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Superintendencia de Industria y Comercio
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Trªmite 011
Evento: 379
Actuación: 444

Referenda : Expediente No. 04.097.209
Asunto : SOLICITUD DE PATENTE PARA: "CONJUGADOS UNIDOS A
- CARBAMATO DE AGENTES TERAPEUTICOS 0 CITOTOXICOS ’t’
PEPTIDOS BIOLOGICAMENTE ACTIVOS"
Solicitante : THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND
Clase : A61 031/027, A61 038/008, A61 047/042, C07K
Fecha de solicitud: Septiembre 29 de 2004

1. Yo, Luz Clemencia DE PAEZ, en ml condición de apoderada de la sociedad solicitante de la

patente de la referenda, me refiero al Oficlo No. 441 notificado por fijación en lista del 15 de enero 2010.

2. Mediante el Oficio No. 441 se puso en conocimiento del solicitante el informe del examen de fondo que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Por medio de memorial radicado el 12 de Abril de 2010, solicitØ plazo de treinta dIas hÆbiles para atender elOficlo No. 441. Al respecto, atiendo a las observaciones de la manera siguiente:

3. Claridad del capitulo reivindicatorio:

3.1 Con el fin de atender las observaciones realizadas en cuanto a la claridad del capItulo reivindicatorio y de conformidad con lo establecido en el artIculo 34 de la DecisiOn 486, se presenta un nuevo capitulo reivindicatorio como sustituto del anteriormente presentado, en el cual se tuvieron en cuenta las objeciones hechas por su Despacho, realizando los siguientes cambios:

3.1.1 Se redacta una nueva reivindicaciOn I en la cual se incluyen los agentes citotOxicos que

se encuentran soportados por la descripciOn de la presente solicitud, se limita la definiciOn

estructural de la secuencia espaciadora hidrofIlica y la definiciÆn de la porciOn objetivo Q de

acuerdo con el contenido de las anteriores reivindicaciones 6, 10 y 12.

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Par Ia anterior, es clara que la materia se encuentra bien definida y tatalmente soportada por
los capItulas descriptivo y reivindicatorio.

4. NIVEL INVENTIVO

Ese Despacho afirrna que el documento Dl (US5948750) afecta el nivel inventivo de la presente invenciOn. A este respecto, queremos manifestar que el nuevo capitulo reivindicatorio va dirigido a un compuesto que incluye un agente citotóxico seleccianada de una lista que se une qulmicamente par medio de un enlace carbamato a un pØptido somatostatina, bombesina un pØptido intestinal vasoactivo (VIP) como porción objetivo. Un enlace carbamato se representa coma:
0

11
R1 0 C N R3

R2

El documento Dl no enseæa asugiere compuestos que tengan un enlace carbamato y mucho menos un compuesto que tenga un enlace carbamato que se una a uno de los agentes citotOxicos que se presentan en el nuevo capitulo reivindicatorio. Por el contrario, Dl presenta conjugados quimicas que comprenden aligopØptidos que tienen secuencias de amino Æcidoque son proteolIticamente selectivos unidos par antIgenos prostÆticosespecificos (PSA) libres y que incluyen un Æcido amino ciclico que tienen un substituyente hidrofilico y agentes citotóxicas conocidos (ver cal. 2, Ilneas 54 a 58 de D1). Por Ia tanto, Dl no enseæa 0 sugiere que la porción oligapØptida del conjugado esta unida al agente citotOxico por media de un enlace carbamato. AdemÆs, ninguno de los ejemplos del documento Dl describe la preparación de un compuesto de acuerdo con el nuevo capitulo reivindicatorio. Par ejemplo, los ejemplos 3, 4 y 5 muestran agentes peptIdicos unidos a daxorubicino par medlo de un enlace amida y no por un enlace carbamato. Asi entonces no es posible concluir que el documento Dl enseæaa sugiere de alguna forma las compuestos de la presente solicitud.

AdemÆs de Ia anterior, los compuestos de la presente solicitud muestran efectas sorprendentes, par ejemplo, en las tablas I y 2 y en la figura 2 de la presente solicitud que se muestran compuestos descritas como en el nuevo capitulo reivindicatorio, que tienen un pØptido somatostatina coma la pardon Q y un captatecina a combretastatina coma Is porción X, muestran vidas medias largas en una solución amortiguadora de fasfata y en suero de rata, son orientados a cØlulas que expresan somatostatina (coma una funciOn de la inhibiciOn del crecimiento hormonal) y demuestran actividad citatOxica con las cØlulas humanas neuroblastoma [MR-32.

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TamblØn adjuntamos al presente memorial algunas publicaciones posteriores a la presente solicitud para probar su nivel inventivo. Dichas publicaciones describen una investigación realizada por los inventores de la presente invención y otros asistentes proporcionando evidencia de los resultados inesperados de los compuestos que se describen en el nuevo capitulo reivindicatorio. En particular el documento de Moody et at. muestra efectos anti tumores e vitro mediados por CPT-L2-BA3, los cuales corresponden a un compuesto en el que
X es camptotecina, n=2, R=N(R1R2), en donde R1 es hidrógeno y R2 es metilo, Y se omite, Z es D-Ser-D-Tyr y X es un pØptido bombesina que tiene la secuencia Gln-Trp-Ala-Val-i3Ala-His-Phe-Nle_NH2 (la estructura quimica de CPT-L2-BA3 se proporciona en la figura I en la pÆgina 23582 del documento de Moody et at.; tambiØn se presenta en el ejemplo 8 de la presente solicitud).

AdemÆs de lo anterior, Moody et al. muestra que un compuesto tal y como se describe en la presente solicitud, en la cual X es camptotecina, n=2, R=N(R1R2), en la que R1 es hidrôgeno y R2 es metilo, Y se omite, Z se omite y X es un VIP, es un citotóxico para cØlulas cancerIgenas
de seno.

Finalmente, el artIculo de Sun et at., muestra que un compuesto tat y como se presenta en el nuevo capitulo reivindicatorio, en el que X es camptotecina, n=2, RN(R1R2), en donde RI es hidrogeno y R2 es metilo, V se omite, Z es D-Ser-NIe-D-Tyr-D-Ser y X es un pØptido somatostatina que tiene la secuencia cIclica [Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-NH2, es citotóxico para neuroblastoma, cancer pancreÆtico, carcinoide pancreÆtico, leucemia y cØlulas cancerIgenas de prOstata, tambiØn muestra actividad anti angiogenica vitro y una habilidad para inhibir la metastasis de los tumores altamente invasivos PC-3.

Los datos en la presente solicitud, as[como los presentados en las publicaciones que se adjuntan, proporcionan evidencia sobre la alta especificidad de los compuestos de la presente solicitud en cØlulas que expresan somatostatina, bombesina VIP receptores (es decir, cØlulas cancerIgenas) en comparaciOn con los compuestos conjugados citotóxicos del arte previo o los agentes quimo terapØuticos, pues los compuestos de la presente solicitud perrnite la union de largas molØculas de fÆrmacos (es decir, camptotecina, homocaptotecina, colchicina, tiocolchicina, combretastatina, dolastatina, doxorubicina, metotrexato, podofilotoxina, rizoxina rizoxina D, un taxol, paclitaxel, CC1065 y maitansinoides) utilizando el enlace carbamato que so define en el nuevo capitulo reivindicatorio sin perder una afinidad receptora y retención de las actividades de anti tumor y anti angiogØnicas. Los compuestos de la presente solicitud tambiØn muestran menos toxicidad sistØmica con respecto a los agentes citotOxicos individuales, la habilidad de inhibir el crecimiento de tumores expresan un receptor al cual el compuesto esta dirigido (es decir, una somatostatina, bombesina o receptor VIP) y la habilidad de bloquear el

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desarrollo de angiogenesis. Los compuestos de la presente solicitud proporcionan una gran especificidad en las cØlulas de tumor con menos toxicidad que las cØlulas normales.
De acuerdo con lo anteriormente expuesto y con la modificaciOn realizada al capItulo reivindicatorio, la objeciOn por nivel inventivo que hizo su Despacho queda subsanada.

5. Conclusion:

Teniendo en cuenta las modificaciones efectuadas en el pliego reivindicatorio, se puede concluir que la presente invención Si cumple con el requisito de claridad establecido en el
artIculo 30 de la Decision 486.

Por otro lado, gracias a los argumentos mencionados a favor del nivel inventivo de la presente solicitud, queda claro que el documento citado por su Despacho no es una anterioridad valida para que se afecte dicho requisito.

Puesto que se ha atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, respetuosamente solicito se continUe con su tramitaciOn.

6. Anexos:

Nuevo pliego reivindicatorio, conformado por trece (13) reivindicaciones.
Formulario para modificaciones.
Recibo por $ 78 000 pesos por concepto de las 3 reivindicaciones en exceso de la dØcima. Moody et al. (J. Pharmacol. Exper. Therap. 318:1265-1272, 2006)
Moody et al. (J. Biol. Chem. 279:23580-23589, 2004)
Moody et al. (Peptides 28:1883-1890, 2007)
Sun et al. (Targeted chemotherapy using a cytotoxic somatostatin conjugate to inhibit tumor growth and metastasis in nude mice) 39 pg.

SeæoraDirectora,

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LUZ CLEMENCIA DE PAEZ
C.C. No. 35.456.344 de Usaqueri T.P.A. No. 23.555

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SOLICITUD DE

Correcciôn de Error Material X Modificación a una solicitud
Nombre: THE ADMINISTRATORS OF THE
TULANE EDUCATIONAL FUND

2 Sociedadconstltuida existente bajo las leyes del
SOILI.#I IUTAITE 1U’I Estado de Louisiana, Estados Unidos de America,

IDENTIFICACION
DirecclOn: 1430 Tulane Ave.,

New Orleans, LI NIT LI Louisiana 70112,

Estados Unidos de America C.EE. LI Otro LI

Domicilio: New Orleans, Nümero:
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IDENTIFICACION 0 Nombre: Luz Clemencia de PAEZ

Dirección: Carrera 41 No. 72-35 C.C. 19 NIT LI
REPRESENTANTE TelØfono: 3473611 Fax: 2179211 C E 0 Otro D
0 APODERADO E-mail: caveIierccaveIier.com
NUmero: 35.456.344 TP 23.555

0 Nümero de expediente: 04.097.209

0 Descripción de Ia corrección o modificación

Presento nuevo capitulo reivindicatorio compuesto por 13 reivindicaciones con el fin de superar las objeciones hechas por Su Despacho a la presente solicitud de patente pido respetuosamente que
el examen de patentabilidad sea realizado sabre estas nuevas reivindicaciones presentadas.

0 Comprobante de pago No. 10-40,401 Fecha: 19 de mayo de 2010
Anexos
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L.J Comprobante de pago de Ia tasa par $114.000, por concepto de modificaciones.
II Comprobante de pago par 3 reivindicaciones adicionales despuØs de la dØcima reivindicaciOn
par $78.000.00.
LI Poderes, si fuere el caso
0 Documenta que acredita Ia existencia representaciOn legal de las partes, cuando sean
personas juridicas.

1

REIVINDICACIONES

I. Un compuesto representado por la siguiente formula:

0
R

en donde:

X es camptotecina, homocamptotecina, coichicina, tiocolchicina, combretastatina, dolistatina, doxorubicina, metotrexato, podofilotoxina, rizoxina, rizoxina D, un taxol, paclitaxol, CCI 065 un maitansinoide;

n es un entero de 0 a 6, en donde (CH2)n es un grupo alquilo o un grupo cIclico;

R es N(R1R2), OR,, a SRI, en donde R1 y R2 son, independientemente, hidrOgeno 0 Ufl grupo aiquilo inferior con menos de II Ætomos de carbono;

Y es una secuencia espaciadora hidrofIlica seleccionada entre un pOptido que incrementa la biodistribución hidrofulica de dicho compuesto y un poilmero hidrofulico, o se omite, en donde:

dicho pØptido que incrementa la biodistribuciOn hidrouulica de dicho compuesto tiene la fOrmula U(V-V)n, en donde U es D-Pro, L-Pro, D-4-OH-Pro, L-4-OH-Pro, Sarcosina, Lys, Ore, Dab, Dap, 4-NH2-Phe, o (NH2-(CH2)mCOOH), donde m=2-I0, inclusive, o es suprimido; cada V es independientemente seleccionado del grupo que consiste de: D-Ser, L-Ser, D-Thr, L-Thr, D-Gln, L-Gln, D-Asn, L-Asn, D-4-OH-Pro, 0 L-4 hidroxi-Pro;
y n=I-50, inclusive, y

dicho polImero hidrofilico es polietilenglicol, acetato de polivinilo, polivinil alcohol, HPMA (N-(2-hidroxipropil) metacrilamida) o copolImeros HPMA, cxj3-poli(N-hidroxietil)- DL-aspartamida (PHEA), o a,13-poli(N-hidroxipropil)-DL-aspartamida;

Z es un pØptido ligador que se une a Q en terminal N o en un grupo amino de cadena lateral compatible de Q y tiene la fOrmula:

D-Ser-Nle-D-Ser-D-Ser, D-Ser-Lys-D-Ser-D-Ser, D-Ser-Lys-D-Tyr-D-Tyr, D-Ser-Lys-D-Tyr-D-Ser, D-Ser-Ser-D-Lys-D-Ser, D-Ser-Ser-D-Lys-Ser, D-Ser-Nle-D-Tyr-D-Ser, D-Ser-Pal-D-Tyr-D-Ser, D-Ser-Thr-D-Tyr-D-Ser, Lys-D-Ser-D-Ser, Ser-D-Lys-D-Ser, Ser-D-Lys-Ser, Nle-D-Tyr-D-Ser, Lys-D-Tyr-D-Ser, Pal-D-Lys-D-Ser, Thr-D-Tyr-D-Ser, D-Ser-D-Lys, D-Ser-D-Tyr, D-Lys-D-Lys, D-Lys-D-Tyr 6 D-Tyr-D-Lys 6 se omite; y

2

Q es una porciOn objetivo seleccionada entre un pØptido somatostatina, bombesina un pØptido intestinal vasoactivo,

2. El compuesto de la reivindicaciOn 1, en donde dicha secuencia de espaciador hidrofIlica es dicho pØptido que incrementa la biodistribuciOn hidrofilica de dicho compuesto.

3. El compuesto de la reivindicación 1, en donde al menos un V es un D-aminoÆcido.

4. El compuesto de la reivindicaciOn 1, en donde V es independientemente D-Ser a L-Ser.

5. El compuesto de la reivindicaciOn 1, en donde dicha secuencia de espaciador hidrofIlica es dicho polImero hidrofIlico.

6. El compuesto de la reivindicación 1, en donde R comprende I a 8 Ætomosde carbono.

7. El compuesto de la reivindicaciOn 1, en donde dicho Q es dicho pØptido somatostatina.

8. El compuesto de la reivindicaciOn 1, en donde dicha pØptido somatostatina es c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-NH2.

9. El compuesto de la reivindicación 1, en donde dicho Q es dicho pØptido bombesina.

10. El compuesto de la reivindicaciOn 1, en donde dicho Q es dicho pØptido intestinal vasoactivo.

II. El compuesto de la reivindicación 1, en donde Y se omite.

12. El compuesto de la reivindicación 1, en donde dicho agente citotóxico (X) es camptotecina, dicho pØptido ligador (Z) es D-Ser-Nle-D-Tyr-D-Ser, dicha porción objetivo
(0) es un pØptido somatostatina que tiene la secuencia ciclo[Cys-Phe-D-Trp-Lys-Thr-Cys]- Thr-NH2, y dicha secuencia espaciadora hidrofIlica (Y) se omite, y donde n=2 y R es N(R1R2), en donde R1 y R2 son hidrógeno y metilo, respectivamente.

13. Un medicamento que comprende un compuesto de acuerdo con cualquiera de las reivindicaciones I a 12 y un vehiculo farmacØuticamente aceptable.

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0022-3565/06/3183-1265-1272
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL TIIRRAPEUTICS Vol. 318, No. 3 U.S. Government work not protected by U.S. copyright 10414113133849 JPET 818:1265-1272, 2006 Printed in U.S.A.

In Vitro and in Vivo Antitumor Effects of Cytotoxic Camptothecin-Bombesin Conjugates Are Mediated by Specific Interaction with Cellular Bombesin Receptors

Terry W. Moody, Li-Chun Sun, Samuel A. Mantey, Tapas Pradhan, L. Vienna Mackey, Nieves Gonzales, Joseph A. Fuselier, David H. Coy, and Robert T. Jensen

Department Health and Human Services, Center for Cancer Research, National Cancer Institute (T.W.M.) and Digestive Diseases Branch, National Institute Diabetes and Digestive andKidney Diseases (S.A.M., T.P., N.G., R.T.J.), National Institutes Health, Bethesda, Maryland; and Department Medicine, Peptide Research Laboratories, Tulane Health Sciences Center, New Orleans, Louisiana (L.-C.S., L.V.M., J.AF., D.H.C.)

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Most human tumors overexpress or ectopically express pep-
tide hormone/neurotransmitter receptors, which are being in-
creasingly studied as a means to selectively deliver cytotoxic agents. Although a number of peptide ligand-constructs dem-onstrate tumor cytotoxicity, the role of specific tumoral receptor interaction in its mediation is unclear. To address this question, we synthesized camptothecin (CP1) bombesin (Bn) analogs, in which CPT was coupled via a novel carbamate linker, 1 2 [N-(N-methyl-amino-ethyl)-glycine carbamate], that were chemically similar but differed markedly in their potency/affinity for human Bn receptors. We then examined their ability to interact with Bn receptors and cause in vitro and in vivo tumor cytotoxicity. CPT-IL2-[D-Tyr6j3-A1a11,D-Phe13,N1e14] Bn (6-14) (BA3) bound with high affinity and had high potency for all three human Bn receptor subtypes, whereas CPT-L2-[D-Tyr6,3-Ala1 1, DPhelR,Nlel4] Bn (6-14)[o-Phe-CPT-L2-BA3] had >1400-fold

Recently, there has been increased interest in the develop-ment of cytotoxic antitumor agents that can be specifically targeted to tumors using cellular receptors highly expressed on the tumor or antigenic sites (Dubowchik and Walker, 1999; de Groot et al., 2001; Sun et al., 2002). Attempts at specific delivery have been sought by using antibodies di-

This work was supported by the intramural Research Program of the National Institutes of Health, National Cancer Institute Center for Cancer Research, National Institute of Diabetes and Digestive and Kidney Diseases, and by the Tulane University Peptide Research Fund.
Article, publication date, and citation information can be found at httpihjpet.aspetjournals.org.
doi:lO.1124/jpot.1O6.104141.

lower affinity/potency. ’251 -CPT-1 2-BA3 but not 1251-o-Phe-CPT-L2-BA3 was internalized by Bn receptor subtype-contain-ing cells. CPT-L2-BA3 displayed significantly more cytotoxicity than o-Phe-CPT-1 2-BA3 toward NCI-1›11299 lung cancer cells in both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazo-hum bromide and clonogenic assays and more potently inhib-ited H1299 xenograft growth in nude mice. CPT-L2-BA3 was also metabolically more stable than its parent peptide and inhibited growth of a number of other tumor cell lines in vitro and in vivo. These results demonstrate that specific tumoral receptor interaction is important in mediating the ability of peptide ligand-cytotoxic constructs to cause cytotoxicity. Be-cause many tumors overexpress Bn receptors, these results also demonstrate that CPT-L2-BA3 will be a useful agent for delivering receptor-mediated cytotoxicity to many different hu-man tumors.

rected against specific tumor antigens or by coupling cyto-toxic agents to ligands that interact with receptors that are ectopically expressed or overexpressed by tumors (Dubowehik and Walker, 1999; Sun et al., 2000a). Peptide receptors have received considerable attention because nu-merous studies demonstrated that most benign as well as malignant human tumors ectopically express or overexpress
one or more of these receptors (Virgolini, 1997; Jensen, 2000;
Kwekkeboom et al., 2000; Hoffman et al., 2001; Reubi et al.,
2002; Reubi, 2003; Schally and Nagy; 2003). Furthermore,
ligands that function as agonists at these receptors are rap-
idly internalized, thus providing a mechanism for site-di-

ABBREVIATIONS: Bn, bombesin; GRPR, gastrin-releasing peptide receptor NMBR, neuromedin B receptor; BRS3, bombesin receptor subtype 3; L2, N-(N-methyl-amino-ethyl)-glycine carbamate; CPT, camptothecin; BA3, [D-Tyr8,13-Ala’1,Phe13,Nle14] Bn (6-14); DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; MU, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bro-mide; hBRS3, human BRS3; hNMBR, human NMBR; GRP, gastrin-releasing peptide; hGRPIR, human GRP receptor IP, inositol phosphate; SIT medium, RPMI 1640 containing 3 x iO M sodium selenite, 5jig/ml bovine insulin and 10 cg/ml transferrin; RIA, radioimmunoassay; s.c., subcutaneous; Lp., intraperitoneal.

