante diferentes, demostrando como ligeras modificaciones estructurales pueden cambiar el objetivo de enlace resultando en un efecto biológico diferente, y en efectos opuestos de los enantiómeros de barbitúricos.

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En este mismo sentido, Roe reporta que estudios iníciales de hidrocarburos policíclicos aromáticos revelaron propiedades que inducen al cáncer en muchos, mientras que compuestos estrechamente relacionados eran inactivos para inducir el cáncer (*véase Anexo 3, página 3, párrafo 3 y los diagramas*).

Lieberman *et al.*, muestra diferentes efectos entre los dos isótopos estables del litio (*p. e.*, Li-7 y Li-6). A ratones que se les suministró ya sea Li-7 o Li-N mostraron menos ganancia de peso que los ratones control. No obstante, a los ratones que se les suministró Li-6 perdieron peso y mostraron un marcado decrecimiento en la motilidad y un aumento en su mortandad. Resultados similares fueron vistos en ratas con respecto a la mortandad. Estudios en gatos indicaron que el efecto diferente del Li-6 pudo ser debido a que este se comporta de forma diferente en la barrera de sangre del cerebro (concentraciones más altas de Li-6 en el plasma y en los fluidos cerebroespinales) *(véase Anexo 4).*

Estos ejemplos resaltan un fenómeno que se encuentra bien documentado en la literatura científica, los cuales ponen en evidencia que la simple similitud estructural entre compuestos (o elementos) no necesariamente indica una actividad biológica similar.

Por lo tanto, toda vez que no existe referencia en D1 o D2 con respecto a los compuestos reivindicados, es un error concluir – diferente a un análisis ex post facto – que la actividad de los compuestos descritas en dichos documentos habría sido preservada por los de la presente invención, teniendo en cuenta las sustitución de sulfonilamido en la posición - 5 del anillo de pirazol.

En conclusión, más allá de evidenciar un amplio número de compuestos, D1 o D2 fallan en motivar a una persona con habilidades comunes en la técnica a reemplazar el grupo carbonilamino que se encuentra en la posición - 5 del anillo de pirazol con un grupo sulfonilamino, como actualmente se reivindica. Adicionalmente, dichos documentos tampoco pone en evidencia datos que demuestren o sugieran que dichos compuestos tendrían actividad parasiticida y plaguicida.

Por consiguiente, toda vez que no existe sugerencia en D1 o D2 con respecto a las modificaciones de los compuestos aquí reivindicados; se puede concluir,

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que D1 y D2 (solos o en combinación) no anticipan la materia de la presente invención y por lo tanto no afectan el nivel inventivo de la misma.

Finalmente, respetuosamente pongo de presente al Despacho que si bien comprendemos que el mismo es autónomo en cuanto al estudio de patentabilidad se refiere, el pliego reivindicatorio de la presente invención ha sido motivo de patente en Europa (Nº concesión: EP-1569910), país que comparte los principios de patentabilidad de la Decisión 486.

**ANEXOS**

1. Nuevo pliego reivindicatorio compuestos por nueve (9) reivindicaciones.
2. KUBINYI, Hugo. "Similarity and Dissimilarity: A Medicinal Chemist's View". Perspective in Drug Discovery and design, 9/10/11:225-252, 1998.
3. ROE, Francis J.C."Cancer Including Agents". Science Journal Reprint. Ref. No. 66/108, May 1966.
4. Liberman et al. "Stable Isotopes of Lithium: Dissimilar Biochemical and Behavioral Effect".

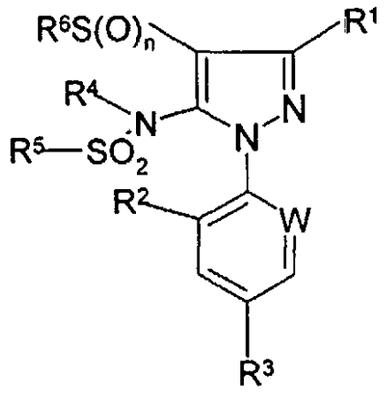
De acuerdo con todo lo expuesto, solicito en forma muy comedida y respetuosa a la Sr. Jefe de la Dirección de Nuevas Creaciones se sirva tener en cuenta las manifestaciones y documentos aportados junto con el presente memorial y, de conformidad con ello, se sirva continuar con el trámite de la solicitud de patente de la referencia.

Del señor Jefe de la División de Nuevas Creaciones,

**LUIS PATIÑO LEYVA**
**C.C. 2.930.616 de Bogotá**
**T.P.A. 2698 del C.S. de la J.**
**Cr 9. # 74 -08 (305)**
**Bogotá D.C.**

# REIVINDICACIONES

1. Un compuesto de fórmula (i):




(I)

En el que

$R^1$ es CN o $CSNH_2$

W es C-halógeno

$R^2$ es halógeno

$R^3$ es $(C_1 - C_6)$ - haloa quilo

$R^4$ es H o $(C_1 - C_6)$ - alquilo no sustituido

$R^5$ es $(C_3 - C_7)$ - cicloalquilo, $(C_2 - C_6)$ - alquenilo, $(C_2 - C_6)$ - haloalquenilo, $R^{13}$, o $CH=CH-R^{15}$; o es $(C_1 - C_{15})$ - alquilo no sustituido o sustitu do por uno o más radicales seleccionados de un grupo que consiste de halógeno, $(C_3 - C_7)$ - ciclo alquilo, $SR^{10}$, $SR^{11}$, $R^{13}$, o $R^{10}$,

$R^6$ es $CF_3$

$R^{10}$ es heterociclilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno

$R^{11}$ es $(C_3 - C_7)$ - cicloalquilo

$R^{13}$ es arilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno, $(C_1 - C_6)$ - alquilo, $(C_1 - C_6)$ - haloalquilo, $(C_1 - C_6)$ - alcoxi, $(C_1 - C_6)$ - haloalcoxi, $NO_2$, $R^{15}$, y $CO_2R^{19}$,

$R^{15}$ es fenilo o naftilo cuyos grupos están no sustituidos o sustitu dos por uno o más radicales seleccionados de un grupo que consiste de halógeno

$R^{19}$ es $(C_1 - C_6)$ - alquilo, y

n es 0, 1, o 2.

2. Un compuesto o una de sus sales como se reivindica en la reivindicación 1 en el o la que

$R^1$ es CN o $CSNH_2$

W es C - halógeno

$R^2$ es Cl

$R^3$ es $CF_3$

$R^4$ es H o $(C_1 - C_6)$ - alquilo no sustituido

$R^5$ es $(C_3 - C_7)$ - cicloalquilo, $(C_2 - C_6)$ - alquenilo, $(C_2 - C_6)$ - haloalquenilo, $R^{13}$, o CH=CH-$R^{15}$; o es $(C_1 - C_6)$ - alquilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno

$R^6$ es $CF_3$

$R^{10}$ es heterociclilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno

$R^{11}$ es fenilo

$R^{13}$ es arilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno, $(C_1 - C_6)$ - alquilo, $(C_1 - C_6)$ - haloalquilo, $(C_1 - C_6)$ - alcoxi, $(C_1 - C_6)$ - haloalcoxi, $NO_2$, y $R^{15}$

$R^{15}$ es fenilo o naftilo cuyos grupos están no sustituidos o sustituidos por uno o más radicales seleccionados de un grupo que consiste de halógeno

n es 0, 1, o 2.

3. Un compuesto o una de sus sales como se reivindica en la reivindicación 1 ó 2 en el o la que $R^1$ es CN.

4. Un compuesto o una de sus sales como se reivindica en la reivindicación 1, 2 ó 3 en el o la que W es C-Cl.
5. Un compuesto o una de sus sales como se reivindica en cualquiera de las reivindicaciones 1 a 4 en donde $R^4$ es H o $CH_3$.

6. Un compuesto o una de sus sales como se reivindica en cualquiera de las reivindicaciones 1 a 4 o 5 en donde $R^5$ es $R^{13}$ o $(C_1 - C_6)$ - alquilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno, $R^{13}$, y $R^{10}$.

7. Un compuesto o una de sus sales como se reivindica en cualquiera de las reivindicaciones 1 a 6, en el o la que

$R^1$ es CN

W es C-Cl

$R^2$ es Cl

$R^3$ es $CF_3$

$R^4$ es H o $CH_3$

$R^5$ es $R^{13}$ o $(C_1 - C_4)$ - alquilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno, $R^{13}$, y $R^{10}$

$R^6$ es $CF_3$

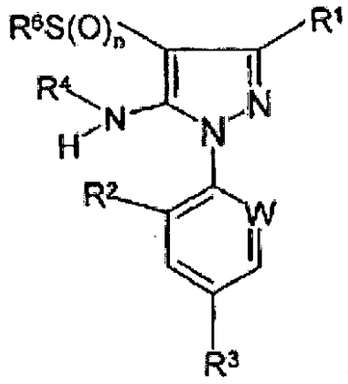
$R^{10}$ es tienilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno

$R^{13}$ es fenilo no sustituido o sustituido por uno o más radicales seleccionados de un grupo que consiste de halógeno, $(C_1 - C_2)$ - alquilo, $(C_1 - C_2)$ - haloalquilo, $(C_1 - C_2)$ - alcoxi, y $(C_1 - C_2)$ - haloalcoxi

n es 0, 1, o 2

8. Un procedimiento para la preparación de un compuesto de la fórmula (I) o una de sus sales como se definen en una cualquiera de las reivindicaciones 1 a 7, cuyo procedimiento comprende:

a) cuando $R^1$, $R^2$, $R^3$, $R^4$, $R^5$, $R^6$, W y n son como se han definido en la reivindicación 1, hacer reaccionar un compuesto de la fórmula (II):




(II)

con un compuesto de la fórmula (III):

$$R^5\text{-}SO_2L^1$$

(III)

en la que $R^5$ es como se ha definido en la reivindicación 1 y $L^1$ es halógeno; en la presencia de una base o solvente a una temperatura de 0 °C a 100°C; o

b) cuando $R^1$, $R^2$, $R^3$, $R^5$, $R^6$, W y n son como se han definido en la reivindicación 1 y $R^4$ es como se ha definido en la reivindicación 1 con la

exclusión de hidrógeno, la alquilación o acilación del correspondiente compuesto de la fórmula (I) en la que $R^4$ es hidrógeno; o

c) cuando $R^1$, $R^2$, $R^3$, $R^4$, $R^5$, $R^6$, W y n son como se han definido en la reivindicación 1, y n es 1 ó 2, oxidar un correspondiente compuesto de fórmula (I) en el que n es 0 ó 1 en la presencia de un perácido en un solvente a una temperatura desde 0 °C hasta la temperatura de reflujo del solvente; y

d) si se desea, convertir un compuesto resultante de la fórmula (I) en una de sus sales aceptables como plaguicidas.

9. Una composición que comprende un compuesto de la fórmula (I) o una de sus sales aceptables como plaguicidas como se han definido en una cualquiera de las reivindicaciones 1 a 8, en asociación con un diluyente o vehículo y/o un agente tensioactivo aceptable como plaguicida.

*Perspectives in Drug Discovery and Design*, 9/10/11: 225-252, 1998.
*KLUWER/ESCOM*


# Similarity and Dissimilarity: A Medicinal Chemist's View

**Hugo Kubinyi**
*Drug Design, BASF AG, D-67056 Ludwigshafen, Germany*

Several 3D approaches discussed in this volume describe methods for the analysis and quantitative description of chemical similarity. The underlying concept is that chemical similarity is reflected by similar biological activities — i.e. chemically closely related analogs should be related in their mode of action, as well as in their relative potencies. This fundamental assumption has, indeed, been used in medicinal chemistry research, and has led to many valuable drugs.

However, chemical similarity may have different facets if a computer chemist or a medicinal chemist look at the compounds. There is no argument that for maximal affinity a ligand of a biological macromolecule has to fit the binding pocket geometrically and that hydrophobic surfaces of the ligand and the binding site have to be complementary. The functional groups of the ligand need a separate consideration. For lipophilicity, there is no significant difference between, e.g. –O– and –NH– in an organic molecule; for ionization, there is a big difference whether the nitrogen atom is part of a basic group (an amine) or a neutral group (e.g. in an amide); and for binding, potency differences of several orders of magnitude may result from the exchange of the hydrogen bond acceptor –O– against a donor function –NH–.

## 1. Similarity as a Design Principle in Lead Optimization

Nearly all drugs result from the optimization of a lead structure. Sources of such leads are natural products from plants or microorganisms, synthetic chemicals or their intermediates, hits from (high-throughput) screening of in-house and combinatorial libraries, rational concepts from a biochemical pathway or the unexpected observation of a therapeutically useful side-effect of a drug. Most often, the biological activity of a lead structure is neither optimal, with respect to its efficacy, nor with respect to specificity, bioavailability, pharmacokinetics, toxic and other side-effects. Chemists perform more or less systematic variations of lead structures, using the experience of about 100 years of medicinal chemistry and the results of (quantitative) structure–activity relationships.

The principle of bioisosteric replacement of functional groups serves as a successful optimization strategy [1–3]. Its systematic application has resulted in a broad variety of therapeutically used drugs, many of them finally having the desired combination of favorable properties. A few examples of typical but different consequences of isosteric replacement of atoms or groups are illustrated by compounds **1**–**3** (Fig. 1), some others with unexpected effects on biological activities are discussed in later sections of this chapter.

In their attempts to optimize lead structures, medicinal chemists intuitively follow the principles of evolution. In genetic and evolutionary algorithms, randomly generated starting models (the lead structures) are reproduced involving random mutations and crossover (the chemical variation of the structures). Better models (compounds) are kept for further modification; worse ones are discarded. The biological activity, in later

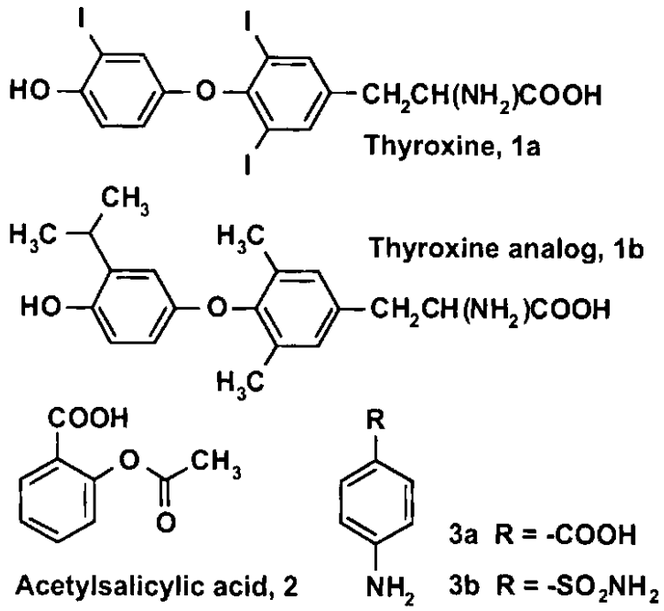



*Fig. 1. Different effects of isosteric replacement. (a) The substitution of all three iodine atoms of thyroxine* **1a** *leads to an analog* **1b** *which still acts as a thyromimetic agent. (b) Replacement of an ester function, -O CO-, by an amide function, NH-CO-, most often produces analogs with higher metabolic stability; if this replacement is done in acetylsalicylic acid* **2**, *an inactive analog results because the amide is no longer able to transfer an acetyl group to a certain serine residue of cyclooxygenase. (c) p-Aminobenzoic acid* **3a** *is an essential metabolite of microorganisms; if the acid group, –COOH, is replaced by a sulfonamide group, $-SO_2NH_2$, the antimetabolite sulfanilamide* **3b** *results (active metabolite of the antibacterial sulfonamide sulfamidochrysoidine).*

stages a selectivity index or some other biological property, serves as the 'fitness function' for the 'survival' of certain structural entities. That genetic and evolutionary algorithms, indeed, reduce the effort in searching the most active analogs has recently been confirmed by dedicated investigations (e.g. [4,5]).

## 2. The Biological Activity of a Ligand Depends on its Flexibility

Other common structural modifications in the optimization of a lead structure are the dissection of rings or the rigidification of flexible molecules. Molecules with several rotatable bonds may adopt many different geometries — some of them being favorable because of low internal energies, others being less favorable because of van der Waals or electrostatic repulsions between non-bonded atoms or groups. If different conformations of such molecules are 'frozen' by closing rings between certain atoms, either one of two very different consequences results. If the frozen conformation differs from the

bioactive conformation of the flexible lead or if the added atoms interfer with the binding, biological activity will be more or less destroyed. If the ring closure stabilizes the bioactive conformation, usually a significant increase in biological activity results. This comes from the fact that the binding of a flexible analog is entropically unfavorable, due to a loss of rotational degrees of freedom, whereas the rigid analog has already lost its flexibility prior to binding. Two examples of highly similar, flexible and rigid analogs are the pairs **4** and **5** [6] and **6** and **7** [7], respectively, where the rigid analogs are significantly more active than their flexible counterparts (Fig. 2).

The computer program CAVEAT was developed for the design of rigid analogs which bear a pharmacophore in a certain geometry [8]. CAVEAT starts from a struc-

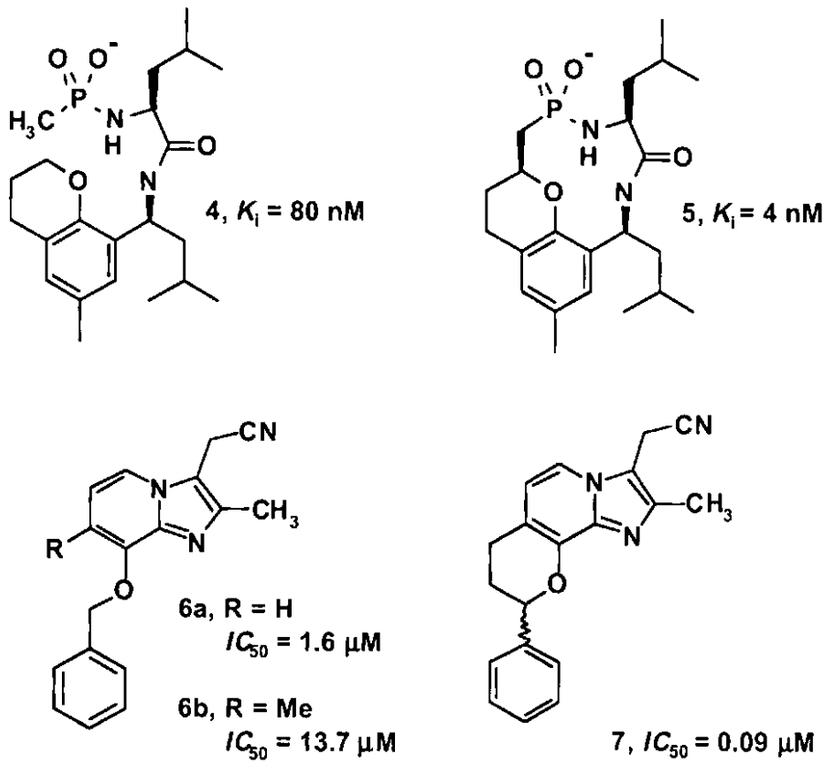



*Fig. 2. Rigidification of a bioactive conformation significantly increases biological activities. (a) If an additional ring is introduced into the thermolysin inhibitor* **4** *to produce analog* **5**, *a 20-fold increase in affinity is observed. (b) Introduction of a methyl group into the $H^+/K^+$-ATPase inhibitor* **6a** *reduces biological activity by a factor of 8, most probably due to a destabilization of the bioactive conformation; if the substituent* R *in* **6b** *is extended to a new ring (compound* **7**), *which fixes the bioactive conformation, the biological activity of* **6b** *is enhanced by a factor of 150.*

tural hypothesis or from the known 3D structure of a ligand, e.g. a polypeptide, and extracts vectors of residues that participate in binding. In a peptide, these vectors are e.g. the $C_\alpha$–$C_\beta$ bonds of the amino acid side chains. Then the program identifies ring systems that are suited to accommodate these residues in exactly the same relative geometries.

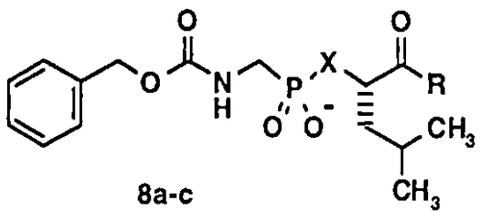
A lead structure, where a significant increase in binding affinity is achieved by steric constraints, not by ring closure, has been described by Kaplan and Bartlett [9]. In the flexible phosphonate tripeptide Cbz–Phe–Ala[$PO_2^-$]–O–CH($CH_3$)COOH (= Z–Phe–Ala$^P$–(O)–Ala; $^P$ indicates a $PO_2^-$ residue instead of a C=O group), inhibitory activities against the zinc protease carboxypeptidase A increase significantly with the stepwise introduction of bulky residues. In the series Z–Phe–Ala$^P$–(O)–Ala, Z–Phe–Ala$^P$–(O)–Phe and Z–Phe–Val$^P$–(O)–Phe, affinities increase from 56 nM to 0.001 nM and 0.000010–0.000027 nMol (i.e. 10–27 fMol). The more active analogs have a higher lipophilicity, due to the additional phenyl group in phenylalanine, as compared to alanine, and due to the isopropyl group instead of the methyl group of the C-terminal lactic acid residue. But in this case, the affinity difference of about six orders of magnitude cannot be attributed only to the increase in lipophilicity which changes by less than three orders of magnitude. It must also result from the conformational constraint imposed on both phenylalanines by the central valine and which, by fortune, stabilizes the bioactive conformation. In terms of 'chemical similarity', all three analogs are closely related; with respect to their internal flexibility and their preferred conformations, they are not.

## 3. Biological Potencies of Similar Molecules

For compounds with comparable flexibility, most often similar analogs have also similar biological potencies. That this need not always be the case can be seen by a comparison of three thermolysin inhibitors **8a–c** (Fig. 3) [10,11]. All analogs **8a** and **c**, with X = –NH– and –$CH_2$–, are about 1000 times more potent than the X = –O– analogs **8b** (R = OH or different amino acids). The explanation for this effect can be easily derived from the 3D structure of the complex of thermolysin with the inhibitor **8a** (R = –Leu–OH). If X is an –NH– group (series **8a**), a hydrogen bond is formed between this group and the oxygen atom of an alanine carbonyl group. In the –O– analog **8b**, this hydrogen bond cannot be formed; in addition, an electrostatic repulsion between the two oxygen atoms results. The biological activity of the –$CH_2$– analog **8c** has been predicted [12] to be comparable to the –NH– analogs and much higher than for the –O– analogs. This was later confirmed by the synthesis of the inhibitors **8c** [11].

A similar but less pronounced effect is observed for the thrombin inhibitors **9a–c** (Fig. 4) [13]; in this case, the non-bonded contact between the –X– group of the ligand and the carbonyl group of Gly 216 in the binding site of thrombin is responsible for the structure–activity relationship. If, on the other hand, the carbonyl group of **10a**, which forms a hydrogen bond with the –NH– group of Gly 216, is replaced by –$CH_2$– (**10b**), the affinity is reduced by more than three orders of magnitude (Fig. 4) [13].

Neutral endopeptidase 24.11 (NEP 24.11; enkephalinase) is a zinc protease with some homology to thermolysin. The NEP inhibitors **11a–d** (Fig. 5) show a different



**8a-c**

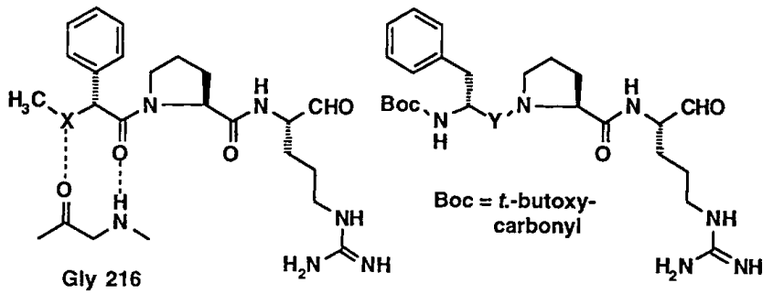
**Binding constants $K_i$ in μM**

| R | X = | 8a -NH- | 8b -O- | 8c $-CH_2-$ |
|---|---|---|---|---|
| -OH | | 0.76 | 660 | 1.4 |
| -Gly-OH | | 0.27 | 230 | 0.3 |
| -Leu-OH | | 0.01 | 9 | 0.01 |

*Fig. 3. The X = –NH– group of the thermolysin inhibitors* ***8a*** *forms a hydrogen-bond with the carbonyl oxygen atom of Ala 113; this affinity-enhancing effect is only moderated by desolvation of the ligand in going from the free to the bound state. All oxygen analogs* ***8b*** *are much less active, because they cannot form such a hydrogen-bond; in addition, there is the unfavorable desolvation effect and an electrostatic repulsion between the oxygen atoms of the ligand and the binding site. Like the –O– analogs* ***8b****, the –$CH_2$– analogs* ***8c*** *cannot form the hydrogen-bond to Ala 113, but this disadvantage is counterbalanced by the effect that no desolvation of the $CH_2$ group of the ligand and no electrostatic repulsion take place. The differences between X = –NH–, –O– and –$CH_2$– are identically observed whether R = –OH, –Gly–OH or –Leu–OH.*

structure–activity relationship, as compared to the preceeding examples. Here the lipophilic –$CH_2$– and –S– analogs are much less active than the –NH– and –O– analogs, which both have comparable biological activities [14]. As long as the 3D structure of NEP 24.11 remains unknown, no explanation can be given for this effect.