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rected intracellular delivery of cytotoxic agents coupled to the agonist (Moody et al., 2004). In recent studies, receptor-mediated tumor localization or cytotoxicity has been reported with analogs of bombesin (Bn) (Carroll et al., 1999; Hoffman et al., 2001; Langer and Beck-Sickinger, 2001; Schally and Nagy; 2003; Moody et al., 2004), melanotropin (Langer and Beck-Sickinger, 2001), leutinizing hormone-releasing hor-mone (Langer and Beck-Sickinger, 2001; Schally and Nagy; 2003), vasoactive intestinal peptide (Virgolini, 1997), neu-ropeptide Y (Langer and Beck-Sickinger, 2001),and soma-tostatin (Kwekkeboom et al., 2000; Langer and Beck-Sick-inger, 2001; Sun et al., 2002, 2004; Schally and Nagy; 2003; Moody et al., 2005).
The Bn family of receptors [gastrin-releasing peptide re-ceptor (GRPR), neuromedin B receptor (NMBR), and the orphan receptor bombesin receptor subtype 3 (BRS3)1 are particularly attractive for the development ofpossible tumor-directed cytotoxic agents because many common neoplasms including breast, prostate, gastric, colon, ovarian, small cell, and nonsmall cell lung cancers ectopically express or overex-press these receptors (Reubi et al., 2002). Although this ap-proach shows promise in a number ofstudies to more specif-ically target cytotoxic agents to the tumor with lower toxicity (Kiaris et al., 1999; Schally and Nagy, 2003), the hypothesis that this specificity is due to tumor-specific targeting of the cytotoxic agent due to specific interaction with the tumor receptors has been difficult to prove, particularly with in vivo experiments.
In the present study, we have attempted to address this question pharmacologically by synthesizing active and inac-tive analogs of Bn that were physicochemically similar and were coupled to camptothecin by a novel carbamate linker with a built-in nucleophile-associated releasing group, L2 (Fuselier et al., 2003; Moody et al., 2004, 2005). Our results show that the active camptothecin (CPT)-Bn construct (CPT-L2-BA3), which is a potent, fully active agonist at each ofthe three human Bn receptor subtypes, binds with high affinity and is rapidly internalized by tumors or cells possessing each Bn receptor subtype, whereas the inactive analog (D-Phe-CPT-L2-BA3) is not. Furthermore, in vitro and in vivo cyto-toxicity studies demonstrate the active CPT-Bn construct causes significantly greater inhibition of growth and inhibits the growth in vivo of a large range of human tumors. The active analog CPT-L2-BA3 had enhanced metabolic stability and caused cytotoxicity in a number of human tumors as-sessed by both in vitro and in vivo growth assays. These results provide direct evidence that interaction with tumor receptors is an important mechanism in mediating this en-hanced cytotoxicity and show that this approach can deliver receptor-directed cytotoxicity to a number of tumors.

Materials and Methods

The following cells and materials were obtained from the sources indicated: Balb 3T3 (mouse fibroblast) and NCI-H1299 (human non-small cell lung cancer) cells were from American Type Culture Collection (Rockville, MD); Dulbecco’s modified Eagle’s medium (DMEM), phosphate-buffered saline (PBS), RPM! 1640, trypsin-EDTA, and fetal bovine serum (FBS) were from Biofluids (Rockville, MD); G418 sulfate was from Life Technologies, Inc. (Grand Island, NY); Na11251 (2200 Cilmmol) and myo-[2-3H]inositol (20 Ci/mmol) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK); camptothecin, formic acid, ammonium formate, disodium tetra-

borate, 4-(dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)- 4-H-pyran, 2-dimethylaminoisopropyl chloride hydrochloride, 1-methyl-2-pyrrolidinone, dimethylaminopyridine, soybean trypsin inhibitor, bacitracin, leupeptin, 4-(2-aminoethyl)-benzenesulfonyl fluoride, poly-L-lysine, and Triton X-100 were from Sigma-Aldrich (St. Louis, MO); 1,2,4,6-tetrachloro-3a,6a-diphenylglycouril was from Pierce Chemical Co. (Rockford, IL); AG 1-X8 resin from Bio-Rad (Hercules, CA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2F1-tetra-zolium bromide (MTI’) and iodo-nitrotetrazolium violet were from Sigma-Aldrich; and agarose was purchased from FMC Corp. (Rock-ford, ME).
Cell Culture. NCI-H1299 cells were cultured in RPM! 1640 me-dium containing 10% heat-inactivated FBS. NCI-H1299 cells were split weekly 1’20 with trypsin-EDTA. Balb 3T3 cells stably express-ing human BRS3 (hBRS3), human NMB receptors (hNMBRs), or human GRP receptors (hGRPRs) made as described previously (Be-nya et al., 1995) were grown in DMEM supplemented with 300 mg/1 G418 sulfate. All the other cell lines were from American Type Culture Collection (Manassas, VA). Hs746T cells were grown in DMEM with 10% FBS. LNCaP cells were maintained in RPMI 1640 medium with 10% FBS, 1% sodium pyruvate, and 1% HEPES. DMS-53 cells were cultured in Waymouth’s MB 752/1 medium with 10% FBS. U-87MG cells were grown in DMEM with 10% FBS, 1% sodium pyruvate, and 1% minimal essential medium nonessential amino acids. All other cells were cultured as described previously (Sun et al., 2004). Cells were mycoplasma free and were used when they were in exponential growth phase after incubation at 37°C in 5% CO2 and 95% air.
Preparation of Peptides. The CPT-Bn conjugates were synthe-sized as described previously (Moody et al., 2004). To synthesize a possible inactive analog that was chemically and structurally very similar to the active conjugate CPT-L2-BA3, we analyzed previously published Bn structure-function studies (Rivier and Brown, 1978; Moody et al., 1982; Leban et al., 1993) that suggested that insertion of a D-Phe in position 13 for Phe would likely produce a Bn peptide with greatly reduced affinity and potency. CPT-Bn conjugates were purified to >97% purity by preparative high-performance liquid chromatography (C18 silica) and characterized by mass spectrometry and amino acid analysis. 1251-CPT-L2-BA3, 1251-BA3, and 1251-CPT-n-Phe-BA3, with specific activities of 2200 Ci/mmol, were prepared as described previously (Moody et al., 2004). The radiolabeled pep-tides were separated using a C18 Sep-Pak (Waters Associates, Mil-ford, MA) and further purified by reverse-phase high performance liquid chromatography on a C18 column. The fractions with the highest radioactivity were pooled, neutralized with 0.2 M Tris buffer, pH 9.5, and stored with 0.5% (wlv) bovine serum albumin at 20°C. Binding of "’I-Labeled Bn-Related Peptides. Binding was performed as described previously (Mantey et al., 1997). The stan-dard binding buffer contained 24.5 mM HEPES, pH 7.4, 98 mM NaCl, 6 mM KC1, 5 mM MgC12, 2.5 mM Nal{2PO4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 0.01% (w/v) soybean trypsin in-hibitor, 1% amino acid mixture, 0.2% (w/v) bovine serum albumin, and 0.05% (w/v) bacitracin. Balb 3T3 cells stably expressing hGRPR (0.3 X 10), hNMBR (0.03 X 10), hBRS3 (0.3 X 10), or NCI-H1299 cells were incubated with 0.05 nM ’251-labeled ligand at 22°C for 60 min. Aliquots (100 l) were removed and centrifuged through 300 l of incubation buffer in 400-,.d Microfuge tubes at 10,000g for 1 mm using a Beckman microcentrifuge B. The pellets were washed twice with buffer and counted for radioactivity in a gamma counter. The nonsaturable binding was the amount of radioactivity associated with cells in incubations containing 50 pM radioligand (2200 CU mmol) and J gM unlabeled ligand. Nonsaturable binding was <10% oftotal binding in all the experiments. Inhibition constants (K) were determined using a least-square, curve-fitting program (Kaleida-Graph; Synergy Software, Reading, PA) and the Cheng-Prusoffequa-tion (Cheng and Prusoff, 1973).
Internalization. Balb 3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 were incubated with raclioligands as stated an-

der Materials and Methods for binding. Internalization experiments were performed as described previously (Moody et al., 2004). In brief, after the indicated incubation times, 100-1l samples were added to a 1.5-ml Microfuge tube with 1 ml of 4°C acid -stripping solution con-taining 0.2 M acetic acid, pH 2.5, and 0.5 M NaCl to remove the surface bound radioligand. After 5 mm, the cells were pelleted, the supernatant was removed, and the cells were washed twice with incubation buffer. In all cases, parallel incubations were conducted in the presence of 1 AM unlabeled ligand to determine changes in nonsaturable binding. Results are expressed as the percentage of saturable ’251-ligand added that is surface bound (acid-stripped) or internalized (not acid-stripped).
Measurement of [3H]Inositol Phosphate. Changes in total [3ll]inositol phosphates ([3H]IP) were measured as described previ-ously (Benya et al., 1995). hBRS3-, hGRPR-, or hNMBR-transfected Balb 3T3 cells were cultured into 24-well plates (5 X cells/well) in media and then incubated for 24 h at 37°C in a 5% CO2 atmosphere. The cells were then incubated with 3 Ci/ml myo-[2-3H] inositol in media supplemented with 2% FBS for an additional 24 h. The 24-well plates were washed by incubating for 30 min at 37°C with 1 ml/well PBS, pH 7.0, containing 20 mM lithium chloride. The wash buffer was aspirated and replaced with 500 Al of IP assay buffer containing 135 mM sodium chloride, 20 mM HEPES, pH 7.4, 2 mM calcium chloride, 1.2 mM magnesium sulfate, 1 mM EGTA, 20 mM lithium chloride, 11.1 mM glucose, and 0.05% (w/v) bovine serum albumin and incubated with or without any of the peptides studied. After 60 min ofincubation at 37°C, the experiments were terminated by the addition of 1 ml of ice-cold 1% (v/v) hydrochloric acid in methanol. Samples were loaded onto Dowex AG1-X8 anion exchange resin columns (Bio-Rad), washed with 5 ml of distilled water to remove free [3H]inositol, then washed with 2 ml of 5 mM disodium tetraborate/60 mM sodium formate solution to remove [2lllglycero-phosphoryhnositol. Two milliliters of 1 mM ammonium formate/100 mM formic acid solution was added to the columns to elute total [3H]IP. Each eluate was mixed with scintillation cocktail and mea-sured for radioactivity in a scintillation counter.
Proliferation Assays Growth studies were performed using the MTT assay (Moody et al., 2004). CFPAC-1, DMS-53, DU-145, Hs746T, HT-29, 1MR32, LNCaP, MCF-7, MOLT-4, NCI-H69, NCI-111299, PC-3, SKNSII, or U-87MG human cancer cells (104/well) were placed in SIT medium (100 Al), and various concentrations of CPT-L2-BA3 or D-Phe-CPT-L2-BA3 were added. After 4 days, 15 Al (1 mg(ml) of MTT was added. After 4 h, 150 Al of dimethyl sulfoxide was added. After 16 h, the optical density at 570 rim was determined using an enzyme-linked immunosorbent assay reader. The prolifer-ation rates were calculated from the optical density readings with various concentrations of CPT-L2-BA3 using the untreated cells as 100.

Clonogenic Assays. The effects of CPT-BN conjugates on the growth of NCI-111299 cells were investigated using a clonogenic assay (Mabmoud et al., 1991). The base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% fetal bovine serum in six-well plates. The top layer consisted of 3 nil ofSIT medium in 0.3% agarose, CPT-BN conjugates, and 5 X 10 NCI-H1299 nonsmall cell lung cancer cells. Triplicate wells were plated; after 2 weeks, 1 ml of 0.1%p-iodonitrotetrazolium violet was added, and after 16 hat 37°C, the plates were screened for colony formation; the number ofcolonies larger than 50 AM in diameter were counted using an Omnicon image analysis system (BioLogics, Inc., Manassas, VA).
Nude Mouse Xenografts. The ability of the CPT-L2-BA3 or r)-Phe-CPT-L2-BA3 to inhibit CFPAC-1, NCI-H69, NCI-H1299, or PC-3 tumor proliferation was investigated in vivo (Mabmoud et al., 1991). Female athymic BALB/c nude mice (Taconic Farms, German-town, NY), 4 to 5 weeks old, were housed in a pathogen-free temper-ature-controlled isolation room with a diet consisting of autoclaved rodent chow and autoclaved water given ad libitum. NCI-H1299 cells (1 X 10) were injected into the right flank of each mouse by s.c. injection. Palpable tumors were observed in approximately 90% of

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the mice after 1 week. CPT-L2-BA3 and D-Phe-CPT-L2-BA3 (1 nmol in 100 Al ofPBS) were injected daily s.c. adjacent to the tumor or, in the case of the studies shown in Table 2, in the flank opposite the tumor or i.p. as indicated. The tumor volume (height X width X depth) was determined twice weekly by calipers and recorded. The animal studies were approved by National Institutes of Health or Tulane University animal care and use committees.
Stability of CPT-L2-BA3. The stability ofCPT-L2-BA3 in mouse plasma was investigated by radioimniunoassay. Mouse blood was obtained from nude mice, and after centrifugation ofthe red cells, the mouse plasma was added to 100 AM CPT-L2-BA3. As a function of time after addition of mouse plasma, 0.2 N HC1 was added and the sample frozen and lyophilized. The sample was resuspended in ra-dioimmunoassay (RIA) buffer (0.25% bovine serum albumin in PBS), and the amount of immunoreactive CPT-L2-BA3 was determined. The radioimmunoassay was performed using a rabbit antibody elic-ited against BA3 linked to keyhole limpet hemocyanin with carbodi-imide. The BA3-carbodiimide-KLH conjugate was initially injected s.c. into rabbits, in Freund’s complete adjuvant, followed by monthly boosts in Freund’s incomplete adjuvant. Plasma samples were incu-bated with rabbit anti-BA3 sera (1:1000) and 125I-CPT-L2-BA3 (10,000 cpm) added in RIA buffer. After incubation at 4°C for 16 h, free 1251-CPT-L2-BA3 was removed by charcoal addition. The sample was centrifuged at 1000g for 5 min, and the ’251-CPT-L2-BA3 bound to the antibody remained in solution and was counted in a LKI3. gamma counter (GE Healthcare).

Results

BA3, CPT-L2-BA3, and n-Phe-CPT-L2-BA3 Binding and Internalization. 1251-CPT-L2-BA3 and ’251-D-Phe-

CPT-L2-BA3 were examined for their ability to interact with cells stably transfected with human Bn receptors (i.e., hGRPR, hNMBR, hBRS3) (Fig. 1). For each of these three human Bn receptor subtypes, 1251-CPT-L2-BA3 but not 1261 a-Phe-CPT-L2-BA3 demonstrated saturable binding (Fig. 1, top). Saturable binding of ’251-CPT-L2-BA3 was time-depen-dent and half-maximal after approximately 3 nun and max-imal by 30 min in each Bn receptor subtype (Fig. 1, top). The ability of human Bn receptor containing cells to inter-nalize 1251-CPT-L2-BA3 and 1251-n-Phe-CPT-L2-BA3 was in-

vestigated. Approximately 40% of the added 1251-CPT-L2-BA3 was internalized by cells containing hGRPR, hNMBR, or hBRS3 after 10 min (Fig. 1, bottom). In all cases, however, the amount of ligand internalized after a 30-min incubation was greater than the amount internalized after a 10-min incubation (Fig. 1, bottom). In contrast, there was no signif-icant internalization of 1251-D-Phe-CPT-L2-BA3 in cells con-

taining hGRPR, hNMBR, or hBRS3 at 10 or 30 min (Fig. 1, bottom). The results indicate that 1251-CPT-L2-BA3 but not ’251-D-Phe-CPT-L2-BA3 internalizes significantly in cells containing each ofthe different human Bn receptor subtypes. To determine the affinities of the various BA3 analogs for each human Bn receptor subtype, dose-inhibition curves were performed with each BA3 analog and 1251-BA3 for each of the three human Bn receptor subtypes (Fig. 2). Specific 1251..BA3 binding to cells containing hGRPR was inhibited in a dose-dependent manner by BA-3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 (Fig. 2, top, left). The IC,, values for BA-3, CPT-L2-BA3, and n-Phe-CPT-L2-BA3 were 0.17 – 0.01, 0.16 – 0.01, and 603 – 16 nM, respectively. Similar IC50 values were obtained using cells containing hNMBR (Fig. 2, top, middle); however, cells containing hBRS3 (Fig. 3, tap, right) bound BA-3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3

23

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10

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I hBRS3 I

0 10 20 30

TIME OF INCUBATION WITH I

10 30 10 30 10

TIME OF INCUBATION

30

Fig. 1. Top, time course of binding of ’I-CPT-L2-BA3 and 1251-n-Phe-CPT-L2-BA3 to cells containing human Bn receptors. The ability of 1251. CPT-L2-BA3 and 1251-o-Phe-CPT-L2-BA3 to bind Balb 3T3 cells stably transfocted with hGRPR, hNMBR, or hBRS3 (0.3 x 10 cells/ml) as a function of time was determined. Results are means – S.E.M. from at least three experiments, and in each experiment, each data point was repeated in duplicate. Bottom, ability of ’251-CPT-L2-BA3 and ’251-n-Plie-CPT-L2-BA3 to be internalized by cells containing human Bn receptor subtypes. Balb/3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 were incubated for 10 or 30 min at 37°C with 0.05 nM ’251-CPT-L2-BA3 or 1251-D-Phe-CPT-L2-BA3 in the presence or absence of 1000 nM CPT-L2-BA3 or n-Phe.CPT-L2-BA3, respectively. Results are expressed as the percentage of the saturably bound radiolabeled peptide internal-ized. Results are mean – S.E.M. from at least three experiments, and in each experiment, each data point was determined in duplicate.

with lower affinity (IC,, values of 8.1 – 0.3, 2.1 – 0.1, and >3000 nM). These results indicate that for each of the three human Bn receptor subtypes, CPT-L2-BA3 had approxi-mately equal high affinity to BA3, whereas D-Phe-CPT-L2-BA3 had a 400- to 3000-fold lower affinity.
BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 Function as Full Agonists. To determine the biological activity of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3, their ability to stimulate phosphoipase C was assessed because activation of each of the human Bn receptor subtypes is known to stimulate this intracellular cascade (Mantey et al., 1997). To assess phospholipase C activation, dose-response curves for BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 to stimulate turnover of [3H:Iinositol phosphates was examined (Fig. 2,

120
110

100

CL, 90
80
70
80

50

40

CD 30
20

10

I I hNMBR I I I I hBRS3 I

- 10 -8 -8 0 -10 -8 -6 0’ -10 -8 -8

PEPTIDE CONCENTRATION (log M)

Fig. 2. Top, relative abilities of BA3, CPT-L2-13A3, and n-Phe-CPT-L2-BA3 to inhibit binding to cells containing human Bn receptor subtypes. Specific binding of ’251-BA3 to cells containing hGRPR, hNMBR, or hBRS3 was determined as a function of unlabeled BA3, CPT-L2-BA3, or D-Phe-CPT-L2-BA3 concentration, From the dose-inhibition curves, the IC50 was determined using the curve-fitting program, KaleidaGraph, and the Ki was calculated using the Cheng-Prusoff equation. Results are means – S.E.M. from at least three experiments, and in each experiment, each data point was determined in duplicate. Bottom, ability of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 to stimulate accumulation of IP in cells containing human Bn receptors. hGRPR-, hNMBR-, and hBRS3-trans-fected Balb/3T3 cells in 24-well plates were labeled with myo-2[3H1 mo-
sitol as described underMaterials and Methods. The cells were incubated for 60 min at 37°C with the indicated concentrations of BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 in IP assay buffer, and total I3UIIP was determined as described under Materials and Methods. Results are ex-pressed as the percentage ofstimulation caused by a maximally effective concentration of agonist (i.e., 1 jiM BA3). Control and 1 jUM BA3-stimu-lated values were: hGRPR, 396 – 50 and 6386 – 818 cpm, respectively; hNMBR cells, 2566 – 191 and 63076 – 2636 cpm, respectively and hBRS3 cells, 3076 – 493 and 14618 – 432 cpm, respectively. Results are means – S.E.M. of at least three experiments, and in each experiment, each value was determined in duplicate.

bottom). BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 were equally efficacious agonists stimulating a 16.1 – 2.1-, 24.1 – 1.4-, and 4.7 – 0.7-fold increase in [3H]IP in cells containing hGRPR, hNMBR, and hBRS3, respectively (Fig. 2, bottom). The EC,, values for BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 were 2.3 – 0.1, 1.0 – 0.1, and 417 – 29 nM, respectively, in cells containing hGRPR (Fig. 2, bottom, left). Similar re-sults were obtained in cells containing hNMBR (Fig. 2, bot-tom, middle). In contrast, in cells containing hBRS3, the EC,, values for BA3, CPT-L2-BA3, and D-Phe-CPT-L2-BA3 were 15.1 – 2.0, 11.1 – 0.8, and 3260 – 210 nM, respectively (Fig. 2, bottom, right). These results indicate that BA3 and

I NCI-H1299 Cionogonic Assaytu I

251- M I BA3

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10

5. **

C) o 1*
0 8 7 8

E - NC1H1 299 MTT Assay I
0.35

LO 0.30 -?

LLI 0.20
z CPT-L2-BA3

0. 10T5

PEPTIDE (log M)
Fig. 3. Comparison ofthe growth effects ofCPT-L2-BA3 and a-Phe-CPT-L2-BA3 in vitro using lung cancer cell line NCI-H1299. Top, effects of BA3, CPT-L2-BA3, and o-Phe-CPT-L2-BA3 on the proliferation of NCI-H1299 cells were investigated using the clonogenic assay. Results are means – S.E.M. ofat three determinations. Bottom, effects ofD-Phe-CPT-L2-BA3 and CPT-L2-BA3 on NCI-H1299 cells were investigated using the MTI’ assay. Results are the means – S.E.M. of eight determinations. Each of these experiments is representative of four others, p < 0.05; *, p < 0.01; **, compared with control using the Student’s t test.

CPT-L2-BA3 both had high potency for activating each ofthe three human Bn receptors and were >200-fold more potent that D-Phe-CPT-L2-BA3 for each receptor subtype.
Ability of BA3, CPT-L2-BA3, and n-Phe-CPT-L2-BA3 to Inhibit Cancer Cell Growth in Vitro. The ability of BA3, CPT-L2-BA3, and n-Phe-CPT-L2-BA3 to alter cancer cell proliferation in vitro was investigated using human can-cer cell lines. CPT-L2-BA3 and D-Phe-CPT-L2-BA3 decreased NCI-H1299 lung cancer tumor growth in a dose-dependent manner (Fig. 3). In the clonogenic assay, CPT-L2-BA3 and D-Phe-CPT-L2-BA3 inhibited NCI-H1299 colony formation with IC50 values of 70 and 500 nM, respectively (Fig. 3, top) In contrast, 1000 nM BA3 had no significant effect on prolif-eration. CPT-L2-BA3 and D-Phe-CPT-L2-BA3 inhibited the proliferation of NCI-H1299 lung cancer cells assessed using the MTT assay with IC50 values of 70 and 600 nM (Fig. 3, bottom). The ability of CPT-L2-BA3 to inhibit the prolifera-tion of a number of different human cancer cell lines was assessed. Using the MTI’ assay, CPT-L2-BA3 was found to inhibit the proliferation of breast cancer (MCF-7), colon can-cer (HT-29), gastric cancer (Hs746T), glioblastoma (U-87MG), leukemia (MOLT-4), lung cancer (NCI-H69), neuro-blastoma (B1R32, SKNSH), pancreatic cancer (CFPAC-1), and prostate cancer (PC-3, DU-145, LNCaP) cell lines with IC50 values ranging from 33 to 2269 nM (Table 1). Cell lines with low IC50 values such as NCI-H1299 (100 nM, Fig. 3) had a higher density of 1251-BAO binding sites than did cells with

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TABLE 1

Ability of CPT-L2-BA3 to inhibit the growth of various human cancer cell lines

Cell Line Cancer Type IC50

nM

CFPAC-1 Pancreatic cancer 625

DMS-53 Lung cancer 828
DU-145 Prostate cancer 240
Hs746T Gastric cancer 821
HT-29 Colon cancer 2269
1MR32 Neuroblastoma 90
LNCaP Prostate cancer 92
MCF-7 Breast cancer 1705
MOLT-4 Leukemia 33
NCI-H69 Lung cancer 1923
PC-3 Prostate cancer 198
SKNSH Neuroblastoma1610
U-87MG Glioblastoma 842

The ability of CPT-L2-BA3 to inhibit the proliferation of the human lung cancer cells was determined by the MVJ’ assay. The mean value of eight determinations, IC51) (nanomolar), is shown. The S.E.M. was loss than 10%.

a high IC50 value such as NCI-H69 (1923 nM, Table 1). These results demonstrate that a CPT-Bn conjugate can inhibit the proliferation of numerous human cancer cell lines and sup-port the conclusion that the inhibition is mediated by a specific Bn receptor-mediated mechanism.
Ability of CPT-L2-BA3 and n-Phe-CPT-L2-BA3 to In-hibit Cancer Cell Growth in Vivo. To assess the ability of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 to inhibit tumor

growth in viva, their ability to inhibit NCI-H1299 xenograft proliferation in nude mice was determined (Fig. 4). Palpable NCI-111299 tumors were present 2.5 weeks after the injec-tion of NCI-H1299 cells s.c. (Fig. 4). CPT-L2-BA3, n-Phe-CPT-L2-BA3 (0.8 mg/kg), and the PBS control were then injected s.c. into nude mice three times a week. At 4.5 weeks, animals injected with CPT-L2-BA3 had significantly smaller tumors (679 – 141 mm’) than did animals receiving D-Phe-CPT-L2-BA3 (1088 – 161 mm’) or PBS (1188 – 227). In addition, at 4 weeks, animals injected with CPT-L2-BA3 had significantly smaller tumors than did animals receiving D-Phe-CPT-L2-BA3 or PBS (Fig. 4). In contrast, animals in-jected with CPT-L2-BA3 had a similar body weight (23.3 – 0.5 g) relative to animals, which received D-Phe-CPT-L2-BA3

1400

1200

LU 1000

_.I 800
0
> 600

400

200
I.

I NC1-H1299 Xenographs in Nude Mice I

PBS

2.0 2.5 3.0 3.5 4.0 4.5 5.0

TIME (weeks)
Fig. 4. Growth effects of CPT-L2-BA3 and D-Phe-CPT-L2-BA3 in vivo. The effects of CPT-L2-BA3, D-Phe-CPT-L2-BA3, and PBS on the prolif-eration ofNCI-H1299 tumorsin nude mice were investigated. Results are means – S.E.M. of eight determinations. This experiment was represen-tative of two others, p < 0.05; *, using analysis of variance.

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(22.9 – 0.5 g) or PBS (22.5 + 0.7 g). Likewise, high doses of CPT-L2-BA3 (8 mg/kg twice a week) injected into nude mice resulted in a 84% inhibition of the growth of pancreatic CFPAC-I tumors (Table 2). When CPT-L2-BA3 was injected i.p. into nude mice (8 mg/kg twice a week), CFPAC-I, NCI-H69, and PC-3 tumor growth was inhibited by 55, 56, and 68%, respectively, relative to control mice injected with PBS. These results indicate that the CPT-Bn conjugate can inhibit xenograft growth in vivo of a number of different human
cancers.

Stability of CPT-L2-BA3. To assess the stability of CPT-L2-BA3, we used a newly developed RIA with a rabbit antibody that was formed against BRS3. Figure 5, top, dem-onstrates that the RIA had high specificity for BA3 and CPT-L2-BA3 (IC50 = 0.5 – 0.1 and 2.1 – 0.6 pmol, respec-tively). Incubation in mouse plasma led to a progressive loss of CPT-L2-BA3 immunoreactivity with a half-time of CPT-L2-BA3 metabolism of 30 min (Fig. 5, bottom). By radioim-munoassay, peak blood concentrations (10 nM) of CPT-L2-BA3 were obtained 30 min after injection into nude mice (T. Moody, unpublished data).

Discussion

One of the principal problems limiting novel treatments (antisense, chemotherapeutic agents with limited solubility, tumor-sensitizing agents, etc.) of many common tumors is the inability to selectively target the therapy to the cancer cell (Dyba et al., 2004). Because many human tumors over-express or ectopically express at least one peptide hormone/neurotransmitter growth factor receptor and in many cases multiple receptors, this is receiving increasing attention as a useful approach for localizing tumors by imaging modalities and/or allowing selective delivery of cytotoxic compounds (Schally and Nagy, 1999, 2003; Langer and Beck-Sickinger, 2001; Reubi et al., 2003). A central premise of these latter studies is that the cytotoxic agent is being delivered specifi-cally to the tumor by a receptor-mediated mechanism. Stud-ies with radiolabel octreotide for imaging of breast cancer in vivo showed imaging results correlated with the expression ofsomatostatin receptors with high affinity for octreotide on the tumor (i.e., sst2 and sst5) (Schulz et al., 2002), and studies with neuroblastomas showed a similar correlation (Orlando et al., 2001). Studies using somatostatin receptor knockout mice (Hofiand et al., 2003) demonstrated decreases in "hn-penetreotide uptake in tissues that normally possess higher densities ofsomatostatin receptors. Conversely, over-expression ofsomatostatin receptor subtype 2 with an adeno-viral vectors in human small cell lung tumors increased

TABLE 2.

Inhibition of tumor proliferation in vivo
Nude mice bearing xenografts ofthe human tumor lines were injected twice a week with the indicated dose of CPT-1,2-13A3. Subcutaneous injections were made in the flank opposite to the tumor implant. The tumor volume for animals treated with CPT-L2-BA3 was compared with that of animals injected with PBS. The mean value – S.E.M. of ii determinations is shown.

Xenograft Injection Dose Tumor Growth Inhibition

CFPAC-1 8 mg/kg S.0 84– 16
CFPAC-1 8 mg/kg i.p. 55–14
NCI-H69 8 mg/kg i.p. 56 – 14
PC-3 8 mg/kg i.p. 68 – 17

2500

2000

Cv) CL 1500
1000

LOtv 500
0
0

CO)

CP 4000

IL
3000
Iu

2000

1000
Z

n

I Radiolmmunossay

B43
cpT-L2.aAa

4 6 B 10 12

PEPTIDE (pmol/tube)

I CPT-1.2-5A3 Metabolism I

CPT-L2.8A3

0 20 40 60 80 100 120 140
TIME

Fig. 5. CPT-L2-BA3 quantitation by radioimmunoassay. Top, ability of unlabeled BA3 or CPT-L2-BA3 to compete for °I-BA3 binding to rabbit antisera (1:1000) generated against BA3 was investigated. Bottom, CPT-L2-BA3 was added to mouse plasma for the indicated amount of time at 37°C. The sample was treated with boiling 2 N acetic acid and lyophilized. The sample was resuspended and tested in the radioinnnunoassay. The mean value – S.E.M. of three determinations is indicated. This experi-ment is representative of four others.

uptake of radiolabeled somatostatin in the tumor implanted in mice (Rogers et al., 2002). Although these results provide evidence for the importance of a receptor-mediated mecha-nism for radionucide-conjugated peptide ligand uptake for imaging, similar evidence is lacking for the possible involve-ment ofthis mechanism in mediating tumor cytotoxicity with peptide receptor agonist chemotherapeutic constructs.
In the present study, we addressed this issue using a pharmacological approach coupled with in vitro and in vivo cytotoxicity studies. Numerous studies demonstrate that many common tumors overexpress or ectopically express Bn receptor subtypes, including 44 to 100% ofsmall cell or non-small cell lung cancers (Guinee et al., 1994; Siegfried et al., 1999), 57% of gastric cancers (Preton et al., 1993), 72% of breast cancers (Reubi et al., 2002), 100% of prostate cancers (Reubi et al., 2002), 100% of head and neck squamous cell tumors (Lango et al., 2002), 40 to 80% of colon cancer (Guinee et al., 1994; Carroll et al., 1999), 100% of ovarian cancer (Sun et al., 2000b; Lango et al., 2002), and 80% of glioblastomas/gliomas (Reubi et al., 2002). Second, many studies also dem-onstrate that radiolabeled analogs ofBn-related peptides can target tumors or tissues containing or overexpressing Bn-receptor subtypes and that these analogs, if agonists, are rapidly internalized by the tumors (Moody et al., 2004). Third, synthetic Bn analogs coupled to various chemothera-peutic agents (i.e., doxorubicin, camptothecin) have been shown to cause cytotoxicity in a number of human tumors (Kiaris et al., 1999; Schally and Nagy, 2003; Moody et al.,

2004). Fourth, each of the three subtypes of human Bn re-ceptors (hGRPR, ILNMBR, hBRS3) can be overexpressed by different tumors; however, each Bn receptor subtype usually has markedly different affinities for different Bn analogs (Mantey et al., 1997). We have recently discovered a syn-thetic Bn analog BA3 that interacts with high affinity with each ofthe three human Bn receptor subtypes (Mantey et al., 1997); thus, if a tumor expresses any ofthe three Bn receptor subtypes, it will bind with high affinity. Furthermore, radio-labeled analogs of this peptide interact with high affinity with Bn receptors on many human tumors. Fifth, we have recently coupled this universal Bn receptor ligand, BA3, through a built-in nucleophile-associated releasing group linker (L2) to the topoisomerase 1 inhibitor, CPT, and the resultant CPT-L2-BA3 peptide not only retained high affinity for each Bn receptor subtype but functioned as a fully effica-cious agonist at each receptor subtype, was rapidly internal-ized, and was cytotoxic for NCI-H1299 lung cancer cells ex-pressing Bn receptors in both an MTI’ and clonogenic growth assay (Moody et al., 2004).
A Bn analog was synthesized that was chemically similar to the active CPT-Bn conjugate (CPT-L2-BA3) but was inac-tive or had much reduced Bn receptor affinity. Previous Bn structure-function studies demonstrated the importance of the penultimate COOH-terminal amino acid (i.e., position 13 in Bn) for high-affinity receptor interaction of high potency (Rivier and Brown., 1978; Moody et al., 1982; Leban et 1993). These studies demonstrated that insertion of a DLeu’3, replacement of the Leu’3 with a rigid amino acid such as proline, or alteration ofthe Leu13-Met" peptide bond all resulted in greatly reduced receptor affinity and loss of or reduced potency. [DTyrS,f3Ala11,DPhe13,Nle14] Bn (6-14), in which a D-Phe was substituted for the leucine in position 13, reduced potency but had identical chemical properties (solubility, lipophilicity, electrochemical charge) to the active peptide, BA3. ’251-D-Phe-CPT-L2-BA3 demonstrated no sat-urable binding to cells expressing any ofthe three human Bn receptor subtypes, was not internalized, and in competition assays, D-Phe-CPT-L2-BA3 had a >1400-fold lower affinity for each of the Bn receptor subtypes than the active analog, CPT-L2-BA3 (i.e., 3400-fold less for hGRPR and hNIVIBR and >1400 fold less for hBRS3).
A number of our results support the conclusion that spe-cific interaction with Bn receptor subtypes is important for mediating the tumor cytotoxicity of the active analog, CPT-L2-BA3. First, in both a clonogenic growth assay and in the MTT growth assay, CPT-L2-BA3 demonstrated significantly greater cytotoxicity and was significantly more potent at causing cytotoxicity than D-Phe-CPT-L2-BA3. Second, CPT-L2-BA3 was significantly more potent than the inactive analog, D-Phe-CPT-L2-BA3, at inhibiting the growth of NCI-H1299 lung cancer xenografts in nude mice. Because CPT-L2-BA3 and -Phe-CPT-L2-BA3 differ principally in their ability to interact with high affinity with human Bn receptor subtypes, these results support the conclusion that this teraction is important for the ability of CPT-L2-BA3 to cause tumoral cytotoxicity in vitro and in vivo. Because D-Phe-CPT-L2-BA3 had weak cytotoxicity, however, for NCI-H1299 cells, it may slowly diffuse across the plasma membrane, as does the hydrophobic CPT, and cause cancer cell apoptosis. CPT, which could only be dissolved in dimethyl sulfoxide, was cytotoxic for NCI-H1299 cells but could not be tested in vivo.

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CPT-L2-BA3 specifically interacts with high affinity with all human Bn receptor subtypes, thus increasing the possi-bility of a specific delivery of CPT to tumoral receptor sites containing any of the three human Bn receptor subtypes. GRP, which has an identical heptapeptide COOH terminus to Bn (Bunnett, 1994), has a half-life of only 1.4 min in the circulation in the pig (Knuhtsen et al., 1984) and <5 min in mouse plasma (Marquez et al., 2000) while being degraded primarily by cleavage at the His"-Leu13 peptide bond and also at the Trp8-Ala’ peptide bond at the COOH terminus (Shipp et al., 1991). CPT-L2-BA3 had a half-life of 30 min in mouse plasma, which exceeded the value for GRP of <5 mm, demonstrating its enhanced stability in plasma. This en-hanced stability could be due to the substitution of Phe’3 for Leu’3, which might interfere with the degradation at the His12Leu18 peptide bond that occurs in GRP or to the cou-pling of a moiety (i.e., CPT-L2) to the NH2 terminus, which can effect degradation of Bn-related peptides (Shipp et al., 1991). Secondly, we found that CPT-L2-BA3 inhibited the proliferation of a large number of human cancer cell lines in vitro using the MTI’ assay and including cancers of the breast, colon, lung, stomach, pancreas, and prostate as well as leukemia and glioblastoma cancer cell lines. Furthermore, we also demonstrated that CPT-L2-BA3 inhibited the growth of pancreatic, lung, and prostate cell lines in vivo in nude mice and was devoid of observed toxicity at relatively high doses.
In conclusion, our results demonstrate that D-Phe-CPT-L2-BA3, which is chemically closely related to CPT-L2-BA3, has a greatly reduced ability to interact with human Bn receptors because it does not interact with high affinity or activate with high potency any of the three mammalian Bn receptor sub-types and is not internalized by any Bn receptor subtype. Compared with the active analog CPT-L2-BA3, D-Phe-CPT-L2-BA3 had a much-reduced cytotoxic effect both in vitro and in vivo, providing support for the conclusion that specific interaction with Bn receptor subtypes is important for medi-ating its tumor cytotoxicity effect.

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References

Benya RV, Kusni T, Pradhan TV, Battey JF, and Jensen RT (1995) Expression and characterization of cloned human bombesin receptors. Mo! Pharmacoi 47:10-20. Bunnett N (1994) Gastrin-releasing peptide, in Gut Peptides (Walsh JH, Dockray GJ eds) pp 428-445, Raven Press, Ltd., New York.

Carroll RE, Matkowskyj KA, Chakrabarti S, McDonald TJ, and Benya 1W (1999) Abberrant expression of gastrin-releasing peptide and its receptor by well- differentiated colon cancers in humans. Am J Physiol 276:G655G665. Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (l() and the concentration of inhibitor which causes 50 percent inhibition (IC50) of an enzymatic reaction. Biochem Pharmacoi 22:3099-3108.
de Groot FM, Damen EW, and Scheeren 11W (2001) Anticancer prodrugs for appli- cation in monotherapy: targeting hypoxia, tumor-associated enzymes, and recep-

tors. Curr Med Chem 8:1093-1122.

Dubowchik GM and Walker MA (1999) Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. Pharmacol Ther 83:67-123.

Dyba M, Tarasova Ni, and Michejda CJ (2004) Small molecule toxins targeting tumor receptors. Curr Pharm Des 10:2311-2334.
FuselierJA, Sun L, Woltering SN, Murphy WA, vasilevich N, and Coy DH (2003) An adjustable release rats linking strategy for cytotoxin-Peptide conjugates. Bioorg Med Chem Lett 13:799-803.

Guinea DG Jr, Fishback NF, Koss MN, Abbondanzo SL, and Travis WD (1994) The

spectrum of immunohistochemical staining ofsmall-cell lung carcinoma in speci- mens from transbronchial and open-lung biopsies, Am JCiin Pat/wi 102:406-414, Hoffman TJ, Quinn TP, and Volkert WA (2001) Radiometallated receptor-avid pep- tide conjugates for specific in viva targeting of cancer cells, Nuci Med Biol 28:527-

539.