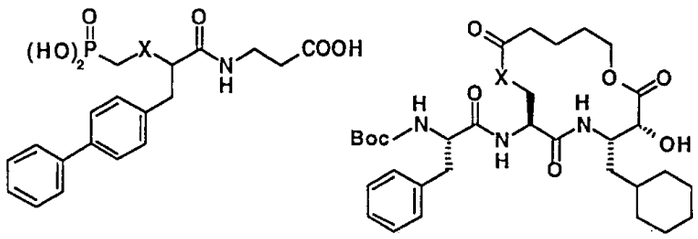
An unexpected structure–activity relationship has been observed for the macrocyclic renin inhibitors **12a** and **b** (Fig. 5) [15]. Modelling of the complex of **12a** with the aspartyl protease renin indicated that X = –NH– should form a hydrogen bond with one of the Asp 226 oxygen atoms. Thus, the replacement of –NH– by –O– should reduce the affinity. The opposite was observed; the affinity of analog **12b** is increased by more than two orders of magnitude. The authors discuss several possible explanations for this observation but finally conclude: 'we have attempted to find a plausible basis for this surprising reversal in potency, but without much success ... the comparison [of both analogs] is therefore intrinsically more difficult, and will constitute a more demanding test for thermodynamic simulation techniques [as compared to the thermolysin inhibitors, Fig. 3]'.




9a, X = -NH- $IC_{50}$ = 0.009 µg/ml
b, X = -O- $IC_{50}$ = 0.13 µg/ml
c, X = $-CH_2-$ $IC_{50}$ = 0.07 µg/ml

10a, Y = -CO- $IC_{50}$ = 0.028 µg/ml
b, Y = $-CH_2-$ $IC_{50}$ = 52 µg/ml

*Fig. 4. The inhibitor 9a forms two hydrogen-bonds with glycine 216 of the serine protease thrombin. As compared to the thermolysin inhibitors 8a–c (Fig. 3), a similar but much less pronounced effect is observed by structural variation of the group X in the thrombin inhibitors 9a–c. If, on the other hand, the carbonyl group of 10a is replaced by a methylene group, the resulting pseudopeptide 10b shows a 2000-fold weaker inhibitory activity.*



11a, X = -NH- $IC_{50}$ = 1.6 nM
b, X = -O- $IC_{50}$ = 10 nM
c, X = -$CH_2$- $IC_{50}$ = 1 000 nM
d, X = -S- $IC_{50}$ = 800 nM

12a, X = -NH- $IC_{50}$ = 590 nM
b, X = -O- $IC_{50}$ = 3.4 nM

*Fig. 5. The NEP 24.11 inhibitors 11a and b are much more potent than the more lipophilic analogs 11c and d. The macrocyclic renin inhibitor 12a is less potent than its oxygen analog 12b, although its NH group should form a favorable hydrogen-bond with the carboxylate of Asp 226 of the enzyme.*

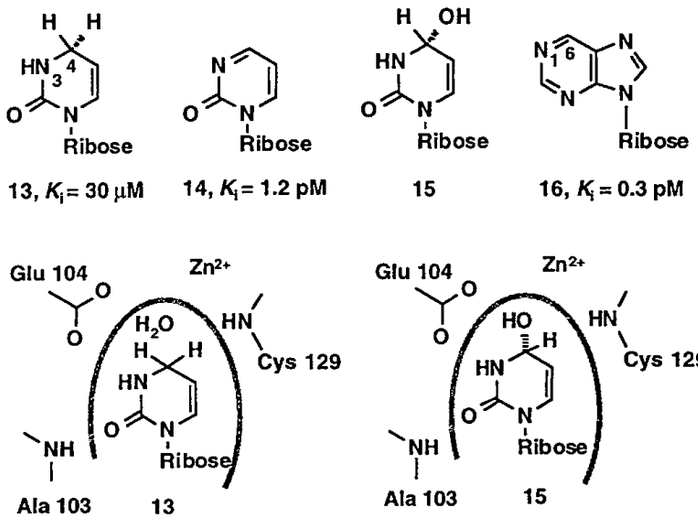



*Fig. 6. 3,4-Dihydrozebularine 13 is a weak inhibitor of cytidine deaminase. Zebularine 14 interacts with the enzyme as the 3,4-hydrate 15, as confirmed by X-ray structure analysis. The favorable interactions of the hydroxy group enhance the biological activity by more than seven orders of magnitude. Nebularine 16, a strong adenosine deaminase inhibitor, is also proposed to interact as the 1,6-hydrate.*

There are many examples in literature where the introduction of an –OH group into a ligand either causes an increase or a decrease of biological affinities. From a theoretical point of view, this is not surprising. If the new –OH group forms hydrogen bonds with polar groups at the binding site (either as a hydrogen bond donor or as an acceptor), the net free energy depends on the balance between the desolvation energies of the water shells at the surfaces of the ligand and the binding site, as compared to the energy of the formed hydrogen bond/s and the entropy gain by the release of some water molecules. Certain tightly bound water molecules in the binding cavity of a protein (usually seen in the X-ray structures), e.g. those which form more than two hydrogen bonds to the protein, are not easily removed. The attempt to introduce an –OH group into the ligand, to replace such a water molecule, must necessarily fail.

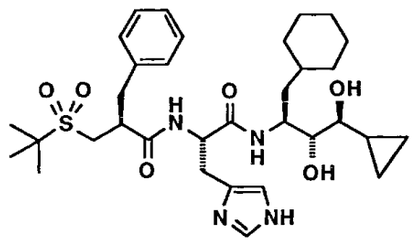
An example where this is not the case, and where significantly enhanced binding affinities result after the introduction of such a hydroxy group, are the cytidine and adenosine deaminase inhibitors **13–16** (Fig. 6) [16,17]. In this special case, the resulting affinity differences are 7 to 8 orders of magnitude!

## 4. Biological Potencies *versus* Similar Biological Targets

Functionally corresponding proteins from different species have identical or very similar 3D structures, but they normally differ in their amino acid composition. Although they always show, dependent on the evolutionary relationship between the two species, a certain degree of homology, structure–activity relationships may significantly differ, even after the replacement of just one single amino acid by another one. This has been shown, for example, by a comparison of rat and human 5-HT receptors [18]. Although both have about 90% identity and more than 95% homology in their amino acid sequences, the binding affinities of a series of 5-HT receptor ligands and $\beta$-adrenergic blocking agents are not related ($n = 10$; $r = 0.27$). If one (!) amino acid of the human receptor, i.e. threonine 355, is replaced by the corresponding amino acid of the rat receptor, i.e. asparagine, the binding affinities of the ligands change significantly. Now the human mutant 5-HT receptor behaves like a rat receptor; all affinities to both receptors are more or less identical ($r = 0.98$) [18].

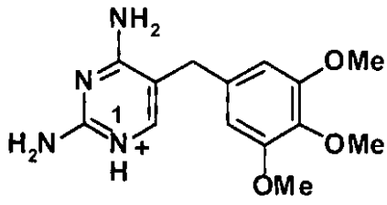
In the past, new compounds have always been tested in animals before they could be applied to humans. Human proteins were not available, except in rare cases. Only for those proteins that could be extracted from human material, e.g. hemoglobin or thrombin from blood, could one predict the human biological activity of a new drug from *in vitro* studies, prior to animal studies. With the progress in gene technology, it is now possible to produce human proteins in sufficient quantities to establish test models. Thus, their biological activity in humans can be forecasted from investigations at the molecular level. How important this is, can be illustrated, e.g. by the activities of the renin inhibitor remikiren **17** against the renins of different species (Fig. 7) [19].

Different QSAR models for dihydrofolate reductase (DHFR) inhibitors, chemically related to the antibacterial drug trimethoprim **18** (Fig. 8), describe the inhibitory activities *versus E. coli* and *L. casei* DHFR [20]. Whereas in the case of *E. coli* DHFR, the 3-, 4- and 5-substituents at the benzyl group contribute to biological activities, only the




17, $IC_{50}$ = 0.8 nM (human)
1.0-1.7 nM (monkeys)
107 nM (dog)
3 600 nM (rat)

*Fig. 7. Remikiren 17 is a much stronger inhibitor versus human and monkey renins, as compared to dog and rat renins. If this compound had only been tested in small laboratory animals (as was the case before gene technology could be applied in drug research), it would not have been considered for further development, due to its weak biological activity.*



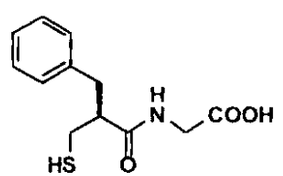

**Trimethoprim, 18**
**(protonated form)**

*Fig. 8. Trimethoprim 18 is proposed to interact with dihydrofolate reductase in its protonated form, where N1 changes its character from a hydrogen-bond acceptor to a hydrogen-bond donor. Due to the positive charge of the ligand, the exchange of negatively charged amino acids of the protein to neutral amino acids and of neutral to positively charged amino acids reduce the affinity of the ligand.*

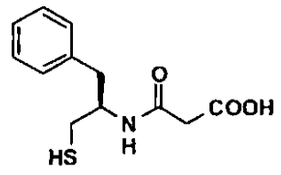
3- and 4-substituents are relevant for the activities against *L. casei* DHFR. Several years later, the X-ray analyses of both enzymes explained these differences: in *L. casei* DHFR, there is a much narrower pocket, due to a bulky leucine in the binding site, as compared to a flexible methionine side chain in *E. coli* DHFR [20].

Whereas many differences in the binding affinities to related biological targets can be attributed to different non-covalent interactions with the amino acids of the binding sites, such effects may also result from more distant changes in a protein. Trimethoprim **18** is thought to bind to DHFR in its positively charged form. An *E. coli* mutant, where the negatively charged glutamate residue 118 is replaced by a neutral glutamine, has a 4.5-fold lower affinity for trimethoprim, despite the fact that the modified group is about 15 Å apart from the charged N1 of trimethoprim. An even more pronounced effect is observed in a double mutant (Glu 118 Gln, Leu 28 Arg): the affinity of trimethoprim is reduced by a factor of 190, although the positive charge of the arginine residue is about 8 Å apart from the positive charge of the ligand [21]. This reduction of binding affinities has been used to explain the selectivity of 5 to 6 orders of magnitude of trimethoprim against bacterial DHFRs, as compared to avian and mammalian DHFRs. In chicken DHFR, seven amino acids in the environment of the binding site (not in the binding site itself) have changed charge from negative to neutral or from neutral to positive, as compared to *E. coli* DHFR. If one compares the effects observed in the *E. coli* double mutant with these figures, the changes seem to be sufficient to explain the observed effect [21].

Thiorphan **19** and its *retro-inverso* peptide, *retro*-thiorphan **20** (Fig. 9), are inhibitors of the structurally related zinc proteases thermolysin and NEP 24.11. Although the affinities of the ligand pair differ, from enzyme to enzyme, by three orders of magnitude, there are no significant differences between them [22]. Thus, they may be considered to be 'similar', which was also confirmed by the X-ray structure analyses of their complexes with thermolysin; both compounds bind in an analogous manner and have corresponding interactions with the protein [23]. On the other hand, their activities

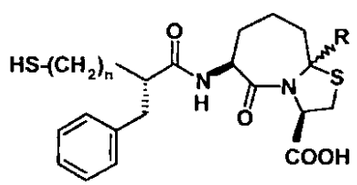


Thiorphan, 19



*retro*-Thiorphan, 20

| | | |
|---|---|---|
| Thermolysin | $K_i$ = 1.8 µM | $K_i$ = 2.3 µM |
| NEP 24.11 | $K_i$ = 0.0019 µM | $K_i$ = 0.0023 µM |
| ACE | $K_i$ = 0.14 µM | $K_i$ > 10 µM |



| | 21 a<br>R = α-H<br>n = 1 | 21b<br>R = α-H<br>n = 0 | 21c<br>R = β-H<br>n = 0 |
|---|---|---|---|
| | $IC_{50}$ values | | |
| NEP 24.11 | 1.1 nM | 11.5 nM | 2 820 nM |
| ACE | 5.5 nM | 16 nM | 11.5 nM |

*Fig. 9. Thiorphan 19 and retro-thiorphan 20 are 'similar' with respect to thermolysin and NEP 24.11, but they are highly 'dissimilar' with respect to angiotensin converting enzyme (ACE). Changing the configuration of 21a and b at the ring junction, from α–H to β–H in 21c, does not influence the ACE-inhibitory activity, but reduces the NEP-inhibitory activity to a large extent: thus, the selectivity against ACE is increased by a factor of 300.*

against yet another related zinc protease, angiotensin converting enzyme (ACE), are significantly different [22]; with respect to this enzyme, the analogs are 'dissimilar'. Whether the differences result from different binding modes or from an unfavorable binding geometry of *retro*-thiorphan in ACE will only be known if the ACE 3D structure becomes available. Corresponding differences in the structure–activity relationships are observed in some other dual zinc protease inhibitors **21a–c** (Fig. 9) [24]. Again, the degree of similarity and dissimilarity of the ligands depends on the biological target.