Hofiand U, Lamberts SW, van Megan PM, Teubi JC, Schaeffer J, Wooijers M, von Koetsveld PM, Srinivasan A, Krenning EP, and Breaven WA (2003) Crucial role

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for somatostatin receptor subtype 2 in determining the uptake of [11 lIn-DTPA-n-Phelloctreotide in somatostatin receptor-positive organs. J Nucl Med 44:1315-1321.

Jensen RT (2000) Somatostatin receptor based scintigraphy and anti-tumor treat-merit: an expanding vista? J Clin Endocrinol Metab 85:3507-3508.
Elena H, Schally AV, Nagy A, Sun B, Armatis P, and Szepeshazi K (1999) Targeted cytotozic analogue of bombesin/gastrin-releasing peptide inhibits the growth of H-69 human small-cell lung carcinoma in nude mice. BrJ Cancer 81:966-971. Knuhtsen S, Hoist JJ, Knigge U, Olesen M, and Nielsen OV (1984) Radioimmuno-assay, pharmacokinetics, and neuronal release of gastrin-releasing peptide in anesthetized pigs. Gastroenterology 87:372-378.
Kwekkeboom D, Krenning EP, and deJong M (2000) Peptide receptor imaging and therapy. J Nucl Med 41:1704-1713.
Langer Mend Beck-Sickinger AG (2001) Peptides as carrier for tumor diagnosis and treatment. Carr Med Chem Anticancer Agents 1:71-93.
Lango MN, Dyer KF, Lui VW, Gooding WE, Gubish C, Siegfried JM, and Grandis JR (2002) Gastrin-releasing peptide receptor-mediated autocrine growth in squamous cell carcinoma of the head and neck. JNatl Cancer Inst 94:375-383.

Leban JJ, Kull FC Jr, Landavazo A, Stockstill B, and McDermed JD (1993) Devel-opment of potent gastrin-releasing peptide antagonists having a n-Pro-psi (CH2NH)-Phe-NH2 C terminus. Proc Nail Aced Sci USA 90.1922-1926. Mahmoud S, Staley J, Taylor J, Bogdan A, Moreau JP, Coy D, Avis I, Cuttitta F, Mulshine J, and Moody TW (1991) [Psi 13,141 bombesin analogues inhibit growth ofsmall cell lung cancer in vitro and in vivo. Cancer Res 51:1798-1802,
Mantey SA, Weber HC, Sainz E, Akeson M, Ryan RR, Pradhan T, Searles RP, Spindel ER, Battey JF, Coy DH, et al (1997) Discovery of a high affinity radlioli-gand for the human orphan receptor, bombesin receptor subtype 3, which demon-strates it has a unique pharmacology compared with other mammalian bombesin receptors. J Biol Chem 272:26062-26071.
Marquez C, Tresten A, Moody E, Jakowlew S, and Moody TW (2000) The metabolism of BW2258U89, a GRP receptor antagonist. Neuropeptides 34:108-115.
Moody TW, Crawley JN, and Jensen RT (1982) Pharmacology and neurochemistry of bombesin-like peptides. Peptides 3:559-563.
Moody TW, FuselierJ, Coy DH, Mantey S, Pradhan T, Nakagawa T, and Jensen RT (2005) Camptothecin-somatostatin conjugates inhibit the growth ofsmall cell lung cancer cells. Peptides 26:1560-1566.
Moody TW, Mantey SA, Pradhan UK, Schumann B, Nakagawa T, Martinez A, Fusilier J, Coy DH, and Jensen RT (2004) Development of high affinity campto-thecin-hombesin conjugates that have targeted cytotoxicity for bombesin receptor-containing tumor cells. J Biol Chem 279:23580-23589.
Orlando C, Raggi CC, Bagnoni L, Sestini K, Brigand V, LaCara G, Bernini G, Tonini GP, Pazzagil M, Soria M, at a]. (2001) Somatostatin receptor type 2 gene expres-sion in neuroblastoma, measured by competitive RT-PCR, is related to patient survival and to somatostatin receptor imaging by indium-ill-pentetreotide. Med Pediatr Oncol 36:224-226.

Preston 5K, Woodhouse LF, Jones-Blackett S, Wyatt JI, and Primrose JN (1993) High affinity binding sites for gastrin releasing peptide on human gastric cancer and Menetrier’s mucosa. Cancer Res 53:5090-5092.

Reubi JC (2003) Peptide receptors as molecular targets for cancer diagnosis and therapy. Endocr Rev 24:389-427.
Reubi JC, Wenger S, Schumuckli-Maurer J, Schaer JC, and Gugger M (2002) Bombesin receptor subtypes in human cancers: detection with the universal ra-dioligand (125)I-[D-TYR(6), beta-ALA(11),PHE(13), NLE(14)] bombesin(6-14). Clin Cancer Res 8:1139-1146.

Rivier JR and Brown MR (1978) Bombesin, bombesin analogues and related pep-tides: effects on thermo regulation. Biochemistry 17:1766-1771.
Rogers BE, Zinn KR, Lin CY, Chaudhuri TR, and Buchsbaum DJ (2002) Targeted radiotherapy with [(90)Y]-SMT 487 in mice bearing human nonsmall cell lung tumor xenografts induced to express human somatostatin receptor subtype 2 with an adenoviral vector. Cancer 84:1298-1305.

Schally AV and Nagy A (1999) Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptors on tumors. EarJ Endocrinol 141:1-14. Schally AV and Nagy A (2003) New approaches to treatment ofvarious cancers based on cytotoxic analogs ofLHRH, somatostatin and bombesin. Life Sci 72:2305-2320. Schulz S, Helniholz T, Schmitt J, Franke K, Otto HJ, and Weise W (2002) True positive somatostatin receptor scintigraphy in primary breast cancer correlates with expression of est2A and sstS. Breast Cancer Res Treat 72:221-226.
Shipp MA, Tarr Gill, Chen CY, Switzer SN, Hersh LB, Stein H, Sunday ME, and Reinherz EL (1991) CD10/neutral endopeptidase 24.11 hydrolyzes bombesin-like peptides and regulates the growth ofsmall cell carcinomas of the lung. Proc Nail Aced Sci USA 88:10662-10666.

Siegfried JM, Krishnamachary N, Davis AG, Gubish C, Hunt JD, and Shniver SP (1999) Evidence for autocrine actions of neuromedin B and gastrin-releasing peptide in non-small cell lung cancer. Pu/rn Pharmacol Ther 12:291-302.
Sun L, Fuselier JA, and Coy DH (2004) Effects of camptothecin conjugated to a somatostatin analog vector on growth of tumor cell lines in culture and related tumors in rodents. Drug Delis 11:231-238.
Sun L, FuselierJA, Murphy WA, and Coy DH (2002) Antisense peptide nucleic acids conjugated to somatostatin analogs and targeted at the n-myc oncogeno display enhanced cytotoxicity to human neuroblastoma IMR32 cells expressing somatosta-tin receptors. Peptides 23:1557-1565.
Sun B, Halmos G, Schally AV, Wang X, and Martinez M (2000a) Presence of receptors for bombesin/gastrin-releasing peptide and mRNA for three receptor subtypes in human prostate cancers. Prostate 42:295-303.
Sun B, Schally AV, and Halmos G (2000b) The presence ofreceptors for bombesin/GRP and mRNA for three receptor subtypes in human ovarian epithelial cancers. Regal Pept 90-.77-84.
Virgolini 1(1997) Receptor nuclear medicine: vasointestinal peptide and somatosta-tin receptor scintigraphy for diagnosis and treatment of tumour patients. Ear J Clin Investig 27:793-800.

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Development of High Affinity Camptothecin-Bombesin Conjugates That Have Targeted Cytotoxicity for Bombesin Receptor-containing Tumor Cells*

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Mammalian bombesin (BN) receptors are among those most frequently overexpressed by a number of common tumors including prostate, breast, lung, and colon can-cers. The aim of this study was to develop a camptoth. ecin-bombesin (CPT-BN) conjugate that interacts with all classes of BN receptors and possibly functions as a prodrug via a labile linker with site-specific cytotoxicity for cancer cells bearing these receptors. CPT was cou-pled to analogs of [n-Tyr6j3.Ala",Phe13,Nle14]BN-(6-14) (BAO) using carbamate linkers (Li and L2) with built-in nucleophile-assisted releasing groups for intracellular cleavage of free cytotoxic agents. One conjugate, CPT-L2-BA3, bound to all three BN receptor classes with high affinity and functioned as a full agonist at each. 1251. CPT-L2-BA3 was rapidly internalized by cells express-ing each BN receptor class and, using fluorescent imag-ing, was found to co-localize with BN receptors initially and later to be internalized in cytoplasmic compart-ments. HPLC analysis of internalized ligand showed that 40% was intact, 25% was metabolized by releasing free CPT, and 35% was metabolized to other breakdown products. CPT-L2-BA3 inhibited the growth of Nd-H1299 non-small cell lung cancer cells in 3-(4,5-dimeth-ylthiazol-2-yl)-2.5-diphenyl.2H-tetrazolium bromide (MTT) and clonal growth assays. CPT-L2.BA3 was cyto-toxic in an lW11 assay for cells transfected with each class of BN receptor however, it had significantly less effect in cells lacking BN receptors. These results in-dicate that CTP-L2-BA3 is a potent agonist that is cy-totoxic for cells overexpressing any of the three EN receptor classes and functions as a prodrug for recep-tor-mediated cytox.icity. It therefore should be a useful prototype to explore the effectiveness of tumor. specific cytotoxicity delivery using a receptor-mediated mechanism.

In order to enhance tumor cytotoxicity and decrease side effects, there has been increased interest in the development of prodrugs that improve site-specific delivery of cytotoxic anti-cancer agents (1, 2). Prodrug conjugates have been described

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utilizing antibodies directed against specific tumor-associated antigens, hydrophilic polymers, and Iieptide or steroid hor-mones that interact with receptors overexpressed or ectopically expressed on the tumor (1, 2). The goal of the prodrug is to deliver the therapeutic agent to the target cell, at which time a tumor-specific process (enzyme activity, specific cellular degra-dation, etc.) will site-specifically release the active drug (1). For peptide hormone receptor prodrugs, this requires coupling of the cytotoxic agent to a peptide hormone through a coupling mechanism that retains high affinity for the peptide hormone receptor and allows the cytotoxic drug to be released after receptor specific internalization and degradation (1, 3, 4). Pep-tide receptor ligands as vehicles to deliver cytotoxic agents are receiving considerable attention, because numerous studies us-ing imaging methods or autoracliographic methods have shown many common malignant tumors either ectopically expressing large numbers of peptide receptors (4-9) or overexpressing them, thus allowing enhanced uptake of selective ligands for these receptors (6, 7).
The mammalian bombesin (BN)’ family of receptors (gas-trin-releasing peptide receptor (GRPR), neuromedin B receptor (NMBR), and the orphan receptor, bombesin receptor subtype 3 (BRS3)) are excellent candidates for possibly targeting cyto-toxic agents to malignant neoplasms. Not only do many com-mon tumors frequently possess and overexpress these recep-tors, but the BN family of receptors is one of the receptor families most frequently expressed by tumors, and the natu-rally occurring agonists for these receptors function as auto-crine growth factors (7, 8, 10). BN receptors have been detected on 40-100% of prostate cancer, breast cancer, lung cancer, gastric cancer, malignant gliomas, colon cancer, and ovarian cancer (5).
Recently, a synthetic analog of BN has been described, [u-Tyr6,3-A1a",Phe’3,Nle14]BN-(6-14) (BAO), which functions as a universal ligand for all three mammalian BN receptors (11, 12). This analog binds with high affinity to each of the three BN receptor classes and is rapidly internalized by each class of BN receptors (11, 12). This is an important property because different tumors may possess different BN receptor

The abbreviations used are: BN, bombesin; CPT, camptothecin;
GRP, gastrin-releasing peptide; BRS3, bombesin receptor subtype 3; hBRS3, human BRS3; GRPR, gastrin-releasing peptide receptor;

hGRPR, human GRPR; NMBR, neuromedin B receptor; hlJMBR, hu- man NMBR; IP, inositol phosphate; NMB, neuromedin B; MT7, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NSCLC, non-small cell lung cancer; BINAR, built-in nucleophile-assisted releas-

ing; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; HPLC, high pressure liquid chromatography.

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classes (5), and this ligand would still interact with high affin-ity with each. Furthermore, recently, a new novel carbamate linker system (3) has been described that allows the conjuga-tion of a peptide to either the topoisomerase I inhibitor, camp-tothecin (CPT), or the tubulin-binding agent, combretastatin. This novel linker system (3) contains a built-in nucleophile-assisted releasing (BINAR) group that enables fine timing of intracellular cleavage rates offree cytotoxic agents containing reactive hydroxyl groups such as CPT or combretastatin (3). In the present study, we have synthesized a number of CPT analogs coupled to universal bombesin agonists through differ-ent carbamate linkers (Li and L2) to identify a potential gen-eral BN receptor cytotoxic prodrug. We have identified one analog, CPT-L2-BA3, which shows high affinity for all three mammalian BN receptors, is fully biologically active at each receptor, and is rapidly internalized by each receptor subtype. Furthermore, CPT-L2-BA3 is cytotoxic for NCI-H1299 non-small cell lung cancer (NSCLC) cells, which possess native GRP receptors (13) and demonstrate greater cytoxicity for Balb/c 3T3 cells containing GRP receptors than those lacking these receptors, suggesting site-selective cytotoxicity.

EXPERIMENTAL PROCEDURES

MaterialsThe following cells and materials were obtained from the sources indicated: Balb/c 3T3 (mouse fibroblast), NCI-11345 (human small cell lung cancer), NCI-H1299 (human NSCLC), and HEK 293 (human embryonic kidney) cells from ATCC (Manassas, VA); Dulbecco’s minimum essential medium, phosphate-buffered saline, RPMI 1640, trypsin-EDTA, and fetal bovine serum from Biofluids (Rockville, MD); G418 sulfate from Invitrogen; Na’251 (2200 Cilmmol) and myo-[2-3H1-inositol (20 Ci/mmol) from Amersham Biosciences; camptothecin, formic acid, ammonium forinate, disodiuni tetraborate, 4-(dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4-H-pyran, 2-dimethylaminoisopropyl chloride hydrochloride, 1-methyl-2-pyrrolidinone, dimethylaminopyri-dine, soybean trypsin inhibitor, bacitracin, leupeptin, 4-(2-aminoethyl)- benzenesulfonyl fluoride, poly-L-lysine, protease inhibitor mixture, and Triton X-100 from Sigma; 1,2,4,6-tetrachloro-3a,6e-diphenylglycouril (IODO-GEN) from Pierce; Fura-2/AM from Calbiochem; AG 1-X8 resin from Bio-Rad; EN, GRP, NMB, and ITyr4IBN from Bachem (Torrence, CA); paraformaldehyde (16% stock solution) from Electron Microscopy Sciences (Ft. Washington, PA); bovine serum albumin from ICN Phar-maceutical Inc. (Aurora, OH); HA antibody from Santa Cruz Biotech-nology, Inc. (Santa Cruz, CA); secondary antibodies and donkey se-rum from Jackson ImmunoResearch Laboratories, West Grove, PA; and Vectorshield from Vector (Burlingame, CA). The mammalian expression vector, pcDNA3, custom primers, and restriction endonucle-ases (BamHI, Hindifi, XbaI, and EcoRI) were from Invitrogen. The ExSite PCR-based site-directed mutagenesis kit was from Stratagene (La Jolla, CA).
Cell CultureNCI-H345 and NCI-111299 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine se-rum (Invitrogen). NCI-111299 cells were split weekly 1:20 with trypsin-EDTA. NCI-H345 cells were diluted 1:1 into new media. Balb/c 3T3 cells stably expressing human BRS3 receptors (hBRS3), human NMB recep-tors (hNMBR), or human GRP receptors (hGRPR) made as described previously (11, 14, 15) were grown in Dulbecco’s modified Eagle’s cell medium supplemented with 300 mg/liter G418 sulfate. HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 gg/ml streptomycin. The cells (Balb/c 3T3 (mouse fibroblast), NCI-11345 (hu-man SCLC), NCI-111299 (human NSCLC), and HEK 293 (human em-bryonic kidney) cells were from ATCC, were mycoplasma-free, and were used when they were in the exponential growth phase after incubation at 37 °C in 5% CO21 95% air.

Preparation of PeptidesThe peptide portions of the peptide-cyto-toxin conjugates were synthesized by solid phase methods using the standard Fmoc (N-(9-fluorenyl)inethoxycarbonyl) protection strategy (3). Sequences were assembled on Rink amide resin (Advanced ChemTech, Louisville, KY), and Trt group protection was employed for the imidazole NH of His, Boc for the indole NH of Trp, and tert-butyl group for the OH groups of Ser and Tyr. Bromoacetic acid was coupled to the N terminus ofthe protected peptide, which was then reacted with a 5excess oftertiary butyloxycarbonyl-1-N-methylamino,2-aminoeth-ane (2 h). CPT (Roche Applied Science) was activated at its position 20

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OH group by treating a suspension in anhydrous dichioromethane with dimethyaminopyridine (4 eq) and a 20% solution ofphosgene in toluene (4 eq) (4 h) followed by removal ofexcess solvent and phosgene in vacuo. A 3 M excess ofthe resulting yellow oil was added to the above resin in anhydrous dichloromethane and allowed to react overnight. After wash-ing and drying of the resin, the camptothecin-peptide conjugate was cleaved using the standard acid mixture, trifluoroacetic acid/H20/ethanedithiol/triisopropylsilane, 95:2:2:1 (2 h), followed by evaporation ofthe trifluoroacetic acid and precipitation and washing of the peptide with ether. Conjugates were purified to >97% purity by preparative HPLC (C15 silica) and characterized by mass spectrometry and amino acid analysis. For the compounds shown in Fig. 1, BAO had a calculated mass of 1133.3 and was found to have a mass of 1132.7; CPT-L1-BA1 had a calculated mass of 1661.8 and was found to have a mass of 1666.3; CPT-L2-BA1 had a calculated mass of 1675.5 and was found to have a mass of 1676.1; CPT-L1-BA2 (mixture of polymers) had an average mass of 3000; and CPT-L2-BA2 had a calculated mass of 1709.9 and was found to have a mass of 1709.1.

Preparation of 12sJ4Q 11-1,2-BA3, 1251-CPT-L2-BA3, ’I-[n-Tyr°JNMB, and 1251-[Tyr4]BNThese radioligands, with specific activ-ities of2200 Ci/mmol, were prepared as previously described (11, 12) or as described below. Briefly, 0.8 Ag ofIODO-GEN solution (0.01 pg1gl in chloroform) was added to a 5-ml plastic test tube, dried under nitrogen, and washed with 100 jsl of 0.5 M potassium phosphate solution (pH 7.4 except for 1251-L2-BA3 and ’251-CPT-L2-BA3, which required pH 7.0). To this tube, 20 pi of potassium phosphate ofthe appropriate pH, 8 pg of peptide in 4 1A of water, and 2 mCi (20 pI) of Na1251 were added and incubated for 6 min at room temperature (Na’251 (2200 Ci/mmol) and myo-[2-3Hlinositol (20 Ci/mmol) were from Amersham Biosciences). The incubation was stopped with 300 Al ofwater. For I-[o-Tyr°JNMB and lnI[Tyr41BN, which have COOH-terminal methionine groups, 300 d of 1.5 M dithiothreitol was added and heated for 1 h at 80 "C. The radiolabeled peptides were separated using a CiS Sep-Pak (Waters Associates, Milford, MA) and further purified by reverse-phase high performance liquid chromatography on a C18 column. The fractions with the highest radioactivity and binding were neutralized with 0.2 M Tris buffer (pH 9.5) and stored with 0.5% bovine serum albumin (w/v) at  20 T.

Binding of’251-Labeled BN-related Peptides to Various CellsBind-ing was performed as described previously (11, 12, 14). The standard binding buffer contained 24.5 miii HEPES (pH 7.4), 98 mm NaCl, 6 mM KCI, 5 miii MgCl2, 2.5 miii NaH2PO4, 5 inn sodium pyruvate, 5 inn sodium fumarate, 0.01% (w/v) soybean trypsin inhibitor, 1% amino acid mixture, 0.2% (w/v) bovine serum albumin, and 0.05% (w/v). Balb/c 3T3
cells stably expressing hGRPR (0.3 x 10), hNMBR (0.03 X lOs), hBRS3 (0.3 X 10), or NCI-H1299 cells were incubated with 50 pie 125I-labeled ligand at 22 "C for 60 min. Aliquots (100 l) were removed and centri-fuged through 300 pJ of incubation buffer in 400-1 Microfuge tubes at 10,000 x g for 1 min using a Beckman Microfuge B. The pellets were washed twice with buffer and counted for radioactivity in a y counter. The nonsaturable binding was the amount of radioactivity associated with cells in incubations containing 50 pm radioligand (2200 Ci/mmol) and 1 pie unlabeled ligand. Nonsaturable binding was <10% of total binding in all the experiments. Receptor affinities (R) were determined using a least-square, curve-fitting program (KaleidaGraph) and the Cheng-Prusoff equation (16).
InternalizationBalb/c 3T3 cells stably transfected with hGRPR, hNMBR, or hBRS3 were incubated with radioligands as stated under "Experimental Procedures." Internalization experiments were per-formed as described previously (14, 15). Briefly, after the indicated incubation times, 100-1kI samples were added to a 1.5-ml Microfuge tube with 1 ml of4 "C acid-stripping solution containing 0.2 M acetic acid (pH 2.5) and 0.5 M NaCl to remove the surface-bound radioligand. After 5 min, the cells were pelleted, the supernatantwas removed, and the cells were washed twice with incubation buffer. In all cases, parallel incu-bations were conducted in the presence of 1 gm unlabeled ligand to determine changes in nonsaturable binding. Results are expressed as the percentage of saturable 1251-ligand added that is surface-bound (acid-stripped) or internalized (not acid-stripped). The internalization of ’I-CPT-BN conjugates was investigated in human NCI-111299 NSCLC cells that naturally possess hGRPR (13). 1I-CPT-L2-BA3 (50 pm) was incubated with NCI-H1299 NSCLC cells for 2 h at 4’C. Two sets of cells were washed three times in receptor binding buffer to remove free peptide. One set was then treated with 0.5 M acetic acid and 0.15 M NaCl for 5 min at 4 "C to remove peptide bound to the cell surface. The supernatant was counted in a y counter. A second set of cells was treated with 0.2 N NaOH to determine total binding at 4 "C. Also, two sets of cells were incubated at 37 "C for 5 min and washed

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three times in receptor binding buffer to remove free peptide. One set
was then treated with 0.5 M acetic acid and 0.15 M NaCl for 5 min at
4 °C to remove peptide bound to the cell surface. The supernatant was
counted in a # counter. A second set of cells was treated with 0.2 N
NaOH to determine total binding at 37 °C.
Characterization ofInternalizedRadioactivityBiib/c 3T3 cells with no transfected bombesin receptors and Balb/c 3T3 cells stably trans-fected with hGRPR (2 x 106/ml) were incubated at 37 °C for 15 min with 0.4 nu 1I-CPT-L2-BA3 in standard binding buffer containing 25 g(ml leupeptin, 0.02% (w/v) bacitracin, 10 jig/mI antipain, 50 jig/mi 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 2 jiu/mi of protease inhibitor mixture (Sigma). Cells were then washed with standard binding buffer with protease inhibitors at 4 °C and underwent acid stripping to remove surface-bound ligand performed for 5 min at 4°C with 0.2 M acetic acid (pH 2.5) and 0.5 N NaCl, as described under "Internalization." After centrifugation, cells were resuspended in 1 ml of standard binding buffer with protease inhibitors containing 1% Triton X-100 (pH 6), and the resuspension was sonicated to solubilize the radioactivity. Nine hundred jil of the sonicated mixture was injected on an HPLC (Waters model 510, Milford, MA) equipped with a C-18 column and eluted with a linear gradient increasing at 0.8% acetonitrile in trifluoroacetic acid (0.1%) per min starting at 12% acetonitrile and ending at 80%. One-ml fractions were collected, and radioactivity in each fraction was deter-mined by counting in a Packard ’ counter.
Measurement ofI3llhlnositol Phosphates (fH1IP)Changes in total [3H]IP were measured as described previously (14, 15). Briefly, hBRS3-, hGRPR-, or hI’MBR-transfected Balb/c 3T3 cells were subcultured into 24-well plates (5 X 10 cells/well) in regular propagation medium and then incubated for 24 hat 37 °C in a 5% CO2 atmosphere. The cells were then incubated with 3 jiCi/ml myo-12-’I]inositol in growth medium supplemented with 2% fetal bovine serum for an additional 24 h. Before assay, the 24-wall plates were washed by incubating for 30 min at 37 °C with 1 nil/well of phosphate-buffered saline (pH 7.0) containing 20 mm lithium chloride. The wash buffer was aspirated and replaced with 500 jil ofIP assay buffer containing 135 mm sodium chloride, 20 mis HEPES (pH 7.4), 2 mu calcium chloride, 1.2 mu magnesium sulfate, 1 mM EGTA, 20 mu lithium chloride, 11.1 mis glucose, 0.05% bovine serum albumin (wlv) and incubated with or without any of the peptides stud-ied. After 60 min of incubation at 37 "C, the experiments were termi-nated by the addition of 1 ml of ice-cold 1% (v/v) hydrochloric acid in methanol. Total [311]IP was isolated by anion exchange chromatography as described previously (14, 15). Briefly, samples were loaded onto Dowex AG1-X8 anion exchange resin columns, washed with S ml of distilled water to remove free E’H]inositol, and then washed with 2 ml of 5 mm disodium tetraborate, 60 mu sodium formate solution to remove [3Hjglycerophosphorylinositol. Two nil of 1mM ammonium formate, 100 mm: formic acid solution were added to the columns to elute total [’H]IP. Each eluate was mixed with scintillation mixture and measured for radioactivity in a scintillation counter.
Cytosolic Calcium [CaC*], MeasurementThe ability of the CPT-BN conjugates to alter cytosolic [Ca2’], was investigated as described pre-viously (17). NCI-H1299 cells and NCI-H345 cells were harvested (2.5 x 106/nil) and incubated with 5 jixs Fura-2/AM at 37 °C for 30 mm. The cells, which contained loaded Fura-2, were centrifuged at 1500 X g for 10 min and resuspended at the same concentration in new SIT medium (RPMI 1640 containing 30 nm sodium selenite, 5 jig/ml bovine insulin, and 10 gglml transferrin). After two washes with the same medium, cells were placed in a Delta PTI Scan 1 spectrofluorometer (Photon Technology International, South Brunswick, NJ) equipped with a magnetic stirring mechanism and temperature (37 °C) -regulated cuvette holder. The fluorescence intensity was continuously monitored at dual excitation wavelengths of 340 and 380 mn, using an emission wavelength of 510 nm prior to and after the addition of BN-like peptides.
Proliferation AssaysGrowth studies were performed using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT) assay. Growth studies in vitro were conducted using MTT (Sigma) colorimetric assays. NCI-H1299 cells, hGRPR-transfected Balb/c 3T3 cells, or Balb/c 3T3 cells (104/well) were placed in SIT medium (100 jil), and various concentrations of CPT-L2-BA3 were added. After 4 days, 15 jil (1 mg/ml) of MTT was added, and after another 4 h, 150 jil of Me2SO was added. After 16 h, the optical density at 570 nm was determined using an ELISA reader. The proliferation rates were calculated from the OD readings with various concentrations of CPT-L2-BA3 using the untreated cells as 100%.
Clonogenic AssaysThe effects of CPT-BN-containing peptides on the growth of NCI-H1299 and NCI-11345 cells were investigated using a clonogenic assay (18). The base layer consisted of 3 ml of 0.5% agarose

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CPT--BA1

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Flu. 1. Structures of BN, various synthetic EN analogs, their CPT conjugates studied, and their affinities for human BN re-ceptors. Top, the synthetic BN analog, BAO, has been shown to be a universal high affinity ligand for all known mammalian BN receptors (5, 11, 12) and was used as the starting peptide to make other BN-related peptides (BA1, BA2, and BA3). These peptides were coupled through nucleophilic carbamate linking groups based on either N-ami-noethyl-glycmne (Li) or the more labile N-(N-Me-amunoethyl)-glycine (L2) (3) (shown in black) to camptothecin. Amino acid identities to BAO in the various peptides are underlined. Bottom, Balb/c 3T3 cells stably transfected with hGRPR (0.3 X 10 cells/ml) (14), hNMBR (0.03 X 10 cells/ml) (14), or hBRS3 (0.3 X 10 cells/nil) were incubated for 60 min at 22 "C with 50 pm ’251-tTyr41BN, 25I-[n °]NMB, or hsIBA0, re-spectively, with concentrations of unlabeled peptides from 0.01 nm to 1 jils. From the dose-inhibition curves, the IC50 was determined using the curve-fitting program, KaleidaGraph, and the K, was calculated using the Cheng-Prusoff equation (16). All values are means – S.E. from at least three experiments, and in each experiment each value was deter-mined in duplicate. Values in parenthesis are the I value in nm for the indicated BN analog.

in SIT medium containing 5% fetal bovine serum in 6-well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose (FMC Corp., Rockford, ME), CPT-BN conjugates, and 5 X 10 NCI-H1299 cells. Triplicate wells were plated; after 2 weeks, 1 ml of 0. 1% p-iodonitrotet-razolium violet was added; and after 16 h at 37 "C, the plates were screened for colony formation. The number of colonies larger than 50 Am in diameter were counted using an Omnicon image analysis system.

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The affinities and potencies-of various synthetic BN analogs and their camptothecin conjugates for human BN receptors
Balb/c 3T3 cells stably expressing hGRPR (0.3 x 10 cells/ml), hNMBR (0.03 X 10 cells/ml), or hBRS3 (0.3 X 10 cells/nil) were incubated with 50 pm iodinated ligand, with or without increasing concentrations of unlabeled ligand for 60 min at 22 ’C as described in the legend to Fig. 2. The affinities were calculated using the Cheng-Prusoff equation (16). To assess phospholipase C activation, the Balb/c 3T3 cells stably transfected with

hGRPR, hNMBR, or hBRS3 were incubated with [3H]inositol, and total [3HJIP was determined as stated in the legend to Fig. 2. A dose-response curve was determined for each ligand with concentrations of 0.001 nm to 1 m. For each ligand, a concentration causing a half-maximal increase

(EC..) was calculated using KaleidaGraph. Each value is a mean – 1 S.E. from at least three experiments.

K for ’°°I-ligands BN receptor cells Peptide EC50 for [3H]IP ’°°I-BAO 12°I-L2-BA3 1251-CPT-L2-BA3 125I-[Tyr4JBN
TiM flM

hGRP BAO 0.32 – 0.012 0.74 – 0.05 0.44 – 0.11 0.19 – 0.14 2.01 – 0.01
L2-BA3 0.22 – 0.013 0.14 – 0.01 0.22 – 0.02 0.18 – 0.02 0.59 – 0.03
CPT-L2-BA3 0.012 – 0.001 0.006 – 0.001 0.028 – 0.007 0.009 – 0.001 0.78 – 0.13
hNMBR BAO 0.74 – 0.05 1.74 – 0.09 2.0 – 0.05 2.2 – 0.18 2.39 – 0.21
L2-BA3 0.14 – 0.01 0.38 – 0.01 0.50 – 0.10 0.59 – 0.04 0.91 – 0.07
CPT-L2-BA3 0.035 – 0.003 0.09 – 0.03 0.035 – 0.01 0.21 – 0.02 1.66 – 0.13

hBRS3 BAO 0.25 – 0.10 0.45 – 0.04 0.47 – 0.03 No binding 1.02 – 0.01
L2-BA3 0.42 – 0.03 1.0 – 0.1 1.2.t 0.02 No binding 0.79 – 0.03
CPT-L2-BA3 0.031 – 0.008 0.21 – 0.05 0.25 – 0.14 No binding 0.62 – 0.10

Preparation ofHA-GRPR-The eDNA of the mouse GRPR used was
identical to that described previously (14, 19). A sequence encoding the
HA epitope tag (YPYDVPDYA) was inserted between the first (Met)
and second (Ala) amino acid residue of the GRPR using the ExSite PCR-based site-directed mutagenesis kit, following the manufacturer’s instructions. Nucleotide sequence analysis of the entire coding region was performed using an automated DNA sequencer (ABI Prism 377 DNA sequencer; Applied Biosystems Inc., Foster City, CA). Fluorescent Microscopy of GRPR-bound CPT-L2-BA3-HEK 293 cells were seeded on 6-well plates at a density of0.5 x 10 cells/well. On the following day, cells were transfected using 3 pl ofFugene 6 reagent and 1 pg of HA-GRPR-pcDNA3 following the manufacturer’s protocol. One day after transfection, the cells were trypsinized and plated on polylysine-coated glass coverslips in 12 wells at a density of 100,000 cells/well. Two days later, cells were washed with phosphate-buffered saline and treated with or without the CPT-L2-BA3 (3 nM) in Dulbecco’s modified Eagle’s medium for various incubation times at 37 ’C. The cells were washed three times in buffer to remove the CPT-L2-BA3. The

cells containing bound CPT-L2-BA3 were fixed (paraformaldehyde 4%, 10 min, 22’C), permeabilized (0.5% Triton X-100, 5 min, 22 ’C), and blocked (2% bovine serum albumin, 2% donkey serum in phosphate-buffered saline, 30 min, 22 ’C). Cells were stained with a polyclonal rabbit anti-bombesun antibody (1:500 dilution) as first antibody and a fluorescein isothiocyanate-labeled donkey anti-rabbit antibody anti-body (1:200 dilution) as secondary antibody. The HA-GRPR was stained using a mouse anti-HA antibody (1:100 dilution) as first antibody and a TRITC-labeled donkey anti-mouse antibody (1:200 dilution) as the sec-ondary antibody. Nuclei were visualized after 4’,6-diamidino-2-phe-nylindole counterstaining. Coverslips were mounted using Vectorshield and fixed on glass slides with nail polish. Imaging was done using a Nikon fluorescent microscope.

RESULTS

CPT-L2-BA3 Binds with High Affinity to hGRPR, hNMBR, and hBRS3 and Has Biological Activity-In developing an agonist that could be coupled to cytotoxic compounds and still bind with high affinity to human BN receptors, we started with the standard BN analog, BAO (Fig. 1, top). Previous studies by us (11, 12) and others (5) have demonstrated that this synthetic BN ligand is a universal high affinity agonist for all human BN receptor subtypes as well as the amphibian BN receptor, BB4. Additionally, a new carbamate linker system was utilized, which consisted ofa series ofBINAR groups, which allows more facile intracellular cleavage offree cytotoxic agents containing reactive OH groups (3). This BINAR linker system, when cou-pled to CPT or combretastatin, allows adjustable rate release of the free cytotoxic agent (3). In the present study, we coupled analogs of the BN receptor universal agonist, BAO, to the two BINAR linkers, which were found previously to have stabilities within a useful range and display the greatest cytotoxicity with camptothecin when coupled to somatostatin analogs (3) (Fig. 1, top). These consisted of an ethylenediamine linker (Li) and

N-methylenedianiine linker (L2) (Fig. 1, top). Each of these BINAR linkers was coupled to the tertiary hydroxyl group in ring position 20 of CPT (Fig. 1, top) and the N112 termini of analogs of the BN receptor universal agonist BAO in which various polar sequences were added in its NH2 terminus (Fig. 1, top). BAO bound with high affinity to 3T3 cells stably trans-fected hGRP, hNMBR, and hBRS3 with Ki values of 0.32, 0.74, and 0.25 mc, respectively (Fig. 1, bottom; Table I). Each ofthe CTP-BN-conjugates was then examined for the ability to inter-act with the three human BN receptor subtypes (i.e. hGRPR, hNMBR, and hBRS3) (Fig. 1, bottom). For each of these three human BN receptor subtypes, only CTP-L2-BA3 retained the high affinity seen with the BAO analog for all three human BN receptor subtypes (Fig. 1, bottom; Table I). In fact, CPT-L2-BA3 had a 30-fold higher affinity than BAO for human GRPR (Ki of 0.012 – 0.002 versus 0.32 – 0.02) (Fig. 1, bottom; Table I), a 21 times higher affinity for the human NMBR (Ki of 0.035 – 0.003 versus 0.74 – 0.05) (Fig. 2, Table I), and an 8-fold higher affinity than BAO for the human BRS3-containing cells (K of 0.03 – 0.01 versus 0.25 – 0.01) (Fig. 1, bottom; Table I). In contrast, CPT-L1-BA2 and CPT-L2-BA1 had 8-fold lower affin-ities than BAO for hGRPR; 252- and 16-fold lower, respectively, for the hNMBR; and 17- and 1260-fold lower, respectively, for hBRS-3. The CPT-L1-BA1 analog retained equal high affinity to BAO for the hGRPR and hNMBR but showed a 17-fold lower affinity than BAO for hBRS-3 (Fig. 1, bottom; Table I). Because of its high affinity for all three BN receptor subtypes, we selected CPT-L2-BA3 as the CPT-BN conjugate for full charac-terization in this study.
In comparing the effect on receptor affinity ofthe addition of CPT with the effect ofthe addition ofthe L2 linker alone to the lead compound, BAO, two different types of pharmacological studies were performed (Table U. First, the ability of unlabeled BAO, the L2-BA3 analog, and CPT-L2-BA3 to inhibit binding to Balb/c 3T3 cells containing hGRPR, hNMBR, and hBRS-3 was determined (Table D. For all three BN receptor subtypes, CPT-L2-BA3 was the most potent in inhibiting binding (Table I). Second, we examined the pharmacology of the radiolabeled analogs of each of these BN-related peptides, because with some peptides, insertions of a radiolabel can alter the pharma-cology from the unlabeled compound (20) (Table D. Each ofthe three peptides (i.e. BAO, L2-BA3, and CPT-L2-BA3) was iodi-nated on the tyrosine residue (Fig. 1), and their affinity for the hGRPR was determined (Fig. 2, Table I) as well as for both hNMBR and hBRS3 (Table I). Each of the radiolabeled com-pounds demonstrated specific binding to the hGRPR-contain-ing cells, and the specific binding of 1251-BAO, 1251-L2-BA3, and

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Camptothecin-Bombesin Conjugates

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- 12 -11 -io -g -8 -7 -6

hGRPRL

100 10

190 9

80 8

70 L2-BA3 7

60- 6

50 CPT-1.2-BA3 5

C1 40 4

30

BAO

20 2

10 1

80

70

60

50

40

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- 12 -11 -10 -9 -8 -7 -6

CONCENTRATION (log M)

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CONCENTRATION (log M)
Fro. 2. Comparison of the ability of BAO, L2-BA3 and CPT-L2-BA3 to inhibit binding of ’I-BAO (top left panel), ’I-L2-BA3 (top middle panel), or 1251.CPT-L2-BA3 (top rightpanel) to hBRPR cells or stimulate accumulation of [311]1P (bottom panel). Top panel, Balb/c 3T3 cells stably transfected with hGRPR (0.3 x 108 cells/mI) were incubated for 60 min at 22 ’C with 50 pm 1I-BA0, 1251-L2-BA3, or ’251-CPT-L2-BA3 and the indicated concentrations of unlabeled peptides. Results are expressed as the percentage of saturable binding without unlabeled peptide. Results are means – S.E. from at least three experiments, and in each experiment each data point was determined in duplicate. Bottom panel, hGRPR, hNMBR, and hBRS3 transfected Bulb/c 3T3 cells in 24-well plates were labeled with myo-[2-3Hlinositol. The cells were

incubated for 60 min at 37 ’C with the indicated concentrations of the various peptides in IP assay buffer, and total [3HIIP was determined as described under ’Experimental Procedures." Results are expressed as the percentage ofstimulation caused by a maximally effective concentration
of agonist (i.e. 1 lim BAO). Results are means – 1 S.E. of at least three experiments, and in each experiment, each value was determined in duplicate. Control and 1 jn BAO-stimulated values for hGRPR were 396 – 50 and 6386 – 818 cpm, respectively; for hNMBR cells, the values were 2566 – 191 and 63076 – 2636 cpm, respectively; and for hBRS3 cells, the values were 3076 – 493 and 14,618 – 432 cpm, respectively.