Corresponding problems are also observed in toxicity predictions for humans, as illustrated by the toxicities of lysergic acid diethylamide (LSD) and 2,3,7,8-tetrachlorodioxin ('dioxin') in different species. LSD is only weakly toxic to mice ($LD_{50}$ = 50–60 mg/kg) and rats ($LD_{50}$ = 16.5 mg/kg) but significantly more toxic to rabbits ($LD_{50}$ = 0.3 mg/kg). An elephant, which was (cautiously?) treated with an LSD dose of 0.3 g (corresponding to about 0.06 mg/kg), died within several minutes [25]! Humans seem to tolerate LSD quite well. Cases of death have been observed as the result of drug-induced suicides, but not by any toxicity of the drug itself.

2,3,7,8-Tetrachlorodioxin is highly toxic to several species — e.g. guinea-pigs ($LD_{50}$ = 0.6–2.5 $\mu$g/kg) and mink ($LD_{50}$ = 4 $\mu$g/kg). It is much less toxic for mice ($LD_{50}$ = 114–280 $\mu$g/kg), rats ($LD_{50}$ = 22–320 $\mu$g/kg), hamsters ($LD_{50}$ = 1150–5000 $\mu$g/kg), rabbits ($LD_{50}$ = 115–275 $\mu$g/kg), dogs ($LD_{50}$ > 100 and < 3000 $\mu$g/kg) and monkeys ($LD_{50}$ < 70 $\mu$g/kg) [26]. If one extrapolates from the monkey, dioxin should be relatively 'harmless' for humans, at least if only acute toxicity is considered. However, the significantly different toxicity *versus* the closely related species guinea pig and hamster puts a caveat on too simple and straightforward extrapolations. Of course, for humans the $LD_{50}$ is not the relevant endpoint, but rather the no-effect $LD_0$ level!

## 5. Similar Structures and Activities, but Different Binding Modes

Comparable biological activities of chemically similar structures do not necessarily result from analogous binding modes of all analogs. Since at least in 3D QSAR, if not in all QSARs, the correct superposition of all analogs within a series is a precondition for reliable results, a better knowledge of the binding modes is most important. Unexpected differences in the binding modes of some analogs certainly produce problems in 3D QSAR studies that are based on such a mutal alignment of the structures. But it is to be expected that also 3D QSAR modifications, which do not require a structural alignment, should have problems in such cases.

Differences in binding modes have been extensively reviewed [1,27–29]. Thus, only some examples will be summarized here, without detailed discussion. Purine nucleoside phosphorylase (PNP) inhibitors **22** and **23** show surprising structure–activity relationships, which can be explained only by inspecting the X-ray structures of the inhibitor complexes (Fig. 10). The exchange of an acceptor nitrogen to a donor nitrogen atom changes not only the interactions with the directly involved asparagine side chain, but also of an 8-amino group with the threonine hydroxyl and methyl groups [30,31].

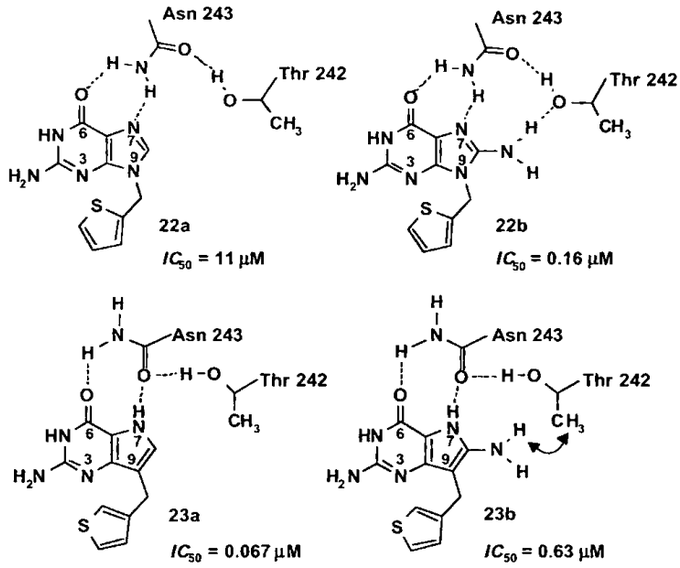



*Fig. 10. The X-ray structures of the purine nucleoside phosphorylase (PNP) complexes with the inhibitors 22 and 23 show that 22a forms less favorable hydrogen-bonds with Asp 243 than the desazapurine 23a. On the other hand, the introduction of an 8-amino group has different consequences in 22a and 23a. Whereas the new amino group of 22b forms an additional hydrogen-bond to Thr 242, it causes unfavorable polar/nonpolar interactions in 23b.*

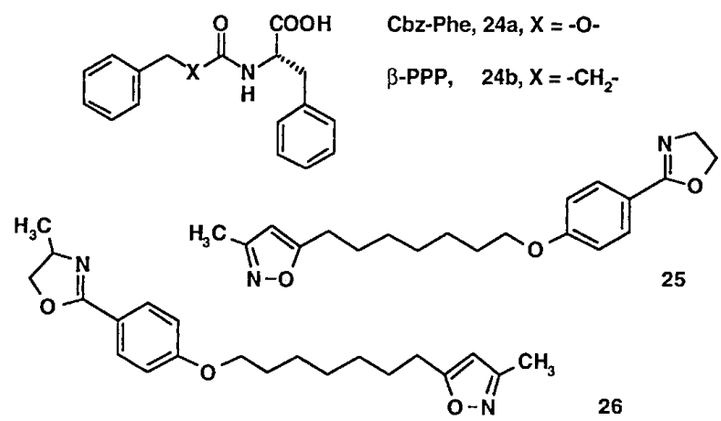



*Fig. 11. Changing an oxygen atom of the thermolysin inhibitor* ***24a*** *to* $CH_2$ *(**24b**) leads to a reversed binding mode. Also the viral coat protein ligands **25** and **26** bind in a reverse manner, as indicated. There are only hydrophobic groups and one hydrogen-bond donor in the binding pocket of the viral protein; this donor can form a hydrogen-bond with either the oxazoline or the isoxazole ring.*

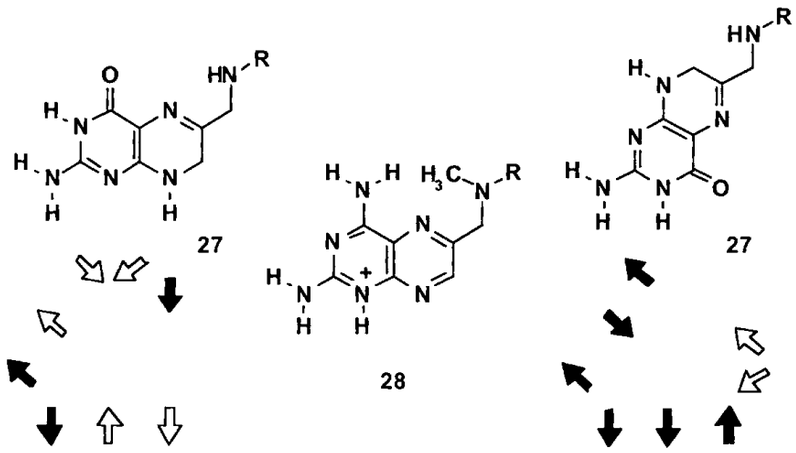



*Fig. 12. Dihydrofolate 27 and the positively charged form of methotrexate 28 (R = p-$C_6H_4$-CONHCH(COOH)$CH_2CH_2$COOH) have different patterns of hydrogen-bond donors and acceptors, if they are superimposed according to their ring systems (left side). If the ring system of 27 is rotated around the bond which connects the ring system with the rest of the molecule, a much better similarity of the hydrogen-bond patterns results (right side). Filled arrows indicate identical directions of the hydrogen-bonds in the analogs 27 and 28.*

Carbobenzoxy-phenylalanine (Cbz–Phe) **24a** and β-phenylpropionyl-phenylalanine (β-PPP) **24b** (Fig. 11) differ only by their X group. Nevertheless, they bind to thermolysin in an opposite direction, as uncovered by the determination of the 3D structures of the complexes [32]. Reverse binding modes are also observed for some structurally related viral coat protein ligands — e.g. compounds **25** and **26** (Fig. 11) [33,34].

Dihydrofolate **27**, the substrate of dihydrofolate reductase (DHFR), and the DHFR inhibitor methotrexate **28** seemingly differ only in minor chemical details (Fig. 12). However, this small difference has significant consequences on the hydrogen-bond donor and acceptor patterns of the compounds. Accordingly, dihydrofolate and methotrexate accommodate the binding site of DHFR in different modes [35], an effect which has been predicted from the X-ray structure of the dihydrofolate/DHFR complex [36].

The isomeric 1-, 2- and 4-phenylimidazoles are inhibitors of the oxidation of camphor by cytochrome $P450_{cam}$. Nevertheless, only 1- and 4-phenylimidazole bind as expected, with one nitrogen atom of the imidazole ring coordinated to the iron atom of the porphyrin system; 2-phenylimidazole cannot be accommodated in such a position and binds in a completely different manner [37]. Structurally related dipeptide inhibitors of the serine protease elastase bind with their corresponding residues in different pockets of the enzyme [1,27,38]. The trypsin inhibitor *p*-guanidiniumbenzoate seems to be one of the rare cases where X-ray crystallography provides clear evidence that one and the same ligand binds in distinctly different orientations [39]. Many other examples of different binding modes of closely related analogs have been described in the literature [1,27–29]. From the observation of such differences, Dagmar Ringe proposed to use this information to design 'hydra-headed' inhibitors, which fill all possible pockets of a binding site.

## 6. Different Mechanisms of Action of Similar Molecules

Several well-known examples of different modes of action of closely related analogs can be found in medicinal chemistry and pharmacology textbooks. Norepinephrine **29a**, epinephrine **29b** and isoproterenol **29c** (Fig. 13) are adrenergic agonists. However, in going from R = H to R = $CH_3$ and R = isopropyl, the mechanism of action gradually changes from a more or less specific α-adrenergic agonism to a pure β-adrenergic agonism. If the two hydroxyl groups of isoproterenol are changed into chloro substituents, the β-adrenergic antagonist dichloroisoproterenol (DCI) **30** results; in fact, **30** was the first β-blocker.

An unexpected effect resulted after the introduction of an isobutyl group into the $AT_1$-specific angiotensin II receptor antagonist **31a** (Fig. 13). The original $AT_1$ selectivity is completely destroyed; in addition, the new analog **31b** is no longer an antagonist, it is a strong agonist at the $AT_1$ and $AT_2$ subtypes of this receptor [40]. A related effect has been observed for some structurally diverse cholecystokinin (CCK) receptor ligands; the introduction of an isopropyl group at a certain nitrogen atom leads from peripheral CCK antagonists to agonists [41,42].

The integrin family of receptors are structurally and functionally related membrane-imbedded glycoproteins that 'integrate' the extracellular matrix with the cytoskeleton.