1251-CPT-L2-BA3 were all inhibited in a similar manner, show- absolute affinities (Table I). These pharmacologic studies dem-
ing similar relative affinities of CPT-L2-BA3 > L2-BA3 - BAO onstrate that each ofthese radioligands can bind to each ofthe
and similar absolute affinities to that seen with the native human BN receptors and show similar relative affinities, with
ligand, ’251-ITyr4]BN (Table I). Similar results were seen with 1251-CPT-L2-]3A3 having the highest affinity for each human
1251-BAO, 1251-L2-BA3, and 1251-CPT-L2-BA3 binding to BN receptor subtype.
hNMBR- and hBRS3-containing cells (Table D. With each of The biological activity ofBAO, L2-BA3, and CTP-L2-BA3 was
these human BN receptor subtypes, similar relative affinities evaluated using Balb/c 3T3 cells transfected with BN receptors
of CPT-L2-BA3 > L2-BA3 BAO were obtained and similar and loaded with L3HhIP (Fig. 2, bottom). Each of the three

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TIME TIME TIME

FIG. 3. Ability of Balb/c 3T3 cells possessing various classes of mammalian BN receptors to internalize asI.BAO, 1"I-12-BA3, or 25ICPT.L2.BA3. hNMBR (0.03 x 10 cells/nil) (4), hBRS3 (0.3 X 106 cells/ml) (B), or hGRPR (0.3 X 10 cells/ml) (C) transfected Balb/c 3T3 cells were incubated with 50 pie 1251-BAO, ’"I-L2-BA3, or 1251-CPT-L2-BA3 for the indicated times. At each time point, the percentage ofthe indicated 1281-ligand saturabiy bound that was internalized was determined using acid-stripping as outlined under "Experimental Procedures." Results are expressed as the percentage of the saturable ligand bound at each time point expressed as the percentage of total counts added to medium that was not removed by acid stripping (i.e. internalized). Results are means – 1 S.E. of at least three experiments, and each point was determined in

duplicate.

ligands (BAO, L2-BA3, and CTP-L2-BA3) were agonists, stim-
ulating increases in 13H1IP in a dose-dependent manner (Fig. 2,
bottom panel). Each BN analog had similar efficacy, stimulat-
ing a 16-, 25-, and 5-fold increase in [311]IP at the hGRPR,
hNMBR, and hBRS3, respectively. Each BN analog caused half-maximal stimulation in the nanomolar range for each of the BN receptor subtypes (Fig. 2, bottom panel). Specifically with the hGRPR, half-maximal stimulation of CPT-L2-BA3, L2-BA3, and BAO occurred at 0.8-2 nM, and with the hNMBR cells and hBRS-3 cells the EC,, values ranged from 0.8 to 2.4 m (Fig. 2, bottom; Table I). These results demonstrate that CPT-L2-BA3 had similar potency and efficacy for activating each of the three human BN receptor subtypes to either the parent compound BAO or the BN linker analog L2-BA3. CPT-L2-BA3 Is InternalizedThe ability of each ofthe three radiolabeled BN analogs to be internalized by each ofthe three human BN receptor subtypes was examined (Fig. 3). With hNMBR cells, each of the three radioligands were rapidly in-ternalized (Fig. 3A, Table II). 1251-CPT-L2-BA3 was internal-ized more rapidly by hNMBR and demonstrated 1.3-fold greater maximal internalization than ’251-BAO. Similar inter-nalization data were obtained using Balb/c 3T3 cells containing

hBRS3 (Fig. 3B, Table II). With hGRPR cells, 1251-BAO, 1261 L2-BA3, and 1251-CPT-L2-BA3 were rapidly internalized with 20-30% ofthe ligand internalized after 2.5 mm (Fig. 30. For 1251-CPT-L2-BA3, maximal internalization occurred at 15.3 – 0.8 min with 42.7 – 2.2% ofthe bound ligand internalized (Fig. 3C, Table II). 1251-CPT-L2-BA3 demonstrated 1.8- and 1.5-fold greater maximal internalization by hGRPR cells than 1251-BAO or 1251-L2-BA3 (Fig. 3C, Table II). These results indicate that 125ICP’L2BA3 is readily internalized by cells containing BN receptors at 37 "C.
To provide direct evidence for the internalization ofCPT-L2-BA3 by GRP receptors, immunofluorescence microscopy tech-niques were used (Fig. 4). Using HEK cells transfected with an

epitope-tagged GRPR (HA-GRPR), the receptor was localized (red) to the plasma membrane with no agonist present (Fig. 48). Two min after CPT-L2-BA3 (10 nM) was added, both the GRP receptors (Fig. 48) and BN-like immunoreactivity (green) (Fig. 41)), were localized primarily to the plasma membrane and were frequently co-localized (yellow) (Fig. 41i’). Some ofthe GRP receptors (red), however, had already internalized after 2 mm (Fig. 4, compare B and E). After a 20-min incubation with 10 nie CPT-L2-BA3, most of the GRP receptors were internal-ized (Fig. 4W. Similarly, the BB-like inimunoreactivity had internalized into intracellular patches (Fig. 4G); however, there was minimal co-localization with BN-like immi.moreac-

tivity (Fig. 41). These results show that after 20 min, the HA-GRP receptor (Fig. 41) and CPT-BN conjugate have largely dissociated. These results demonstrate directly that the BN conjugate CPT-L2-BA3 is internalized by a GRPR-mediated process and accumulates rapidly in intracellular organelles after dissociating from the internalized GRPR.
To characterize the nature of the internalized radioactivity, Balb/c 3T3 cells not containing hGRPR and Balb/c 3T3 cells stably transfected with hGRPR were incubated with 0.4 nie 1251-CPT-L2-BA3 for 15 min, and the surface-bound ligand was removed by acid stripping (data not shown). The internalized radioactivity was solubilized and sonicated and characterized by analyzing HPLC fractions. With the hGRP-containing Balb/c 3T3 cells, 40% ofthe internalized radioactivity eluted in the same fraction as intact 1251-CPT-L2-BA3, 25% as 1251-L2-BA3, and 35% as two uncharacterized metabolites (data not shown). In contrast, with the Balb/c 3T3 cells not containing hGRPRs, no internalization or metabolism of the ligand oc-curred. These results demonstrate that intact CPT-L2-BA3 is

internalized and metabolized by releasing CPT and generating L2-BA3 as well as other metabolites. Furthermore, the pres-ence of hGRPR is required for internalization and metabolism of CPT-L2-BA3.

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TABLE II

Comparison of the kinetics and the maximum internalization of ’251BA0 1251-L2-BA3, or 1251-CPT-L2-BA3 by human BN receptors
hGRPR-, hNMBR-, and hBRS3-transfected Balb/c 3T3 cells were incubated with the indicated ligands, and the rate and magnitude of internalization were determined. Results show the percentage ofmaximal saturable ligand binding at each time point that was not acid-stripped (i.e. maximum percentage internalized). T,,,,, refers to the time in minutes for maximal internalization to occur. Results are from the data shown in Fig. 3 and are the mean – S.E. of at least six experiments. Each point was determined in duplicate.

Ligand

hGRPR hNMBr’.

T,, Maximum internalized Tm,,, Maximum internalized

hBRS

Tm,. Maximum internalized
mm % mm % rain %
5I-BA0 7.5.t 0.6 24.2 – 2.1 30 – 3 26 – 3 7.5 – 0.7 31 – 3
1251-L2-BA3 15.2 – 1.2 28.9 – 2.3 30 – 3 34 – 4 7.5 – 1.1 40 – 6

1251cp’2L2BA3 15.3 – 0.8’ 42.7 – 2.2" 15 – Ib 34 – 2h 7.5 – 1.2 42 – 6

"p < 0.001 compared with ’251-BAO. b p < 0.05 compared with 1251-BAO.

FIG. 4. Internalization of the cainptothecin-bombesin conju-
gate in HEK 293 cells transiently expressing HA-tagged GRP
receptors. HEK 293 cells (0.5 x 101 cells/6-well plate) were transfected
with 1 jug of HA-GRPR-pcDNA3 and split to polylysine-coated 12-well

plates the following day. Three days after transfection, cells were treated with (D-1) or without (A-C) CPT-L2-BA3 (3 me) for the indi-

cated periods of time. After removal of free CPT-L2-BA3, cells contain-

ing bound CPT-L2-BA3 were fixed, permeabilized, and stained with a polyclonal bombesin antibody (left) (A, D, 0) or an HA antibody against

the receptor (middle) (B, E, II), as described under ’Experimental Procedures." Merged images (C, F, 1) ofthe pictures are shown to reveal

colocalization of the BN analog and the receptor (right). Shown are representative pictures of three independent experiments.
CPT-L2-BA3 Causes Elevation of Cytosolic Ca21 in Nd-111299 Cells’251-CPT-L2-BA3 bound with high affinity to NCI-111299 cells and was internalized (data not shown). CPT-L2-BA3 (K = 0.8 – 0.2 me) had a 5-fold greater affinity for hGRP receptors on these cells than BAO (K = 4.3 – 0.4 rim). CPT-L2-BA3 (10 me) stimulated a rapid increase in cytosolic Ca" in Fura-2-loaded NCI-H1299 cells (Fig. 5). BW2258U89 (10 j.tM), a specific GRP receptor antagonist (17), had no effect on cytosolic Ca2 but completely inhibited the increase in cy-tosolic Ca2 caused by 1 or 10 nM CPT-L2-BA3 (Fig. 5). The subsequent addition of 1000 nM CPT-L2-BA3, however, over-came the inhibition caused by the GRP receptor antagonist (Fig. 5). Similar results were obtained using NCI-H345 (SCLC) or hGRPR Balb/c 3T3 cells (data not shown). Because this antagonist binds with high affinity to hGRPR but not hNMBR or IBRS3 (11, 17), this result indicates that CPT-L2-BA3 is stimulating an increase in cytosolic calcium by activation of hGRPR on NCI-H1299 cells. CPT-L2-BA3 (0.1 nM) had no effect on cytosolic Ca2, but the subsequent addition of 10 me GRP to

NCI-H1299 cells increased cytosolic calcium (Fig. 5). CPT-L2-BA3 (1 me) caused an increase in cytosolic Ca" 20% of that seen with ionomycin (Fig. 5). A maximally effective concentra-tion of CPT-L2-BA3 (i.e. 10 na’s) caused a marked increase in cytosolic Ca2, and the subsequent addition ofGRP (10 ni’s) had no effect (Fig. 5).
CPT-L2-BA3 Inhibits Cellular ProliferationThe ability of CPT-L2-BA3 to inhibit cellular proliferation was investigated in vitro. Using the MIT assay, CPT-L2-BA3 caused a dose-de-pendent inhibition of proliferation by NCI-H1299 cells and hGRPR-transfected Balb/c 3T3 cells with concentrations from

100 to 3000 me (Fig. 6). CPT-L2-BA3 had a half-maximal (IC50) inhibitory growth effect in NCI-111299 cells of 190 – 20 nM, whereas the IC50 was 500 – 40 na’s and 2450 nI’s for GRPR-transfected Balb/c 3T3 and control Balb/c 3T3 cells not contain-ing human BN receptors, respectively (Fig. 6). Also, the IC50 was 800 and 600 aM for Balb/c 3T3 cells transfected with hNMBR or hBRS3 (data not shown). The results indicate that CPT-L2-BA3 was more potent at inhibiting the proliferation of cells containing BN receptors than cells lacking BN receptors. Using a clonogenic assay, CPT-L2-BA3 also inhibited cellu-lar proliferation ofNCI-H1299 NSCLC cells in a concentration-dependent manner (Fig. 6). Large robust NCI-111299 colonies formed in the absence of CPT-BN conjugates (control). The colo-nies decreased in number and size using 100 or 300 ni’s CPT-L2-BA3 (0.1 or 0.3 jIM) and were absent using 1000 nj’s CPT-L2-BA3 (1 iLM). For the NCI-111299 cells, the IC50 for CPT-L2-BA3 to inhibit proliferation was 170 – 20 na’s. In contrast, 1000 na’s BAG did not alter proliferation (data not shown). CPT-L2-BA3 (1000 rim) decreased the viability ofNCI-H1299 cells based on trypan blue exclusion and decreased I31-1lleucine incorporation into proteins as well as [311]thymidine incorporation into DNA (data not shown). These results indicate that CPT-L2-BA3 was cyto-toxic for NCI-H1299 cells.

DISCUSSION

Recent studies, primarily using radiolabeled somatostatin analogs (6, 7), demonstrate that the frequent ectopic expression or overexpression ofpeptide hormone receptors by tumors (4, 7) can be utilized to administer tumor-selective cytotoxicity (6, 7). A similar approach is now being investigated using peptide receptor ligands coupled to chemotherapeutic agents for a num-ber of peptide hormone receptors frequently present on com-mon malignant tumors (i.e. prostate, breast, central nervous system tumors, lung cancer, various gastro-intestinal malig-nancies, and ovarian cancer) (4, 8, 21, 22). A cytotoxic agent using bombesin receptors as a molecular target would be useful for a number of reasons. First, BN receptor expression and/or overexpression occurs on almost all prostate cancers (5, 23), 33-72% of breast cancers (23, 24), 44-100% of small cell or non-small cell lung cancers (23, 25), 57% ofgastric cancers (26), 100% ofsome types ofmalignant gliomas (24), 100% of ovarian cancer (27), 100% of squamous cell carcinoids of the head and

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FIG. 5. Effect of CPT-L2-BA3 alone 1.5

or in combination with a GRPR re- ceptor antagonist on [Ca2], in Nd-
111299 cells. Top, NCI-111299 cells were loaded with 5 jkm Fura-2/AM as described
under "Experimental Procedures," and Uf

changes in cytosolic Ca" ([Ca2]) were determined in a Delta PTI Scan 1 spec- 1.0 trofluorometer using a cell density of
2.5 X 10" cells/ml. In the top panel are shown results with different concentra-

tions of CPT-L2-BA3 in the presence or absence of an hGRPR antagonist (10 gm),

(3-phenylpropanoyl-6)- [His7,-Ala11,-
Pro12,ifrl3-14,Phe14]-BN-(6 -14) (BW

2258489) (17). CPT-L2-BA3 stimulates g increases in [Ca2], and its effect at lower
concentrations is inhibited by the GRPR

antagonist but overcome at higher con- 1-5 centrations (i.e. 1000 nM) of CPT-L2-BA3.

These experiments are representative of
three others. Bottom, the effects of in-
creasing concentrations of CPT-L2-BA3,
10 nra GRP, or ionomycin are shown.
These experiments are representative of
three others. 10

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GRPR

CPT-L2-BA3 Antagonist CPT-LT-BA3 (nM) (10 nM) (10 PM) 1 10 1000

 3min }.u-3 min›oj

CPT-L2- CPT-L2-

BA3 GRP BA3 lonomycin BA3 GRP
(0.1 nM) (10 nM) (1 nM) (5 jig/mI) (10 nM) (10 nM)

. * 4 }.4 - -4

neck (28), and 40-80% of colon cancer (23, 29). Second, pep-tides, which function as agonists but not antagonists for each of the three BN receptor subclasses, are rapidly internalized (15, 30-32). Third, recent studies using radiolabeled synthetic an-alogs of BN-related peptides demonstrate that these can be used to target tumors/tissues containing BN receptors, and these analogs are internalized in high amounts by these tu-mors (9).
To develop a conjugated bombesin analog that would be selectively cytotoxic to tumor cells overexpressing mammalian BN receptors, two important obstacles needed to be overcome. First, the BN cytotoxic conjugate should bind with high affinity to hGRPR, hNMBR, and hBRS3. This is important because different tumors may express different classes ofBN receptors in different amounts (5, 7). This requirement was met by using the novel bombesin analog, [u-Tyr6,-Ala11,Phe13,Nle14}BN-(6-14), which binds with high affinity for each of the three classes of mammalian bombesin receptors (11, 12). Second, a coupling system needs to be used that can be cleaved by intra-cellular enzymes to selectively release the cytotoxic agent within the tumor cell (1). Various carbamate linkers were uti-lized (3), which contained BINAR groups that allow coupling to chemotherapeutic agents that contain a reactive hydroxy group. This BINAR linkage system, when used to couple soma-tostatin analogs to combretastatin or CPT, was found to retain high affinity binding for somatostatin receptors, be stable in plasma, and have tumor cytotoxicity at doses below the maxi-mum tolerated equivalent dose of cytotoxic agent alone (3). In a previous study (3), different BINAR groups had different effects on receptor affinity or peptide stability. Therefore, we utilized two different BINAR carbamate linkers, an ethylenedi-amine-containing linker (Li) and anN-methylethylenediamine (L2) CPT conjugate, in combination with different BN analogs. Both BINAR linkers and the amino acids at the N-terminal of

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BAO had a profound effect on binding affinity to BN receptors. Only one combination of BINAR linker and peptide conjugate coupled to camptothecin (CPT-L2-BA3) retained high affinity for all three BN receptors, and this analog was fully character-ized in the present study.
A number of our results support the conclusion that the BINAR bombesin analog, L2-BA3, has the necessary charac-teristics to be an effective compound for site-specific delivery of chemotherapeutic agents to cells possessing mammalian bomb-esin receptors. These characteristics include the following. The bombesin analog, L2-BA3, functions as a high affinity agonist at each class of mammalian bombesin receptor and is rapidly internalized by each receptor class, and the subsequent conju-gation of camptothecin has no detrimental affect on any of these properties. This bombesin analog had equal high affinity to the original universal ligand, BAO (Fig. 1) (11, 12), for each of the three classes of mammalian bombesin receptors. Fur-thermore, activation of each of the mammalian bombesin re-ceptors results in phospholipase C stimulation (15, 19, 33, 34), and when this was assessed, this analog had an equal or greater potency for activating each receptor and stimulating generation of phosphoinositides as well as being fully effica-cious to the original universal ligand, BAO.
Using iodinated analogs of the bombesin analog, L2-BA3, and the universal ligand, BAO, we were able to show that 1251-L2-BA3 was internalized by cells possessing each of the three mammalian bombesin receptors, and the internalization rates and magnitude ofinternalization were similar for the two bombesin analogs. Not only did the addition of camptothecin to the linker bombesin analog, L2-BA3, not decrease the receptor affinity, its addition in fact resulted in a 30-fold increase in affinity for GRP receptor, a 20-fold increase for the NIVIB re-ceptor, and an 8-fold increase in affinity for the BRS-3 receptor. At present, the molecular basis for this increased affinity is

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Flu. 6. Ability of CPT-L2-BA3 to in-

hibit proliferation of various BN re- ceptor-containing and non-BN recep-

tor-containing cells. Top, in the MIT
assay, each of the cells (104Iwell) was in-
cubated with the indicated concentration

of CPT-L2-BA3 for 3 days, at which time
MTT (1 mg/ml) was added. After this, 150

pJ was added, and after 16 h the optical density was determined at 570 nm. Re-

sults are expressed as the percentage of
proliferation seen with untreated cells.
The mean value – S.E. of eight determi-
nations is indicated. This experiment is
representative of four others. Bottom, in

the clonogenic assay, colony number was determined 2 weeks after seeding with
5 x 104 NCI-111299 cells. The colonies were determined in the absence of CPT-
L2-BA3 (i.e. control) or presence of the
indicated concentration of CPT-1,2-13A3.
This experiment is representative ofthree
others.