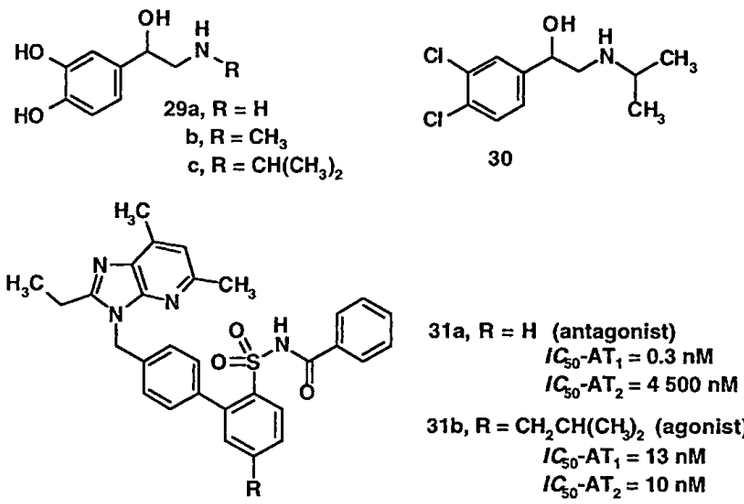






*Fig. 13. The change from norepinephrine 29a to epinephrine 29b and isoproterenol 29c gradually changes the mode of action from α-agonism to β-agonism. Dichloroisoproterenol (DCI) 30 was the first β-adrenergic antagonist. Introduction of an isobutyl group into the $AT_1$-selective antagonist 31a produces the potent, unselective $AT_1/AT_2$ agonist 31b.*

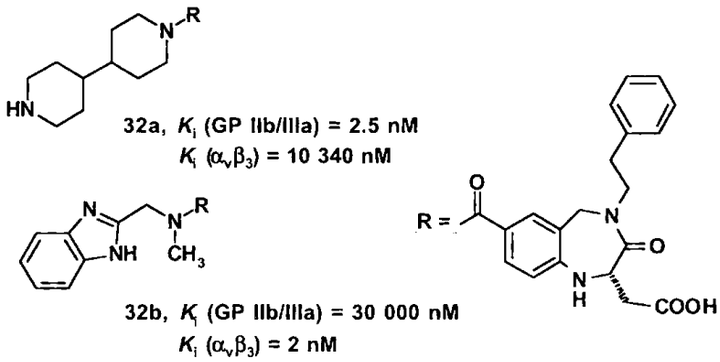



*Fig. 14. The chemically closely related integrin antagonists 32a and 32b differ in their selectivity towards the fibrinogen (GPIIb/IIIa) and the vitronectin ($\alpha_v\beta_3$) receptors by nearly eight orders of magnitude.*

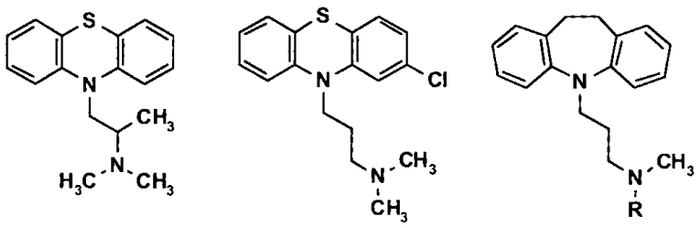
Important members are the GPIIb/IIIa receptor (GP for glycoprotein, IIb/IIIa for the $\alpha_{IIb}\beta_3$ integrin) and the vitronectin ($\alpha_v\beta_3$) receptor [43,44]. Whereas the GPIIb/IIIa receptor binds, *inter alia*, fibrinogen and mediates blood platelet aggregation, the $\alpha_v\beta_3$ receptor binds vitronectin and is responsible for angiogenesis, vascular smooth muscle migration and adhesion of osteoclasts to the bone matrix. Thus, both receptors are important for drug design, the GP IIb/IIIa receptor for the development of antithrombotic agents and the vitronectin receptor for the development of drugs for the treatment of cancer, as well as against restenosis and osteoporosis. However, both fibrinogen and vitronectin interact with their receptors using an identical structural domain, the so-called RGD motif (RGD = Arg-Gly-Asp), but obviously in different conformations. This was first confirmed by the investigation of different cyclic pentapeptides, containing D-amino acids in different positions [44,45]. How far structure-based design (in this case, based only on the structures of ligands) can be advanced is illustrated by compounds **32a** and **32b**, which are highly selective GPIIb/IIIa and vitronectin receptor antagonists, respectively (Fig. 14) [46]. The selectivity of these analogs against the two receptors differs by nearly eight orders of magnitude, despite their close chemical similarity! The only major difference is the distance between the carboxylic group, which mimics the Asp, and the basic nitrogen atom, which mimics the Arg of the RGD motif.

A well-known example of different modes of action of closely related analogs are the anti-allergic agent promethazine **33**, the neuroleptic chlorpromazine **34**, and the antidepressants imipramine **35a** and desipramine **35b** (Fig. 15). Despite their very similar structures, **33** acts mainly as a histamine H1 antagonist, **34** is a dopamine antagonist, **35a** is an unspecific norepinephrine- and serotonin-uptake inhibitor and **35b** is a norepinephrine-specific uptake inhibitor. Steroid hormones — e.g. **36–39** (Fig. 16) — give another example of strikingly different biological effects of chemically closely related analogs.

Several therapeutically used drugs bind to more than one receptor and are correspondingly termed 'promiscuous' ligands. However, whether a certain (balanced) unspecificity of their mode of action is advantageous for therapy or not still remains uncertain.

## 7. Chirality and Biological Activities

Due to the chiral nature of amino acids (except glycine), drug binding sites of proteins are asymmetric. In the past, the different actions of enantiomers of chiral molecules on enzymes and receptors were often neglected [47]. For economic reasons, racemates of synthetic drugs were used in therapy. Today, researchers and drug companies are more aware of the different effects of enantiomers and diastereomers [1,2] in their biological activities, as well as in their pharmacokinetics. Enantiomers can even be differentiated by their odor — e.g. the monoterpenes (*R*)- and (*S*)-limonene **40** and (*R*)- and (*S*)-carvone **41** (Fig. 17) [48]. Butaclamol **42** is mentioned as just one example of significantly different eudismic ratios (i.e. ratios of affinities of the different enantiomers) *versus* different receptors (Fig. 17) [47].



**33, Promethazine ($H_1$ antagonist)** **34, Chlorpromazine (D antagonist)** **35 a, R = $CH_3$, Imipramine b, R = H, Desipramine (uptake inhibitors)**

*Fig. 15. Despite their chemical analogy, the tricyclic compounds 33, 34 and 35 have different mechanisms of action and different therapeutic effects; 33 is an antiallergic agent, 34 is used for the treatment of schizophrenia and 35a and b for the treatment of depression.*

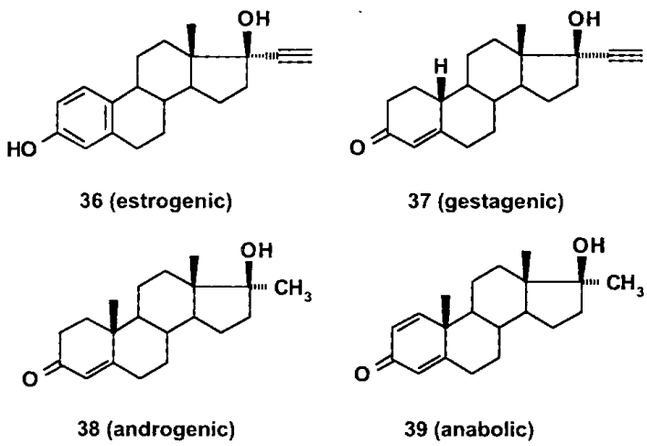



*Fig. 16. In the steroid series, minor chemical differences produce very different effects on the biological properties of the compounds. Compounds 36–38 correspond in their biological actions to the different female and male sex hormones, compound 39 is a male sex hormone-related anabolic.*

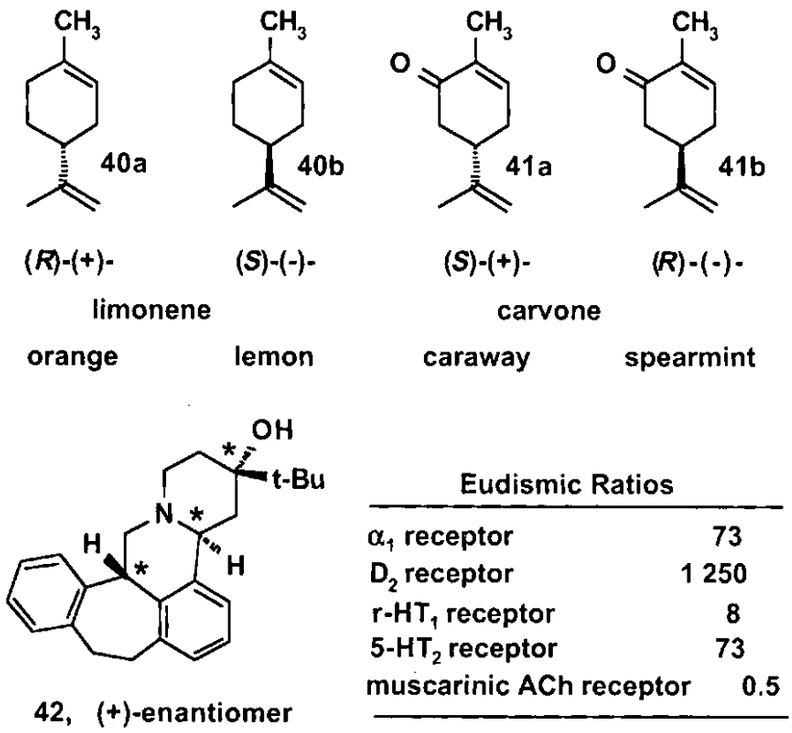
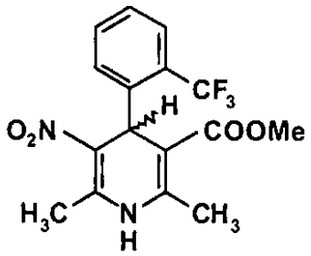



*Fig. 17. Enantiomers are different: the olfactory receptors of our nose can even distinguish such minor structural differences as between **40a** and **b**, as well as between **41a** and **b** (the typical odors of the compounds are indicated below the structures). Activity differences between enantiomers are defined by their eudismic indices, the ratios of biological activities of the individual enantiomers. They significantly depend on the biological target. Whereas both enantiomers of butaclamol **42** look very 'similar' to the muscarinic acetylcholine receptor, they differ by more than three orders of magnitude in their affinitities to the $D_2$ receptor.*

Enantiomers may even have opposite biological effects. Certain chiral barbiturates show sedative activities in the one enantiomer and convulsive activities in the other one. The nifedipine analog Bay K 8644, **43** (Fig. 18) was originally synthesized as a racemate. *In vitro* tests with isolated smooth muscle strips showed it to be more or less inactive. However, several years later its high affinity to calcium channels was discovered; this demanded a separation of the racemate into the two enantiomers. One is a calcium channel agonist, the other a weak antagonist. Höltje explained these differences by the asymmetry of the molecular electrostatic potentials of the enantiomers, if the molecules are superimposed by their ring systems [49].




**43a, (*S*)-(-)-form: agonist**

**43b, (*R*)-(+)-form: weak antagonist**

*Fig. 18. The enantiomers of Bay K 8644 have opposite biological effects. Whereas 43a stabilizes the calcium channel in its open form, 43b stabilizes its closed form.*

## 8. The Similarity Principle in QSAR and 3D QSAR

Quantitative descriptions of the specific binding of ligands to a protein surface are based on the additivity principle of activity group contributions to the overall affinities. This has been extensively proven by dedicated investigations performed by Andrews, Goodford and Bohacek [50], but also by Böhm's scoring function of the *de novo* design program LUDI [51]. Some other approaches are reviewed in this book. However, despite the differences between enantiomers, discussed above, classical QSAR has no good tools to distinguish different conformers or enantiomers. 3D QSAR methods are much more efficient in this respect.

The very first approach to describe structure–activity relationships by N × N similarity matrices (N = number of compounds) was presented by Rum and Herndon in 1991 [52]. More systematic investigations, using 3D similarity indices, were performed by Richards et al. in 1993 [53,54]. N × N Distance matrices are even appropriate for the quantitative description of nonlinear structure–activity relationships [50,55,56].

A very promising method, based on similarity fields, is the Comparative Molecular Similarity Indices Analysis (CoMSIA) approach [57,58]. As in CoMFA, all molecules are aligned according to a common pharmacophore; similarity indices of certain probes to the different molecules are then calculated in the positions of a regular grid. These fields, not just pairwise similarity indices of the molecules, are correlated with the biological activities. Due to the 'soft' character of the Gaussian potentials that are used to derive the similarity fields, smoother and more contiguous contour maps result from CoMSIA analyses [57].

As compared to the CoMFA and CoMSIA methods, the use of N × N 3D similarity matrices in QSAR has several advantages. Instead of a mutual alignment of all active and inactive molecules to a common reference frame, only pairwise alignments need to be performed [56,58]. This leads to a matrix with individual 'similarity vectors' for each analog. Inactive analogs do no longer distort the alignment of the active analogs. If SEAL similarity indices [59,60] are used in the generation of the N × N matrix, not even a grid is needed. Thus, the frequently observed disturbance of the CoMFA results, after translation or rotation of the box around the molecules (e.g. [61]), are not ob-

served. A disadvantage of the calculation of pairwise similarity coefficients is the fact that no contour maps can be generated from the resulting QSAR model.

## 9. Conclusion

There are many lessons to be learned for 3D QSAR applications. Firstly, a correct alignment is the most important basis of each structure–activity analysis. Errors that are produced there can never lead to correct models.

An overreliance in target-independent similarity indices has to be questioned, because of the dependence of 'similarity' on the biological macromolecule to which the analogs bind. Sophisticated investigations on the 'dissimilarity' of chemical databases are most often futile. Similar compounds may have very different actions and different molecules can be very similar in their biological activities. Considering the examples presented in this chapter and the many more cases in the literature, one has to admit that we are far from a deeper understanding of the details which underline the observed structure–activity relationships. Applying the results from one series of analogs to another one may lead to completely wrong conclusions.

In the past, the use of molecular connectivity parameters in QSAR studies has led to highly controversial discussions. The same may happen to the BCUT, WHIM and EVA parameters, all described in other chapters in this volume. But one fact can be taken for sure: all these methods describe the similarity between molecules to a different extent. Thus, one should not be surprised that these approaches work, in some cases, even as well as other, more 'rational' methods.

Besides a correct alignment, the selection of the training and test sets is critically important to the results of a 3D QSAR study [58]. Only if both sets cover approximately the same range of structural space, can one be sure that the analysis and the results are meaningful. Cross-validation methods do not help in this respect. If the data set is highly redundant, even cross-validation in groups will produce 'good' results, whereas in clearly non-redundant datasets even a simple leave-one-out cross-validation must fail. One has to come back to the original goal of QSAR studies: to derive a working hypothesis and to design new analogs, based on one or several alternative working hypotheses. In this sense, QSAR is a theoretical tool which cannot solve all our problems but which definitely should accompany the experiments of the medicinal chemists and biologists.

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SCIENCE JOURNAL REPRINT

# Cancer inducing agents

Francis J. C. Roe

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Francis J. C. Roe

# Cancer inducing agents

It has become increasingly difficult to state whether a given agent will or will not induce cancer. It is possible that, given the appropriate conditions, all substances are carcinogenic

**Some carcinogens in industry and every day life**

Aniline dyes

Antioxidants such as benzidine and β-naphthylamine

Asbestos

Certain metals: arsenic, beryllium, cadmium, chromium, cobalt, nickel and selenium

Certain medicines, particularly some of those used in the treatment of cancer; also some hormones

Creosote

Epoxides, used widely as adhesives and in the manufacture of plastics

Exhaust gases from petrol and diesel engines

Smoked foods and charcoal steaks

Soot, coal tar and smoke

Tobacco smoke and raw tobacco used by 'chewers'

Ionizing radiation

Sunlight

Cancer producing viruses ?

**WIDELY DIFFERING agents have been shown to be capable of causing cancer in man. In the past they have been arbitrarily divided into chemical, physical and viral. For many reasons this division is not very meaningful. Thus asbestos may cause cancer because of its physical rather than chemical nature**

FRANCIS JOHN CALDWELL ROE is Head of the Department of Experimental Pathology at the Chester Beatty Research Institute, and Reader in Experimental Pathology at the University of London. His current research deals with the mechanism of carcinogenesis, the detection of environmental cancer hazards and the application of such studies to the prevention of cancer in man.

CANCER can be induced in laboratory animals by deliberately exposing them to a wide variety of physical, chemical or viral agents some of which are known to cause cancer in man. It has become customary to regard the agents concerned as 'carcinogens'. Yet this may not be the best way of looking at the situation, because it suggests too strongly that the many different cancer inducing agents share a common feature which enables them to bring about the induction of cancer by a specific mechanism.

Instead it may be better to regard carcinogenesis as an intimate '*pas de deux*', with the living tissue and the cancer inducing agent as the principal dancers on a stage which is the environment provided by the multi-cellular organism in which cancer will eventually arise. Both the dancers can appear in other roles at other times and the stage can be used for other purposes. Other dancers can act as substitutes, but the *pas de deux* itself always requires two dancers and a stage on which to appear.

THE ANIMAL BODY consists of a complex community of cells all derived originally from one cell, the fertilized ovum. Within each cell, coded in the arrangement of bases in its nucleic acids (deoxyribonucleic acid, DNA, and ribonucleic acid, RNA), is a plan of the whole body but, under normal circumstances, only a fragment of the plan is expressed by each cell. It may be presumed that the expression of the majority of the plan is actively suppressed by a pattern of control mechanisms. These include long distance hormonal and neural systems; local systems involving tissue antigen-antibody reactions and cell to cell contacts; and intracellular gene-suppressor systems, built-in to cells during their differentiation from the undifferentiated state (see "The control of genes", SCIENCE JOURNAL, March 1966).

The normal steady state is maintained in the body by the continual operation and interplay of these control mechanisms, each acting as a negative feed-back system. Repair following injury is controlled by the same mechanisms, the combined function of which is to maintain, or in this case, re-establish the *status quo*. It is convenient, therefore, to apply the adjective 'homeostatic' to these control mechanisms.

Each body cell may be regarded as an individual living unit of which the genetic potential is constantly fettered by the chains of homeostatic controls.

Whatever else carcinogenesis may involve, it certainly involves an effective breaking out from these chains.

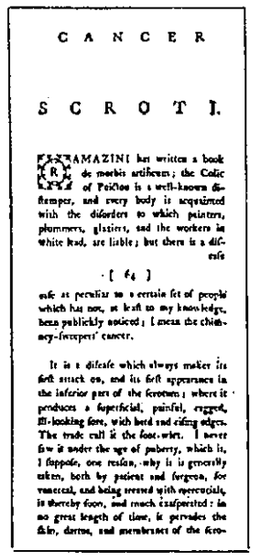
Observation indicates that, even under normal circumstances, the two daughter cells resulting from the division of a tissue cell are not completely identical. Their inequality may be trivial, non-heritable and correctable during subsequent cell divisions. But if inequitable cell division proceeds on a large scale, and if sequential abnormal divisions occur because the mechanism for getting rid of defective cells cannot operate quickly enough, then a mixed population of cells will arise. Under the pressure of the limited availability of nutrients, and the operation of homeostatic mechanisms, a process of natural selection will then operate: the most vigorous cells—those least susceptible to homeostatic suppression—and their progeny will become relatively more numerous than the less vigorous members of the population. Once a cell which carries a heritable defect has gained a measure of autonomy, natural selection operating among its progeny will tend to lead to the dominance of progressively more vigorous and autonomous cells. In keeping with this view is the fact that it is a characteristic of cancer that it is progressive—in the sense that cancerous tissue tends to become more malignant with time. Furthermore it is usual for a time interval, which may be very long, to separate exposure to carcinogenic stimulus and the appearance of cancer. It now seems likely that this interval is occupied by the process of natural selection and the slow emergence of cells sufficiently autonomous to be regarded as cancerous.

This is the background against which carcinogenesis should be viewed. In theory, at least, cancer may be induced either directly by an effect on the genetic material of cells or indirectly by interference with extracellular homeostatic control mechanisms. This distinction is far more meaningful than one based on the chemical, physical or viral nature of the agents concerned and it may in fact throw light on the mechanisms by which these agents operate.

IN 1775 PERCIVALL POTT recorded an association between the occupational exposure of chimney sweepers to soot and cancer of the scrotum: a majority of young men in their teens or twenties with this form of cancer had a history of having been employed as 'climbing boys'. One hundred and forty years later two Japanese workers reported the induction of skin cancer in rabbits by the repeated application of coal tar. In 1930, a team under the late Sir Ernest Kennaway detected and synthesized the first chemical carcinogens of the type present in coal tar. These were aromatic polycyclic hydrocarbons made up of four or five benzene rings. In time many hundreds of such substances were synthesized. Some proved active in the induction of cancer in animals, others—often closely related chemically to active compounds—proved quite inactive. Theoretical chemists developed hypotheses associating structure and activity. Calculation of the electron charge densities of each of the various active molecules showed that all possessed a region of high density, the 'K-region', in their double bond system. In these areas the probability of bond formation occurring with other molecules through charge transfer is greatest. However, not all 'K-region' positive compounds proved to be active as carcinogens: high electron density in a second area of the molecule, the 'L-region', rendered such compounds inactive. By the use of this theory, and subsequent modifications of it, it was possible to predict carcinogenic activity within the limited class of the polycyclic hydrocarbons.

In the meantime, however, a wide variety of quite different chemical agents were found to be carcinogenic. Their extreme diversity led to the abandonment of the hope that the theoretical chemists would quickly solve the cancer problem.

ANOTHER LANDMARK in the history of the subject was the observation of Alexander Haddow in the late 1930s that there is an association between carcinogenic activity and ability to inhibit growth, including tumour growth. Not only this, but it seemed that carcinogenic compounds often had a selective toxic effect on the process of cell division, and were able to produce mutations in the cells. During the past 20 years this observation has led to the discovery of new carcinogens such as the aminostilbenes and to substances such as the biological alkylating agents which both induce cancer and inhibit cell multiplication. The essential property of alkylating agents is their ability to introduce alkyl



CANCER

SCROTI.

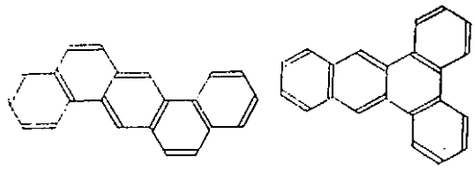
RAMAZINI has written a book de morbis artificum; the Colic of Poictou is a well-known distemper, and every body is acquainted with the disorders to which painters, plummers, glaziers, and the workers in white lead, are liable; but there is a disease

[ 64 ]

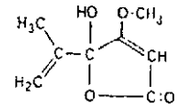
safe at peculiar to a certain set of people which has not, at least to my knowledge, been publickly noticed; I mean the chimney-sweepers' cancer.

It is a difeafe which always makes its firft attack on, and its firft appearance in the inferior part of the fcrotum; where it produces a fuperficial, painful, ragged, ill-looking fore, with hard and riding edges. The trade call it the foot-wart. I never faw it under the age of puberty, which is, I fuppofe, one reafon, why it is generally taken, both by patient and furgeon, for venereal, and being treated with mercurials, is thereby foon, and much exafperated: in no great length of time, it pervades the fkin, derma, and membranes of the fcro-

**PERCIVALL POTT first described the association between exposure of chimney sweeps to soot and cancer of the scrotum in 1775. His book, first page of text illustrated, was the first in a long and continuing line of works on occupational and industrial medicine**

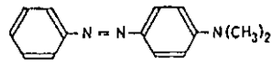


**SIMILAR COMPOUNDS can have dissimilar effects. Thus 1, 2, 5, 6-dibenzanthracene, left, is a potent carcinogen whereas the related substance 1, 2, 3, 4-dibenzanthracene, right, is carcinogenically inactive**

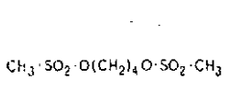


**PENICILLIC ACID is the basis of a variety of penicillins. Causes cancer at injection site in rats**

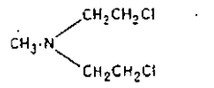
**NITROSOUREA causes tumours at various sites including the brain in some laboratory animals**



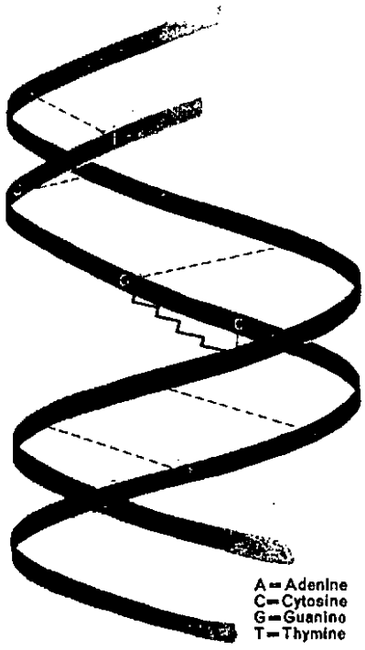
**4-DIMETHYLAMINOAZOBENZINE (butter yellow) was formerly used to give colour to margarine. However, it has since been found to cause liver cancer in rats**



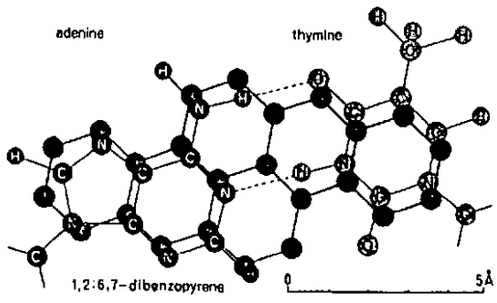
**MYLERAN (BUSULFAN) is of value in the treatment of myeloid leukaemia —a blood cell cancer**



**NITROGEN MUSTARD has been found of value in the treatment of several different types of cancer**




**TWO STRANDS OF DNA may become linked together by a bifunctional alkylating agent such as myleran, the molecule of which becomes attached at each end to a nucleotide base (A, C, G or T). The effect of such a linkage may lead either to a reduction in the capacity of affected cells to reproduce or to the induction of cancer**




**SOME CARCINOGENS, such as 1, 2: 6, 7-dibenzopyrene, shown coloured, are similar in chemical structure to parts of the DNA molecule, for example adenine and thymine, black. This suggests that this type of compound may be able to cross link the DNA**

radicals, such as $-CH_3$ or $-C_2H_5$, into molecular components of cells. The effect of such introduction leads to a dramatic reduction in the capacity of cells to reproduce and, in some cases, to the induction of cancer. The former effect is immediate and the latter delayed. Alkylating agents capable of introducing a single alkyl radical are termed monofunctional; those able to introduce two or more are termed bifunctional or polyfunctional respectively. When bifunctional or polyfunctional compounds react with two or more molecules the end result may be that the molecules become linked together by the alkylating agent.