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unknown. Previously, GIn121, Phe185, Ala198, Pro’99, Arg288 and Ala308 of the GRPR were found to be important for high affinity binding by GRP (35, 36), and possibly CPT-L2-BA3 interacts with higher affinity with these amino acids or others due to the increased hydrophobicity caused by the addition of CPT to the L2-BA3. Furthermore, the camptothecin-coupled L2-BA3 had equal potency and efficacy to the uncoupled L2-BA3 for stimulating phospholipase C and, therefore, functioned as a full agonist. The coupling of camptothecin to the bombesin analog, L2-BA3, not only did not interfere with its internaliza-tion by the three mammalian bombesin receptors; its addition, in fact, significantly increased the amount internalized by 76% for the GRP receptor and 31% for the NMB receptor. Previous studies demonstrate that GRPR agonists are rap-idly internalized and also demonstrate that the BN agonist-GRPR complex is initially colocalized in endosomes and then moves to vesicles in the perinuclear space, where it is degraded (19, 32, 37, 38). Our results using fluorescent probes are con-sistent with the conclusion that CPT-L2-BA3 is being processed ma similar manner by the GRP receptor-bearing cells. Specif-ically, we found that the CPT-L2-BA3 colocalized on the cell surface with the GRPR was subsequently internalized in vesi-cles largely separate from the GRPR. These results indicate that CPT-L2-BA3 is internalized as a result of hGRPR-medi-

ated endocytosis. After internalization, GRP receptor ligands are degraded intracellularly by lysosome-dependent and -inde-

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[CPT-L2 -9A3 (log M)

pendent mechanisms (39, 40). This could result in CPT-L2 being released into the cytosol and being metabolized by cyto-chrome P450 enzymes to release intracellular CPT. Our HPLC results of internalized radioactivity by hGRPR-containing Balb/c 3T3 cells after incubation with 1251-CTP-L2-BA3 and removal of surface-bound ligand support this proposal. These results demonstrate that intact CPT-L2-BA3 was internalized, and it was metabolized to L2-BA3 by releasing free CPT. This internalization was dependent on the presence of GRPR on the Balb/c 3T3 cells and thus provides evidence that CPT-L2-BA3 was functioning as a prodrug by delivering the cytotoxic con-jugate to GRPR-containing cells, where the cytotoxic agent could be released by intracellular metabolism. Unfortunately, we were unable to directly label caniptothecin in our analogs without loss of activity, and, therefore, we could not directly study the kinetics ofthe intracellular generation offree camp-tothecin. Carbamate linkages are known to be metabolized by cytochrome P450, which is abundant in cancer cells (41). Cyto-chrome P450 is thought to metabolize CPT-somatostatin ana-logs, leading to cytotoxicity (3). Furthermore, P450 is known to be present in numerous human tumors including colon, breast, lung, liver, kidney, and prostate as well as to be up-regulated by human tumors (42).
A number of our results and those in the literature support the conclusion that the camptothecin-BINAR-bombesin analog, CPT-L2-BA3, will be a useful prototype agent for investigating

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the ability of site-specific delivery of this chemotherapeutic agent to cause cytotoxicity in tumors overexpressing mamma-lian bombesin receptors. First, CPT-L2-BA3 bound with high affinity and was rapidly internalized by native hGRPR on NCI-H1299 cells. Second, the intact CPT-L2-BA8 was internal-ized and metabolized intracellularly, releasing free CPT. Third, CPT-L2.-BA3 was biologically active in NCI-1›11299 cells in that it elevated cytosolic Ca", and its action was blocked by specific hGRPR antagonists. Fourth, using both the MTF and clone-genic growth assay, CPT-L2-BA3 inhibited the growth of NCI-H1299 cells. In contrast, a bombesin analog not coupled to CPT had no effect on growth. Fifth, in Balb/c 3T3 cells that were transfected with any one of the three classes of mammalian bombesin receptors, the CPT-L2-BA3 analog caused greater cytotoxicity than in the same cells not possessing bombesin receptors, demonstrating the ability of the bombesin receptor to cause enhanced cytotoxicity. Sixth, a number ofproperties of CPT-L2-BA3 and results ofsimilar compounds in the literature would suggest in vivo that it should have an acceptable safety profile. CPT-L2-BA3 is more hydrophilic than native campto-thecin, allowing greater aqueous solubility, and thus, greater concentrations should be available to act on targeted tissues (i.e. bombesin receptor-containing cells). Its selectivity for tis-sues overexpressing bombesin receptors should help target the analog, allowing greater cytoxicity with lower dosing. This proposal is supported by such results with doxorubicin analogs coupled to peptide delivery systems (8, 21).
In conclusion, our results show that CPT-L2-BA3 binds with high affinity to all mammalian BN receptor subtypes, functions as a fully potent and efficacious receptor agonist at each receptor class, is internalized, is metabolized intracel-lularly to L2-BA3-releasing camptothecin, and is cytotoxic for both tumor cells and cells transfected with human BN recep-tors. Its availability should be useful to explore the effective-ness of tumor-specific cytotoxicity delivery using a receptor-mediated mechanism.

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REFERENCES

1. deGroot, F. M., Damon, E. W., and Scheeran, H. W. (2001) Curr. Med. Chem. 8,1093-1122
2. Dubowchik, C. M., and Walker, M. A. (1999) Pharmacol. Ther. 83,67-123
3. Fuselier, J. A., Sun, L., Woltering, S. N., Murphy, W. A., Vasilavich, N., and Coy, D. H. (2003) Biaorg. Med. Chem. Lett. 13, 799-803
4. Langer, M., and Beck-Sickinger, A. G. (2001) Curr. Med. Chem. Anti-Cancer Agents 1, 71-93
5. Reubi, J. C., Wenger, S., Schwnuckli-Maurer, J., Schaer, J. C., and Gugger, M. (2002) Clin. Cancer Res. 8,1139 -1146
6. Kwekkeboom, D., Krenning, E. P., and deJong, M. (2000) J. Nuci. Med. 41, 1704-1713

7. Reubi, J. C. (2003) Endccr. Rev. 24, 389-427

23589

S. Schally, A. V., and Nagy, A. (2003) Life Sci. 72, 2305-2320
9. Hoffman, T. J., Quinn, T. P., and Volkert, W. A. (2001) Nucl. Med. Dial. 28,
527-539

10. Moody, T. W., Chan, D., Fahrenkrug,J., and Jensen, B. T. (2003) Curr. Pharm. Des. 9, 495-509
11. Mantey, S. A., Weber, H. C., Sainz, E., Akeson, M., Ryan, R. R., Pradhan, T. K., Searles, R. P., Spindel, E. H., Battey, J. F., Coy, D. H., and Jensen, B. T. (1997) J. Biol. Chem. 272, 26062-26071
12. Pradhan, T. K, Katsuno, T., Taylor, J. E., Kim, S. H., Ryan, H. H., Mantey, S. A., Donohue, P..1., Weber, H. C., Sainz, E., Battey, J. F., Coy, D. H., and Jensen, R. T. (1998) Ear. J. Pharmacol. 343, 275-287
13. Moody, T. W., Zia, F., Venugopal, H., Fagarasan, M., Oie, H., and Hu, V.(1996) J. Cell. Biachem. Suppl. 24, 247-256
14. Benya, H. V., Kusui, T., Pradhan, T. K, Battey, J. F., and Jensen, B. T. (1995) Mal. Pharmacol. 47,10-20
15. Benya, H. V., Wada, E., Battey,J. F., Fathi, Z., Wang, L. H., Mantey, S. A., Coy, D. H., and Jensen, B. T. (1992) Mel. Pharmacol. 42,1058-1068
16. Chang, Y., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099-3108
17. Moody, T. W., Venugopal, H., Zia, F., Patierno, S., Leban,J. J., and McDermed, J. (1995) Life Sal. 56, 521-529
18. Mahmoud, S., Staley, J., Taylor,J., Bogdan, A., Moreau, J. P., Coy, D., Avis, I., Cuttitta, F., Muishine, J. L., and Moody, T. W. (1991) Cancer Res. 51, 1798-1802

19. Benya, B. V., Fathi, Z., Pradhan, T., Battey, J. F., Kusmi, T., and Jensen, H. T. (1994) Mol. Pharmacol. 46,236-245
20. Jensen, K T., Charlton, C. G., Adachi, H., Jones, S. W., O’Donohue, T. L, and Gardner, J. D. (1983) Am. J. Physiol. 245, G186-G195
21. Schally, A. V., and Nagy, A. (1999) Ear. J. Endacrinot. 141, 1-14
22. Varga, J. M., Asato, N., Lande, S., and Lerner, A. B. (1977) Nature 267,56-58
23. Guinea, D. G., Jr., Fishback, N. F., Koss, M. N., Abbondanso, S. L., and Travis, W. D. (1994) Am. J. Clin. Pat/wi. 102,406-414
24. Schally, A. V., Comaru-Schally, A. IL, Nagy, A., Kovacs, M., Szepeshazi, K, Plonowski, A., Varga, J. L, and Halmos, G. (2001) Front. Neuroendocrinot. 22,248-291
25. Siegfried,J. M., Krishnaniachary, N., Davis, A. G., Gubish, C., Hunt, J. 1)., and Shriver, S. P. (1999) PuIm. Pharmacol. Ther. 12,291-302
26. Preston, S. K., Woodhouse, L F., Jones-Blackett, S., Wyatt,J. I., and Primrose, J. N. (1993) Cancer Des. 53, 5090-5092
27. Sun, B., Schally, A. V., and Holmes, G. (2000) Regal. Pept. 90, 77-84
28. Lango, M. N., Dyer, K F., Lw, V. W., Gooding, W. E., Gubish, C., Siegfried, J. M., and Grandis, J. H. (2002) J. Nail. Cancer Inst. 94, 375-383
29. Preston, S. R., Woodhouse, L F., Jones-Blackett, S., Miller, G. V., and Prim-rose, J. N. (1995) Br. J. Cancer 71, 1087-1089
30. Mantey, S., Frucht, H., Coy, D. H., and Jensen, R. T. (1993) Mol. Pharmacol. 45,762-774
31. Bunnett, N. W., Payan, D. C., and Grady, E. F. (1996) Histochem. J. 28, 811-826

32. Slice, L. W., Yea, H. F., Jr., and Walsh, J. H. (1998) Receptors Channels 6, 201-212

33. Wu, J. M., Nitecki, D. H., Biancalana, S., and Feldman, H. L (1996) Mol. Pharmacol. 50,1355-1363
34. Kroog, C. S., Jensen, H. T., andBattey, J. F. (1995) Med. Res. Rev. 15,389-417
35. Akeson, M., Sainz, E., Mantey, S. A., Jensen, H. T., and Battey, J. F. (1997) J. Biol. Chem. 272, 17405-17409
36. Tokita, K, Hocart, S. J., Coy, D. H., and Jensen, R. T. (2002) Mol. Pharmacol. 61,1435-1443
37. Grady, B. F., Slice, L. W., Brent, W. 0., Walsh, J. H., Payan, D. C., and Bunnett, N. W. (1995) J. Biol. Chem. 270,4603-4611
38. Slice, L W., Wang, H. C., Sternini, C., Grady, H. F., Bunnett, N. W., and Walsh, J. H. (1994) J. Blot. Chem. 260, 21755-21782
39. Williams, B. Y., Dion, S. B., and Schonbrunn, A. (1998) Mol. Pharmacol. 54,
889-898

40. Zachary, I., and Hozengurt, B. (1987) EMBO J. 8, 2233-2239
41. Cha, S. W., Cu, H. K, Lee, K P., Lee, M. H., Han, S. S., and Jeong, T. C. (2000) Toxwol. Lett. 115,173-181
42. Patterson, L. H., McKeown, S. H., Robson, T., Gallagher, H., Raleigh, S. M., and Orr, S. (1999) Anticancer Drug Des. 14, 473-486

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Vasoactive intestinal peptide-camptothecin conjugates inhibit the proliferation of breast cancer cells

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Abstract

The effects ofvasoactive intestinal peptide camptothecin (VIP-CPT) conjugates were investigated
on breast cancer cells and cells transfected with VIP receptors (R). (Ala2’8’9’19’242527, N1e17,
Lys28)VIP, (A-NIL-K)VIP, was synthesized and Lys28 was coupled to a linker, N-methyl-amino-ethyl-glycine, L2, which formed a carbamate bond with CPT. The resulting (A-NL-K)VIP-L2-CPT
was cytotoxic for MCF7 breast cancer cells, which have VPAC1-R, with IC50 values of 380 and 90 nM using the MTT and clonogenic assays, respectively. (A-NL-K)VIP, (A-NL-K)VIP-L2 and (A-NL-K)VIP-L2-CPT inhibited specific binding of 1251-VIP to 3T3 cells transfected with VPAC1-R with IC50 values of 1.9, 56 and 126 nM respectively. In contrast, (A-NL-K)VIP, (A-NL-K)VIP-L2 and (A-NL-K)VIP-L2-CPT inhibited specific binding of 1251-Ro25-1 553 to 3T3 cells transfected with VPAC2-R with IC50 values of 3.9, 3162 and 2690 nM respectively. (A-NL-K)VIP, (A-NIL-K) VIP-L2 and (A-NL-K)VIP-L2-CPT caused increased cAMP after addition to MCF7 cells. 125I-(A-NL-K)VIP-L2-CPT was internalized by MCF7 cells at 37°C but not 4°C. These results indicate that (A-NL-K)VIP-L2-CPT is a VPAC1-R agonist which is cytotoxic for breast cancer cells.

Keywords

camptothecin; VIP receptors; cellular proliferation; breast cancer; receptor-mediated cytotoxicity

1. Introduction

Breast cancer, which kills approximately 40,000 women annually in the United States, utilizes
numerous growth factors to stimulate proliferation. Approximately 30% of breast cancer
tumors over-express Her2/Neu, a tyrosine kinase [7]. Patients, with amplified Her2/Neu, can
be treated with the monoclonal antibody Herceptin [44]. Herceptin delays progression ofbreast
cancer in 90% ofthe treated patients, allowing time fortreatment with other agents such as the chemotherapeutics adriamycin and taxol [47]. Approximately halfofthe breast cancer tumors

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express the estrogen receptor, and these patients can be treated with the anti-estrogens
tamoxifen or raloxifene [10]. There is a need, however, to develop additional modes of
treatment for patients whose tumors are estrogen receptor and Her-2/Neu negative.

The vasoactive intestinal peptide (VIP) receptor (R), in addition to Her-2/Neu and the estrogen
receptor, is a molecular target for breast cancer. High densities ofVPAC1-R and mRNA are
present in all breast cancer cells tested [15,45,48]. VIP, which is a 28 amino acid peptide, binds with high affinity to VPACI-Rand VPAC2-R [18,22,41]. VIP is structurally related to pituitary adenylate cyclase activating polypeptide (PACAP), which binds with high affinity to VPAC1-R, VPAC2-R and and PAC1-R [1,38,42]. VPAC1-R, VPAC2R and PAC1-R interact with a stimulatory guanine nucleotide binding protein after activation by agonist, resulting in increased adenylyl cyclase activity [28]. The elevated cyclic AMP causes activation ofprotein kinase A, resulting in the phosphorylation ofprotein substrates such as CREB [46]. This causes increased expression on nuclear oncogenes such as c-fos or c-jun, leading to elevated levels of vascular endothelial cell growth factor [3]. Using in vitro autoradiographic techniques, VPAC1-R has been detected in numerous tumors including neuroblastoma as well as bladder, breast, colon, liver, lung, stomach, thyroid, uterus and prostate cancer [40]. VPAC2-R predominates in gastric leimyomas, whereas PAC1-R is abundant in glioblastoma, adrenal and pituitary cancer [39].

The overexpression of VIP receptors, as well as other cell surface receptors, is increasingly being used to develop new drugs for the treatment of cancer patients. VIP receptor antagonists such as VlPhybrid or (N-Stearyl1, N1e1)VIP not only inhibit the proliferation oflung and breast cancer cells [17,34], but they potentiate the cytotoxicity of chemotherapeutic agents [14,31]. The overexpression ofpeptide receptors by the tumor can be used for receptor-targeted
delivery ofpeptide receptor cytotoxic agents [35]. Previously, we reported that VIP-ellipticine
(E) conjugates were cytotoxic for lung and breast cancer cells [26]. VIP-LALA-E was
internalized by cancer cells containing VPAC1-R, and subsequently metabolized by proteolytic
enzymes. The cytotoxic ellipticine (E), which was released after proteolytic metabolism ofthe
VIP-E conjugate, inhibited topoisomerase enzymes leading to cellular cytotoxicity. In the
cn present study we have extended these studies and synthesized VIP-camptothecin (CPT)
conjugates which have a novel carbamate linker with a built-in nucleophile associated releasing
group, L2. [11,27,33]. After receptor-mediated endocytosis, (Ala2’8’9"9’242527, N1e17, Lys28)
VIP-L2-camptothecin ((A-NL-K)VIP-L2-CPT) is metabolized by cytochrome P450 enzymes,
releasing CPT. Our results show that (A-NL-K)VIP-L2-CPT is a selective VPAC1-R agonist,
which is cytotoxic for breast cancer cells.

2. Materials and Methods

2.1 Materials

The following cells and materials were obtained from the sources indicated: BALB 3T3 (mouse
fibroblast), T47D and MCF7 (human breast cancer) and SupTl cells were from American Type
Culture Collection (ATCC), Rockville, MD; Dulbecco’s minimum essential medium
(DMEM), phosphate-buffered saline (PBS), Roswell Park Memorial Institute (RPMI-1640),
0 tiypsin-EDTA and fetal bovine serum (FBS), from Biofluids, Rockville, MD; G418 sulfate
from Life Technologies, Inc., Grand Island, NY; Na1251 (2200 Ci/mmol) was from Amersham
Pharmacia Biotech; CPT, formic acid, ammonium formate, disodium tetraborate, 4-

(dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4-H-pyran (DCM); 2- cn dimethylaminoisopropyl chloride hydrochloride (DIC); I -methyl-2-pyrrolidinone (NMP);
dimethylaminopyridine (DMAP); soybean trypsin inhibitor, bacitracin, leupeptin, 4-(2-
aminoethyl)-benzenesulfonyl fluoride (AEBSF), poly-L-lysine and triton X- 100 were from
1 Sigma-Aldrich, St. Louis, MO; I,2,4,6-tetrachloro-3a,6a-diphenylglycouril (IODO-GEN)
from Pierce Chemical Co., Rockford, IL; VIP and Ro25- 1553 were purchased from Bachem,

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Torrance, CA; AG 1-X8 resin from Bio-Rad, Richmond;[3-(4,5 dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide] (MY!’) and iodo-nitrotetrazolium violet was from Sigma
Chemical Co., St. Louis, MO; Agarose was purchased from FMC Corp., Rockford, ME.

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2.2 Cell culture
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MCF7, SupTi and T47D cells were cultured in RPMI-1640 medium containing 10% heat-
inactivated FBS (Life Technologies, Rockville, MD). Cells were split weekly 1/20 with
trypsin-EDTA. BaIb 3T3 cells stably expressing human VPAC1-R or VPAC2-R were made as
10 described previously [21] and were grown in DMEM supplemented with 300 mg/liter ofG418 sulfate. Cells were mycoplasma free and were used when they were in exponential growth
phase after incubation at 37°C in 5% CO2, 95% air.

2.3 Preparation of peptides

(A-NL-K)VIP, which is an analog ofthe selective VPAC1-R agonist,

(Ala2’8’9’1 1.1924252728)VIP, was synthesized using solid phase techniques. The VIP-CPT
conjugates were synthesized by coupling the CPT to the Lys28 of(A-NL-K)VIP-L2 as described previously [19]. The product (A-NL-K)VIP-L2-CPT had >97% purity, by preparative HPLC (C18 silica), mass spectrometry and amino acid analysis. 1251-VIP, 1251-(A-NL-K)VIP-L2-CPT and 1251-Ro25-1 553 (Ro) had specific activities of2200 Ci/mmol, were prepared as previously described [19]. The radiolabeled peptides were separated using a Cl 8 Sep-Pak (Waters Associates, Milford, MA) and fractions with the highest radioactivity were pooled, neutralized with 0.2 M Tris buffer(pH 9.5) and stored with 0.5% bovine serum albumin (w/v) at 20°C.

2.4 Binding of 1251 -labeled VIP-related peptides

Binding was performed as described previously [21]. The standard binding buffer contained 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCI, 5 mM MgCl2, 2.5 mM NaH2PO4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 0.01% (w/v) soybean trypsin inhibitor, 1% amino acid mixture, 0.2% (w/v) bovine serum albumin (BSA), and 0.05% (w/v) bacitracin. BALB 3T3 cells stably expressing human VPAC1-R (0.3 x 106), VPAC2-R (0.03 x 10), SupTi or T47D cells were incubated with 50 pM 1251-labeled ligand at 22°C for 60 min. Aliquots (100 .d) were removed and centrifuged through 300 pJ ofincubation buffer in 400 j.tl microfuge tubes at 10,000 xg for I min using a Beckman micro-centrifuge B. The pellets were washed twice with buffer and counted for radioactivity in a gamma counter. The nonsaturable binding was the amount ofradioactivity associated with cells in incubations containing 50 pM radioligand (2200 Ci/mmol) and 1 ItM unlabeled ligand. Nonsaturable binding was <10% of total binding in all the experiments. Inhibition constants (K1) were determined using a least-
square, curve-fitting program (KaleidaGraph) and the Cheng-Prusoff equation [5].

The internalization of 1251-(A-NL-K)VIP-L2-CPT was investigated. 1251-(A-NL-K)VIP-L2-
CPT (0.1 nM) was incubated with MCF-7 cells at 4°C for 2 h in PBS containing 2% BSA and
200 pg/mI bacitracin in the presence or absence of 1 tM VIP. One ofthe plates was incubated
at 37°C for 5 mm. The plates were washed in cold PBS containing 2% BSA and 200 big/ml
0 bacitracin three times to remove free 1251-(A-NL-K)VIP-L2-CPT. The cells were treated with 150 mM NaCl/100 mM acetic acid for 5 min at 4°C. The supernatant, which contained
radiolabeled peptide bound to the cell surface, was counted. The cells, which contained
radiolabeled peptide that was internalized, were dissolved in 100 mM NaOH and counted in a
gamma counter. -’
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2.5 Cyclic AMP assays

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Cyclic AMP assays were conducted by radioimmunoassay. NCI-H1299 cells in 24 well plates
z were incubated with VIP-CPT conjugates in the presence of250 pi of SIT medium containing
50 tM IBMX, 1 mg/ml bacitracin and 1% BSA for 5 min at 37°C. After 5 mm, the samples
-!D were treated with 250 tl of cold ethanol. The samples were mixed and stored at 80°C until
> assayed. The samples were assayed in duplicate by radioimmunoassay (New England Nuclear,
> Boston, MA). The mean value + S.D. of4 determinations were calculated.