It is now believed that both the carcinogenic and tumour inhibiting effects are due to reactions of this type with the genetic material of the cell, deoxyribonucleic acid (DNA). In recent years an ingenious theory has been developed that the most significant effect of the polyfunctional alkylating agents is to cross-link the two strands of DNA. If this were true, it would be possible to explain, in terms of a single chemical mechanism, all three types of biological activity of these agents: carcinogenicity, mutagenicity and growth inhibiting activity. As a result of similar thinking a new suggestion was made with regard to the possible mode of action of carcinogenic polycyclic hydrocarbons, and their distinction from inactive analogues. It was pointed out that some of the carcinogenically active compounds, such as 1, 2:6, 7-dibenzopyrene, were similar in chemical structure to part of the DNA molecule. This suggests that this type of compound might also cross-link the two strands of DNA.

These theories are still actively stimulating new work but, clearly, they do not apply to all forms of carcinogenesis. As more compounds have been examined, the association between carcinogenicity, growth inhibition and mutagenicity has become less clear cut. Monofunctional alkylating agents, capable of reacting with DNA but not of cross-linking its two strands, have been shown to be carcinogenic, though less so than related polyfunctional compounds.

There are grounds for believing that most potent carcinogens affect the DNA or RNA of cells more or less directly and specifically. The most potent cancer inducing agents of all, namely the tumour viruses, consist simply of DNA or RNA. Carcinogens may be weak either because their reaction with nucleic acids is non-specific, or because they affect nucleic acids indirectly rather than directly. A third possibility is that their primary effect is not on cells at all, but on their environment by interference with the homeostatic control systems.

Two facts concerning chemical carcinogenesis deserve special mention. First, several metals are known to be capable of inducing cancer, either in men exposed to them in industry, or in laboratory animals under experiment. A human cancer hazard is now recognized in relation to arsenic, beryllium, chromium and nickel. In the laboratory the latter three, together with cadmium, cobalt and iron, have been shown to induce cancer. Curiously, no one has yet succeeded in inducing cancer by exposing laboratory animals to arsenic. Secondly, in recent years a number of naturally occurring carcinogens have been recognized. These include cycasin, an alkaloid present in the cycads—types of palm the leaves, stems and roots of which provide starch, for example sago, and form a staple item of diet in some parts of the world; the senecio alkaloids present in some species of ragwort; safrole, a component of several essential oils including sassafras oil, which is extensively used in North America to flavour 'root beer'; and aflatoxin, a lactone formed by some species of the mould *Aspergillus flavus* when it grows in ground nuts and cereals stored under the hot, humid conditions of the tropics.

IT HAS LONG BEEN PUZZLING that hormones, produced naturally by the body, act as carcinogens under certain circumstances. For instance, cancers of many kinds may be induced in animals by giving them excessive amounts of oestrogen, one of the hormones normally produced by the ovary. Many attempts have been made to show structural resemblances between particular hormones and carcinogens of the polycyclic hydrocarbon type. Indeed, some of the latter have been shown to exhibit weak oestrogenic activity. Nevertheless, the links between carcinogenesis by hormones and by other agents are very tenuous. It is much more likely that the primary action of hormones is not on cells but on one of the homeostatic mechanisms which control their growth and self-expression. The continued presence of excessive amounts of a hormone may, by blocking a normal suppressing mechanism, permit the proliferation of a tissue. This may favour the emergence of autonomous cells in one of two ways. First, the con-

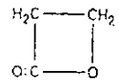
ditions existing in a proliferating tissue may be more favourable to the survival of cells which deviate from normal. Secondly, if because of previous exposure to a carcinogen the cancerous process has been started and there is already a mixed population of cells, the process of selection of more vigorous and autonomous cell lines will operate more quickly under conditions of active proliferation.

IT HAS BEEN SHOWN in the laboratory that cancer may be induced by exposing an animal to two agents, A and B, in that order, whilst exposure to A alone, B alone, or B followed by A does not lead to cancer. Despite several attempts to do so, no one has yet found an agent which has only A-type activity (tumour initiating activity) or only B-type activity (tumour promoting activity). According to the theory presented here, tumour initiators act primarily on cells and tumour promoters primarily on homeostatic control mechanisms. Doses of carcinogens too low to complete the process of carcinogenesis may, nevertheless, be sufficient to initiate it. It is not surprising that pure promoters have not been demonstrated since it is not possible to escape background exposure to initiating agents in the form of low doses of environmental chemical carcinogens and ionizing radiation. It is reasonable to suspect that homeostatic control mechanisms are weakened as a result of the ageing process. The tumour promoters may do no more than bring forward in time a process which would eventually occur spontaneously as a result of ageing. There is some experimental evidence that this is the case. The term 'co-carcinogen' has been applied to an agent which enhances the activity of a carcinogen. There are many possible mechanisms by which such enhancement may be brought about; for example, the co-carcinogen may aid the absorption of carcinogen or block its breakdown to non-carcinogenic metabolites. All tumour promoters are co-carcinogens, but not all co-carcinogens are tumour promoters.

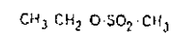
UNTIL THE PUBLICATIONS of F. C. Turner in 1941 and of B. S. and E. T. Oppenheimer and A. P. Stout from 1948 onwards the term physical carcinogenesis was applied, for the most part only, to the induction of cancer by agents such as ultraviolet light, ionizing radiation, physical trauma and chronic irritation of various kinds. It was fairly easy to interpret the effects of both ultraviolet and ionizing radiation in chemical terms, and the interpretation was supported by the introduction of the idea of 'radiomimetic agents' in connection with chemical agents whose effects, in general, resembled those of ionizing radiation.

The fact that physical trauma seems to be able to initiate or precipitate cancer has never been very palatable to those who seek a neat chemical explanation of all forms of carcinogenesis. To them the discovery by Peyton Rous that wounding the ears of rabbits enhanced carcinogenesis by coal tar, though it did not induce cancer on its own, was a windfall. Physical trauma could then be dismissed as a co-carcinogenic factor and need not be explained directly in chemical terms. On the other hand, the demonstration by first Turner and later the Oppenheimers that cancer may be induced by the implantation of chemically inert materials into the tissues made it necessary once more to consider physical carcinogenesis seriously in its own right. At the Chester Beatty Research Institute we have induced cancer in the bladders of mice by the implantation of simple glass beads. Some have argued that nothing is so chemically inert that chemical carcinogenesis is ruled out. Nevertheless, the induction of cancer by the implantation of objects of various shapes, and the simultaneous failure to induce cancer by implanting the powdered chemicals from which the objects are made, have compelled the acceptance of the view that the implanted objects induce cancer because of their physical and not their chemical characteristics. In our researches at the Institute we have found that a high proportion of a group of rats developed cancer at the site of implantation in the subcutaneous tissues of pieces of polyvinyl sponge (as used in plastic surgery) measuring $20 \times 20 \times 5$ mm, whereas only 1 out of 24 rats did so in response to implants measuring $33 \times 33 \times 2$ mm. The amount of sponge was the same in both cases but the shape was different. What possible relation can this type of cancer induction have to that due to the various chemical agents discussed above?

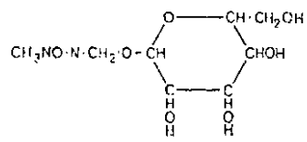
In the past the distinction between chemical and physical carcinogenesis has tended to be artificial. A better distinction is between agents which primarily affect cells and those which primarily affect homeostatic control mechanisms.



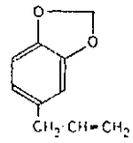
**β-PROPIOLACTONE** is an alkylating agent with virus killing properties. It induces cancer on injection into rats or when it is applied to the skin of mice



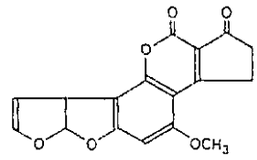
**ALKYLATING AGENT** (ethyl methanesulphonate) has been shown to induce formation of tumours of the lung when injected into infant mice



**CYCASIN**—an alkaloid found in various species of cycad palm which are an important source of starch in many tropical countries—can induce cancers in rats



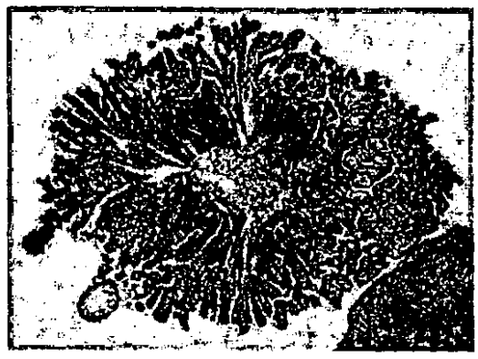
**4-ALLYL—1, 2-METHYLENEDIOXYBENZENE** (safrole) is a natural ingredient of sassafras tea and has been used to flavour 'root beer' in North America. It has been shown to cause liver tumours when fed to rats



**AFLATOXIN B** is one of a group of toxins produced by strains of the mould *Aspergillus flavus* which grows in ground nuts and cereals stored under hot, humid conditions. Besides acting as a poison—it killed more than 100,000 turkeys and game birds in 1960—it can induce cancers of the liver and kidney in rodents

**CANCER**, arrowed, in the bladder of a mouse following the implantation of a glass bead 40 weeks earlier

**IMPLANTED OBJECTS** can induce cancer because of their physical characteristics. The cancer, arrowed, arose in the region of a piece of plastic sponge implanted in the subcutaneous tissue of the rat several months earlier. A similar piece of sponge of the same weight but of different shape was found to induce cancer far less frequently



**ASBESTOS** induces cancer possibly because of its physical rather than chemical properties. This nodule of cancerous tissue from the abdominal cavity of a rat arose several months after the last of a series of four subcutaneous injections of asbestos

It is now suggested that, as in the case of the induction of cancer by the administration or withdrawal of hormones, carcinogenesis by inert objects is brought about by interference with an extracellular homeostatic mechanism. It is possible, for instance, that the presence of the object interrupts tissue communication and negative feed-back control mechanisms over an area. From studies on cells grown in tissue culture it is apparent that an environment in which cells may divide and move freely leads sooner or later to the appearance of cancer-like cells.

It is possible that this is also the explanation of some examples of what has up to now been regarded as chemical carcinogenesis. Certain iron-carbohydrate complexes, such as iron-dextran, iron-dextrin and saccharated iron oxide, induce local cancer when injected subcutaneously or intramuscularly into animals. The carbohydrate moieties of the complexes do not themselves induce cancer, nor does iron in other forms do so. The complexing of iron with the carbohydrates leads to the formation of very large molecules. These are ingested by scavenging white blood cells which may remain for long periods at the site of injection, particularly in animals which are not generally deficient of iron. The question is do these large masses of iron complex loaded cells act in the same way as implanted inert objects and do they interfere with trans-tissue communication in the same way as such objects? Certainly the whole sequence of events which precedes the development of cancer is very similar in the two instances.

AT FIRST SIGHT it may seem that this is a merely academic problem but, in fact, it has important practical implications. In most countries, governments now require that constituents of cosmetics and pharmaceutical preparations, substances added to food, and substances such as pesticides or herbicides which may contaminate food should be tested for carcinogenicity in laboratory animals. A positive result in any carcinogenicity test renders a substance, if not completely unacceptable, then at least suspected of being dangerous for man. Should a potentially useful food additive or drug, intended for oral administration, be banned on the grounds that it induces cancer at the site of its subcutaneous or intramuscular injection in animals? Might not the latter be an implantation-induced effect rather than an example of true chemical carcinogenesis?

AS MORE AND MORE different types of agent—chemical, physical and viral—have been shown to be capable of inducing cancer, the avoidance of exposure to known carcinogens which was once easy became at first difficult and then impossible. Substances capable of inducing cancer in laboratory animals are present in vehicle exhausts, tobacco smoke and the general atmosphere—even where there is no marked pollution. They have also been isolated from certain cooked foods and even from so called 'health' foods. Dangerous materials are used in many branches of industry. They are present in freshly mined minerals, such as asbestos, in the materials used for household or garden maintenance, such as creosote, and in a variety of pharmaceutical preparations. No man, be he primitive or civilized, can completely avoid exposing himself to potentially carcinogenic factors. Why then bother to try? Why stop smoking? Why take precautions? The answer is that it is a matter of dose and probability: the larger the quantity of carcinogen taken into the body the greater the risk of cancer.

In the consideration of an environmental factor the question "Is this carcinogenic?" is the wrong one—since it only permits of two answers, "Yes" or "No". The better question is, "What is the risk that exposure to a measured dose of this particular agent by a specified route of exposure will lead to cancer during a stated interval of time after exposure?" The question framed in this way takes into account the vital part played by the exposed tissue in the carcinogenesis process. It also stresses the distinction between major and minor carcinogenic hazards. Such a distinction can be made tentatively on incomplete evidence and given as an expression of informed opinion, whereas the inflexibility of the 'all' or 'none' distinction between 'carcinogenic' and 'non-carcinogenic' stultifies clear thinking and paralyses legislative action. For those who insist on an 'all' or 'none' distinction perhaps the most reasonable one is that there are two types of substances, materials or agents, those which have been shown to induce cancer and those which have not *yet* been shown to do so. In other words there may be no agents in the 'none' category.

## FURTHER READING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CHEMICAL CARCINOGENESIS AND CANCERS by W. C. Hueper and W. D. Conway (*Charles C. Thomas, Springfield, Illinois, 1964*)



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# Stable isotopes of lithium: dissimilar biochemical and behavioral effects

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*Summary.* Lithium, which is used routinely in the treatment of mania, is composed of two stable isotopes, lithium-7 (92.58%) and lithium-6 (7.42%). Usually there is minimal physiological or biochemical differentiation between isotopes of an element, but lithium is an exception. Data derived from a variety of biochemical and behavioral experiments are reviewed to support this idea. Additionally, the clinical implications of this work are presented.
*Key words.* Lithium; isotopes; lithium-6; lithium-7; mania.

Use of isotopes of an element has been invaluable in studies of a variety of problems including metabolic pathways, enzyme reaction mechanisms and the distribution of administered drugs. Isotopes of lighter elements have significant mass differences, which could be manifested by unexpected differences in biological systems. However, when isotopes are incorporated into comparatively large molecules, differences between their masses (i.e. hydrogen, deuterium and tritium) are reduced because of high overall molecular weights. In the case of lithium (Li) it is the ion, $Li^+$, that is biologically active.

As a consequence of its small mass, $Li^+$ possesses physicochemical properties different from the other alkali metal ions, sodium ($Na^+$), potassium ($K^+$), rubidium ($Rb^+$) and cesium ($Cs^+$), with respect to solubility, formation of complexes and magnitude of the radius of hydration. Li exhibits properties intermediate between the alkali and alkaline earth elements; it is similar to magnesium (Mg) with respect to ionic radius and calcium (Ca) with respect to charge density. The uniqueness of $Li^+$ is primarily a result of the small size of its ionic radius and a capability of polarization superior to the other alkali metal ions, which leads to solvation and covalent bond formation[7]. As would be expected $Li^+$ has both the biggest charge/radius ratio and radius of hydration of the alkali metal ions; these combined effects have a profound influence on the transport of $Li^+$ ions across the cell membrane.

Naturally occurring lithium (Li-N) is composed of two stable isotopes: lithium-7 (Li-7) is the major (92.6%) and lithium-6 (Li-6) is the minor constituent (7.4%). Nuclei of Li-6 contain three protons and three neutrons, Li-7 three protons and four neutrons. Radii of hydration of $(Li\text{-}6)^+$ and $(Li\text{-}7)^+$ are not the same because of differences in the charge/mass ratios of the unhydrated ions. The associated changes in the electrostatic interaction between the isotope ions, water molecules and negatively charged membrane species could contribute to dissimilarities in transport of the two Li isotopes across membranes. Isotopically pure Li-6 and Li-7 salts are commercially available.

Previous references to the utilization of Li-6 in biological systems are not abundant. Thellier et al.[21] used Li-6 to determine the distribution patterns of Li in the rat with a technique related to conventional autoradiography. Li-6 was also used as a tracer for Li-7 in a pharmacokinetic study by Birch et al.[4]. A limited number of reports is found in the literature dealing with the chronic toxicology of the stable Li isotopes and their possibly different effects. Rats maintained on small quantities of Li-6 showed no obvious signs of toxicity and four human subjects received Li-6 without experiencing any ill effects[4,5]. However, recent studies have demonstrated differential effects of the Li isotopes in a variety of systems using biochemical, pharmacological, behavioral and toxicological approaches. An internal consistency is apparent with respect to the data obtained in these studies, regardless of the specific methodology used and an isotope effect is clearly indicated.

Until recently it has been tacitly assumed that toxicity of the two Li isotopes was equivalent primarily because isotopically pure Li-6 administered for a year in drinking

water produced no obvious effects in rats[5]. However, when massive amount of the two Li isotopes were administered i.p. in equal doses in mice, Li-6 proved to be more lethal than Li-7[1]. Li-N had a lethality that was intermediate between Li-6 and Li-7. When the toxicity data was expressed in terms of 50% lethal dose (LD-50), the LD-50's for Li-6, Li-N and Li-7 were 13 meq/kg, 15 meq/kg and 16 meq/kg, respectively. Mice given Li-6 became lethargic 5–10 min after administration; mice treated with Li-7 appeared to be more alert.

Four groups of Swiss-Webster mice were given drinking fluid containing the chlorides of Li-6, Li-7 and Li-N or water for six weeks[2]. Li taste was masked with saccharin. The Li-6 group displayed greater changes than the Li-7 group. All Li-treated mice showed less gain in weight than controls, but only Li-6 mice showed weight loss. Li-6 mice experienced a greater decrease in spontaneous motility. Mortality was higher in Li-6 than Li-7 animals (80% versus 20%) and deaths occurred earlier than in Li-6 animals.

Spontaneous motor activity in experimental animals is known to be decreased after treatment with Li. Rats given Li-N for up to ten days showed a significant decrease in exploratory behavior and motility[9,10,15,19]. Isotopically pure Li-6 and Li-7 (1.5 meq/kg twice daily) administered i.p. to rats decreased motility; Li-6 initially produced a more profound effect than Li-7[11]. The differential behavioral effect of the isotopes proved to be time dependent, being most evident on the third day of treatment and disappearing by the fifth treatment day. On the third day of treatment the mean motility of the Li-6 group was 52% of the pre-Li value ($p < 0.02$); the mean motility of the Li-7 group was 82% of the pre-Li value ($p < 0.05$). Of interest was the difference in motility on the third treatment day between ranked pairs of Li-6 and Li-7 animals, which was statistically significant at a confidence level of $p < 0.05$.

Pharmacokinetic studies were performed in vivo in cats to determine the distribution of the Li isotopes between plasma and CSF. Adult cats were given a single dose acutely of either Li-6 or Li-7 in the chloride form by intubation (1 meq/kg)[20]. The mean plasma level of Li-6 was consistently higher than Li-7 between two and nine hours post-administration. The elimination curve for the isotopes was biphasic; the slow component for Li-6 had a mean half-life of $12.9 \pm 0.69$ (SE) h and for Li-7, $15.9 \pm 1.28$ (SE) h ($p < 0.01$). The determinations of simultaneous CSF and plasma kinetics provided a convincing demonstration of the differential handling of Li-6 and Li-7. The plasma and CSF concentrations of Li-6 were higher than those of Li-7. The CSF/plasma ratio for Li-6 was greater than Li-7 ($p < 0.05$), indicating a differential distribution of the two isotopes at the blood-brain barrier.

In studies of the effects of Li in pregnancy and early development in rats, chlorides of Li-6, Li-7 or Li-N were given orally to 3-month-old females prior to and during gestation and lactation[17]. Two dosage schedules were used, 2 meq/kg and 4 meq/kg. At the low dose alterations in maternal behavior and delays in early development were found. Effects were more pronounced at the higher dose, primarily in the development of visual depth perception. Li-6 at both doses appeared to have a stronger effect on maternal behavior and development than Li-7. Maternal effects were transient, gradually disappearing after cessation of Li ingestion; effects in offspring persisted for at least four months after treatment was terminated.

With an in vitro uptake technique it was demonstrated that the human erythrocyte membrane differentiated Li-6 from Li-7[12]. The quantity of Li-6 which accumulated in the erythrocyte was found to be greater than Li-7 with the ratio of concentrations of Li-6/Li-7 in the erythrocyte ranging from 1.05–1.08. On the basis of the uptake data an isotope effect of 5.4–8.5% appeared to exist between Li-6 and Li-7. More Li-6 was taken up by the erythrocytes than Li-7 at the end of three and four hours of incubation in homologous plasma from either males or females. The difference was statistically significant (males: 3 h, $p < 0.025$; 4 h, $p < 0.01$; females: 3 h, $p < 0.025$; 4 h, $p < 0.05$) using paired comparison t tests. The combined data for erythrocytes from both males and females also indicated a statistically significant larger accumulation for Li-6 than Li-7 for both incubation times (3 h, $p < 0.01$; 4 h, $p < 0.05$) using paired comparison t tests.

Li salts are routinely administered in the treatment of manic-depressive illness[3]. Taken for long periods of time these salts may produce toxic side effects, including kidney damage[3]. Intake during pregnancy, while discouraged, does occur and raises questions of possible deleterious effects on offspring development[16,23]. Of course salts used in therapy contain both Li-6 and Li-7. No data on therapeutic effectiveness of isotopically pure preparations are available. However, it has been established at the membrane level that manic patients given Li-N chloride were able to distinguish Li-6 from Li-7[14]. Thirty manic patients 21–77 years of age treated with Li from 4 to 156 months participated in the study. Using a modification of the method of Brost et al.[6] the isotopic abundances in erythrocytes and plasma were determined by atomic absorption spectrophotometry. Had no isotopic fractionation occurred, the ratio of Li-6 abundances in erythrocytes and plasma would have been unity. The ratio of abundances was $1.271 \pm 0.041$ (SE) with a significance of $p < 0.01$, demonstrating that the erythrocyte membrane had the in vivo capability of distinguishing Li-6 from Li-7. The percentage of Li-6 in the patient erythrocytes was enhanced by a factor greater than 25%. Sherman et al.[18] corroborated the differential treatment of Li-6 and Li-7 by a biological membrane. They found that the rat cerebral cortex was able to distinguish between the isotopes of Li. After a s.c. administration of equal doses of the two isotopes the ratio of Li-6/Li-7 in the cerebral cortex was 1.5. Furthermore, Li-6 enhanced myoinositol-1-phosphate activity more than Li-7, 26.8% versus 17.7%, respectively. It was concluded that the differential effects of Li-6 and Li-7 on inositol metabolism can be explained by the ability of brain tissue to take up the two isotopes differentially.

A possible unifying concept in all these studies may be the relationship between the observed effects and $Li^+$ ions crossing a membrane or series of membranes. Accumulated Li-N transport data indicate that a $Li^+$ ion crossing a membrane requires energy-dependent and passive diffusion mechanisms[15]. An assumption is made that iso-

topes of Li are initially transported in the hydrated form. Based upon lattice constant measurements the ionic volume of $(Li\text{-}6)^+$ is greater than $(Li\text{-}7)^+$[8,22]. However, it is the hydrated form $(Li\text{-}7)^+(H_2O)$ that has a greater ionic volume than $(Li\text{-}6)^+(H_2O)$. Because of its smaller ionic volume, the passage of $(Li\text{-}6)^+(H_2O)$ by passive diffusion through a membrane channel would be more rapid than the passage of $(Li\text{-}7)^+(H_2O)$. An energy dependent mechanism of transport would of necessity make an explanation more complex.

Because of the differential treatment of the two Li isotopes by membrane systems in vitro and in vivo, the possibility was raised that harmful sideeffects of Li during prolonged clinical use in manic-depressive patients might be reduced by administering only Li-7, the less toxic of the two stable isotopic forms. There is ample justification for clinical trials of this hypothesis because of the data indicating both the greater in vivo toxicity and the more rapid penetration in vitro of Li-6 than Li-7. Whether Li-7 alone would have therapeutic efficacy equivalent to Li-N remains to be established.

The finding that biological systems can discriminate between isotopes of a given element is in itself provocative. Relative rates at which isotopes cross biological membranes might be used to provide further information regarding the mechanisms of ion transfer. They could also provide further information pertinent to explaining the mechanisms of the differential effects of these isotopes on neuronal activity and behavior. Finally, these observations may have clinical relevance for ameliorating toxicity in clinical disorders for which Li treatment is indicated.


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# Full Papers

## Long-term motor activity recording of dogs and the effect of sleep deprivation

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*Summary.* Motor activity of laboratory dogs was recorded for several weeks with an ambulatory monitoring device. The effect of 24 h sleep deprivation (SD) on motor activity during recovery was investigated. A clear rest-activity rhythm was established. The dogs exhibited a similar mean daily rest-activity pattern: 1) rest occurred mainly in the dark; 2) the animals were most active after light onset; activity increased during the last two dark hours; 3) a rest period was found at noon and reduced activity during afternoon hours. There was a marked difference in total activity