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2.6 Proliferation assays

Growth studies were performed using the [3-(4,5dimethylthiazol-2-yI)-2,5-diphenyl]-2H-
tetrazolium bromide (MTT) assay [24]. MCF7 human breast cancer cells (104/well) were
placed in SIT medium (100 pi) and various concentrations of(A-NL-K)VIP-L2-CPT, (A-NL-K)VIP or (A-NL-K)VIP-L2 added. After 4 days, 15 tl (1 mg/ml) ofMTT was added. After 4 h, 150 i1 ofDMSO was added. After 16 h, the optical density at 570 nm was determined using an ELISA reader. The proliferation rates were calculated from the OD readings with various
concentrations of VIP-CPT conjugate using the untreated cells as 100.

The effects ofVIP-CPT conjugates on the growth ofMCF7 cells were investigated using a
clonogenic assay. The base layer consisted of3 ml of0.5% agarose in SIT medium containing
5% fetal bovine serum in 6 well plates. The top layer consisted of3 ml ofSIT medium in 0.3%
agarose, VIP-CPT conjugates and 5 x 104 MCF7 cells. Triplicate wells were plated and after
C 2 weeks, I ml of0.1% p-iodonitrotetrazolium violet was added and after 16 hours at 37°C, the
plates were screened for colony formation; the number of colonies larger than 50.im in
diameter were counted using an Omnicon image analysis system.

3. Results

3.1 (A-NL-K)VIP, (A-NL-K)VIP-L2 and (A-NL-K)VIP-L2-CPT bind to cells containing VPAC1-R
or VPAC2-R

I25I-VIP and 1251-Ro25-1553 (Ro) were examined by radioreceptor assay for their ability to
interact with human cancer cells and cells stably transfected with human VIP receptors (Fig.
1). 1251-VIP bound specifically to 3T3 cells stably transfected with VPAC1-R (total binding
was 5057 – 263 cpm and nonspecific binding was 225 –27 cpm) and VPAC2-R,
whereas 1251-Ro bound specifically to 3T3 cells stably transfected with VPAC2-R (total binding was 2945 – 176 and nonspecific binding was 220 – 107 cpm) but not VPAC1-R. Also, 1251-VIP bound specifically to T47D breast carcinoma cells (total binding was 1127 –
52 cpm whereas nonspecific binding was 68 – 5 cpm), which have VPAC1-R [19], whereas 1251-Ro bound with high affinity to SupTl cells (total binding was 764–39 cpm and
nonspecific binding was 15 – 1 cpm), which have VPAC2-R [21].

(A-NL-K)VIP inhibited specific binding of 1251-Ro with high affinity to cells containing
VPAC2-R or 1251-VIP binding to cells containing VPAC1-R. Little specific 1251-Ro binding
C to 3T3 cells or SupTi was inhibited by 0.1 nM (A-NL-K)VIP, but almost all specific binding
0 was inhibited by 100 nM (A-NL-K)VIP (Fig. 1, bottom). The IC50 values for (A-NL-K)VIP
and 3T3 containing VPAC2-R or SupTI cells were 1.9 – 0.1 or 4.3 – 0.2 nM respectively.
Similarly, (A-NL-K)VIP inhibited specific 1251-VIP binding to 3T3 cells containing VPAC1-
R with an IC50 value of 1.9 + 0.1 nM (Fig. 1, top). Surprisingly, (A-NL-K)VIP inhibited
CO specific 1251-VIP binding to T47D cells with an IC50 value of 0.13 +.01 W. These results
indicate that (A-NL-K)VIP binds with high affinity to cells containing either human VPAC1-
R or VPAC2-R.

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The ability of(A-NL-K)VIP-L2 or (A-NL-K)VIP-L2-CPT to bind to cells containing
VPAC2-R or VPAC1-R was investigated. Specific 1251-Ro binding to 3T3 cells containing
VPAC2-R was inhibited by (A-NL-K)VIP-L2 or (A-NL-K)VIP-L2-CPT with IC50 values of
372 – 15 and 589 – 23 nM, respectively (Fig. 1, bottom). Similarly, specific 1251-Ro binding

jo to SupT-1 cells was inhibited by (A-Nt-K)VIP-L2 or (A-NL-K)VIP-L2-CPT with 1050 values of 3162 – 126 and 2690 – 800 nM, respectively. Specific 1251-VIP binding to 3T3 cells
transfected with VPAC1-Rs was inhibited by (A-NL-K)VIP-L2 or (A-NL-K)VIP-L2-CPT with
IC50 values of 56 – 2 and 126–11 nM, respectively (Fig. 1, top). Specific 1251-VIP binding to
T47D cells was inhibited by (A-NL-K)VIP-L2 or (A-NL-K)VIP-L2-CPT with IC50 values of
13.2 – 0.7 and 39.8 – 2.3 nM, respectively. Similar results were obtained using MCF7 cells
which have VPAC1-R. (data not shown). These results indicate that (A-Nt-K)VIP-L2 and (A-NL-K)VIP-L2 CPT bind with 5-30 fold higher affinity to cells containing VPAC1-Rs than cells containing VPAC2-Rs.

1251(A..NLK)VIPL2CPT bound specifically to MCF7 cells. Table I showsthat at 4°C, most ofthe specific 1251-(A-NL-K)VIP-L2-CPT bound to the cell surface and only 22.1% was internalized. In contrast at 370C, most ofthe specific 1251(A-NL-K)VIP-L2-CPT bound to MCF7 cells was internalized (61.8%). Similar results were obtained using 1251-(A-NL-K)VIP and MCF7 cells (data not shown).

3.2 (A-NL-K)VIP, (A-NL-K)VIP-L2 and (A-NL-K)VIP-L2-CPT are VPAC1-R agonists

The ability of VIP-CPT conjugates to elevate cAMP was investigated in MCF7 breast cancer
cells, which have VPAC1-R [48], by radioimmunoassay. Table 2 shows that addition of 10 nM
VIP to MCF7 cells increased the cAMP 7.4-fold from 7 to 52 flnol. Forskolin, which interacts
with Gs resulting in maximal adenylyl cyclase activity, increased the cAMP 10.9-fold from 7
to 76.3 fmol. (A-NL-K)VIP, (A-NL-K)VIP-L2 and (A-NL-K)VIP-L2-CPT (1000 nM)
03 increased the cAMP in MCF-7 cells 10.1 -fold, 8.3 -fold and 8.7-fold respectively. These results indicate that (A-NL-K)VIP, (A-NL-K)VIP-L2 and (A-NL-K)VIP-L2-CPT are VPAC1-R
agonists.

3.3 (A-NL-K)VIPL2-CPT inhibits the growth of breast cancer cells

The ability ofVIP-CPT conjugatesto inhibit the growth ofMCF7 cells was investigated. Using
the MIT assay, 30 nM (A-NL-K)VIP-L2-CPT had little effect on proliferation, whereas 1000
nM (A-Nt-K)VIP-L2-CPT strongly inhibited MCF7 proliferation (Fig. 2). The IC50 value was 380 nM for (A-NL-K)VIP-L2-CPT, whereas (A-NL-K)VIP had little effect on MCF7 growth at concentrations up to 1000 nM. Similarly, (A-NL-K)VIP-L2 did not affect MCF7 proliferation (data not shown). These results indicate that (A-NIL-K)VIP-L2-CPT inhibits the growth of breast cancer cells.
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Using the clonogenic assay, (A-NL-K)VIP-L2-CPT reduced MCF7 proliferation with an IC50 value of 90 nM (Fig. 3). In contrast, (A-NL-K)VIP had little effect on MCF7 growth. Similarly, (A-NL-K)VIP-L2 had little effect on MCF7 colonies (data not shown). Figure 4 showsthat a large number ofrobust MCF7 colonies formed in the absence (Fig. 4A) or presence of 30 nM (A-NL-K)VIP-L2-CPT (Fig. 4B). In the presence of 100 or 300 nM (A-Nt-K)VIP-L2-CPT, MCF7 colonies were smaller and absent (Fig. 4C and 41), respectively). These results indicate that (A-NL-K)VIP-L2-CPT but not (A-NL-K)VIP or (A-NL-K)VIP-L2 is cytotoxic for MCF-7 cells.
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4. Discussion

VIP-like peptides are autocrine growth factors for some cancer cells [30]. Pre-pro-VIP and pre-pro-PACAP mRNA are present in lung cancer cells [16]. Also, VIP and pro-VIP

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immunoreactivity are present in lung cancer cells [25]. PACAP mRNA and immunoreactivity
are present in breast cancer biopsy specimens. Pre-pro-PACAP but not PACAP-38 immunoreactivity was present in peri-tumoral and tumoral breast tissues as well as alveolar
epithelial cells and leukocytes in connective tissue [121. Fully processed VIP-like peptides and
their precursors have biological activity [9].

Basic amino acids of VIP such as Arg14, Lys15 and Lys21 may interact electrostatically with acidic amino acids such as G1u36 on the VPAC1-R [36]. Substitution of Ala for VIP amino
acids at positions 1, 6, 12, 14 or 23 reduced binding affinity to VPAC1-R by over 2-orders of
magnitude [19,36]. Substitution ofAla for VIP amino acids at positions 3, 5, 7, 10, 15, 20 or
21 reduced binding affinity to VPAC1-R by over 1 -order ofmagnitude [19,36]. The analog
(A1a2’8’9’11’19’24’25’27’28)VIP is a potent VPAC1-R agonist that is resistant to degradation
[20].

70 VIP receptors were detected in 68 of 68 breast cancer biopsy specimens, as determined using
in vitro autoradiographic techniques [40]. Specific 1251-VIP binding was displaced by a
selective VPACI-R agonists but not Ro25- 1553, a VPAC2-R agonist. Some arterial vessels in
the smooth muscle also had VPAC1-R and VPAC2-R. By RT-PCR, mRNA for VPAC1-R was
detected in 20 out of20 breast cancer biopsy specimens whereas VPAC2-R mRNA was only
present in 45% ofthe samples [29]. By immunocytochemistry, VPAC1-R and VPAC2-R were
present in ductal and glandular epithelial cells both in the normal and malignant breast in 13
patients [13]. Immunoreactive VPAC1-R and VPAC2-R were present in carcinomatous tissue
7: at the stromal level in leukocytes. Because 100 nM VIP, increases adenylyl cylase activity in
breast membranes, the VIP receptors are biologically active.

Breast cancer VIP receptors bind radiolabeled VIP with high affinity [48]. Ala was substituted
for VIP amino acids at non-essential positions 2,8,9,19,24,25,27, Nle was substituted at

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Met17 and Lys substituted at position 28 for conjugation to CPT. The resulting (A-NL-K)VIP
bound with high affinity to VPAC1-R and VPAC2-R. (A-NL-K)VIP bound with higher affinity
(Kd = 0.1 nM) to T47D cells than 3T3 cells transfected with VPAC1-R (Kd = 1.9 nM). This

may result because T47D cells have fewer receptors (resulting in less ligand internalization 10 and degradation) or have less guanine nucleotides (GTP impairs VIP receptor binding).
Addition ofL2 or L2-CPT to the Lys28 c-amino group reduced the affinity ofbinding by
approximately 1 -order ofmagnitude to VPAC1-R and 2-orders ofmagnitude to VPAC2-R.
Previously, we found that VIP and VIP-E analog had IC50 values of 10 and 100 nM using cells
containing VPAC1-R [26]. The results indicate that position 28 of VIP or the C-terminal of
VIP can be modified with retention ofhigh affinity binding. Because addition of(A-NL-K)
VIP, (A-NL-K)VIP-L2 or (A-NL-K)VIP-L2-CPT to MCF7 cells increased cAMP, each ofthe VIP analogs functioned as VPAC1-R agonists. 125I-(A-NL-K)VIP-L2-CPT was internalized by MCF7 cells at 37°C but not 4°C. In other cellular systems, peptide-CPT conjugates are

7: rapidly internalized after binding to cell surface receptors [23,32].

VIP-CPT or VIP-E conjugates may be internalized after binding to VPAC1-R by receptor-
mediated endocytosis. After targeting to lysosomes, VIP-LALA-E may be metabolized by

endopeptidases, which degrade amide bonds. In contrast, (A-NL-K)VIP-L2-CPT is OZ - metabolized by P450 enzymes which metabolize carbamate bonds [4]. P450 enzymes are
present in numerous human tumors including breast, colon, kidney, liver, lung and prostate,
as well as to be up-regulated by human tumors [37]. A major difference, however, is that E
targets topoisomerase II, whereas CPT targets topoisomerase I. Because (A-NL-K)VIP-L2-
CPT is more resistant to degradation by proteases than is VIP-LALA-E, it is better at killing MCF7 cells.

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VIP-CPT conjugates inhibited the proliferation of MCF7 cells. In the MTT assay, (A-NL-K)
VIP-L2-CPT at 300 and 1000 nM significantly inhibited MCF7 proliferation, whereas (A-NL-
K)VIP or (A-NL-K)VIP-L2 did not. In the clonogenic assay, (A-NL-K)VIP-L2-CPT
significantly inhibited MCF7 proliferation at 100, 300 and 1000 nM, whereas (A-NL-K)VIP
or (A-NL-K)VIP-L2 did not. The clonogenic assay may be more sensitive than the MiTassay,
because MCF7 cells are exposed to VIP-CPT conjugates for 2 weeks as opposed to 3 days. Theses results indicate that CPT is essential for VIP-chemotherapeutic conjugatesto kill breast cancer cells, however, it is also possible that VIP-chemotherapeutic conjugates will be cytotoxic for normal cells having high densities of VPAC1-R.

Prodrugs are being used to decrease drug associated side-effects and enhance tumor cytotoxicity [6]. A wide spectrum ofprodrugs have been developed, however, the use ofligands for hormone/growth factor receptors that are overexpressed by tumors has received considerable attention [8]. Recent studies report that bombesin, luteinizing hormone-releasing hormone, somatostatin and VIP receptor ligands can be coupled to cytotoxic agents or radiolabels and have potent antitumor effects [2,32,35,43]. The results ofthese studies with other peptide receptors, as well asthe present study with VIP indicate that the chemotherapeutic cytotoxic analogs coupled to peptide receptor ligands can be developed that are biologically active. These analogs not only allow receptor-targeted delivery but hydrolyzed release ofthe cytotoxic agent within the cancer cell. The availability ofpeptide-chemotherapeutic analogs such as the VIP-CPT conjugates should allow a better assessment ofthis approach using in
vivo models.

Acknowledgments

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Abbreviations

VIP

CPT

PACAP

vasoactive intestinal peptide

camptothecin

pituitary adenylate cyclase activating polypeptide

(A-NL-K)VIP
(Ala2’8-9-19-2425-27, N1e17, Lys28) VIP

K

L2

’P

RPM!

SIT medium

inhibitory constant

N-(N-methyl-amino-ethyl)-glycine carbamate

inositol phosphate

Roswell Park Memorial Institute

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MTT

VPAC1-R

VPAC2-R

PAC1-R

References

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RPMI-1640 containing 3 x 10 M sodium selenite, 5 Vg/ml bovine insulin and
10Vg/ml transferrin

[3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]

VIP-1 receptor

VIP-2 receptor

PACAP receptor

1. Arimura A. Pituitary adenylate cyclase activating polypeptide (PACAP): Discovery and current status ofresearch. Regul Peptides 1992;37:287-303.
2. Buchholz S, Keller G, Schaily AV, Halmos G, Hohia F, Heinrich E, Koester F, Baker B, Engel JB. Therapy ofovarian cancers with targeted cytotoxic analogs ofbombesin, somatostatin, and luteinizing hormone-releasing hormone and their combinations. Proc Nat! Aced Sci USA 2006;103:10403-7. [PubMed: 16801542]
3. Casibang MC, Purdom 5, Zia H, Jakowlew 5, Neckers L, Ben-Av P, Hla T, You L, Jablons D, Moody TW. Prostaglandin E2 and VIP increase VEGF mRNAs in lung cancer cells. Lung Cancer 2001;31:203-212. [PubMed: 111653991
4. Cha SW, Gu HK, Lee KP, Lee MH, Han SS, Jeong TC. Immunotoxicity ofethylcarbamate in female BALB/C mice: Role of esterase and cytochrome P450. Toxicol Left 2000;115:173-181. [PubMed: 10814887]
5. Cheng Y, PrusoffWH. Relationship between the inhibition constant (1(i) and the concentration of inhibitor which causes 50 percent inhibition (IC50) of an enzymatic reaction. Biochem Pharmacoi 1973;22:3099-3108. [PubMed: 4202581]
6. deGroot FM, Damen EW, Scheeren HW. Anticancer prodrugs for application in monotherapy: targeting hypoxia, tumor-associated enzymes, and receptors. Curr Med Chem 2001;8:1093-1122. [PubMed: 11472243]
7. Dickson, RB.; Pestell, RG.; Lippman, ME. Molecular biology ofbreast cancer. In: DeVita, VT.; Hellman, S.; Rosenberg, SA., editors. Cancer Principles and practice of oncology. Philadelphia: Lippincott, Williams and Wilkins; 2006. p. 1399-1414.
8. Dubowchik GM, Walker MA. Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. Pharmacol Ther 1999;83:67-123. [PubMed: 10511457]
9. Fahrenkrug J. Glycine-extended processing intermediate ofproVIP: A new bioactive form ofVIP in the rat. Biomed Res 1992;13(Sup2):19-23.
10. Fisher B, Constantino JP, Wickerham DL, Cecchini WM, Cronin WM, Robidoux A, Bevers TB, Kavanah MT, AtkinsiN, Margolese RG, Runowicz CS, James JM, Ford LG, WolmarkN. Tamoxifen forthe prevention ofbreast cancer. Currentstatus ofthe National Surgical AdjuvantBreast and Bowel Project P-I Study. JNati Cancer Inst 2005;97:1652-1662. [PubMed: 16288118]
11. Fuselier JA, Sun L, Woitering SN, Murphy WA, Vasilevich N, Coy DH. An adjustable release rate linking strategy for cytotoxin-peptide conjugates. Bioorg Med Chem Left 2003;13:799-803. [PubMed: 12617894]
12. Garcia-Fernandez MO, Bodega G, Ruiz-Villaespesa A, Cortes J, Prieto JC, Carmena MJ. PACAP expression and distribution in human breast cancer and healthy tissue. Cancer Let 2004;205:189-
195. [PubMed: 15036651]
13. Garcia-Fernandez MO, Collado B, Bodega G, Cortes J, Ruiz-Villaespesa A, Carmena MJ, Prieto JC. Pituitary adenylate cyclase-activating peptide/vasoactive intestinal peptide receptors in human

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normal mammary gland and breast cancer tissue. Gynecol Endocrinol 2005;20:327-333. [PubMed: 16019382]
14. Gelber E, Granoth R, Fridkin M, Dreznik Z, Brenneman DE, Moody TW, Gozes I. A lipophilic vasoactive intestinal analog enhances the antiproliferative effect of chemotherapeutic agents on cancer cell lines. Cancer 2001;92:2172-2180. [PubMed: 11596035]
15. Gespach C, Bawab W, Decremox P, Calvo F. Pharmacology, molecular identification and functional characteristics ofvasoactive intestinal peptide receptors in human breast cancer cells. Cancer Res 1988;48:5079-5083. [PubMed: 2842044]
16. Gozes I, Davidson A, Draoui M, Moody TW. The VIP gene is expressed in non small cell lung cancer cell lines. Biomed Res 1993;13:37-40.
17. Gozes 1, McCune SL, Jacobson L, Warren D, Moody T, Fridkin M, Brenneman DJ. A hybrid antagonist to vasoactive intestinal peptide. Effects on cellular function in the central nervous system.
J Pharmacol Exp Ther 1991;257:959-966. [PubMed: 1646331]
18. flarmar T, Lutz EM. Multiple receptors for PACAP and VIP. Trends in Pharmacological Sciences 1993;15:97-9. [PubMed: 79124621

19. Igarishi H, Ito T, Hou W, Mantey SA, Pradhan TK, Ulrich HC, Hocart SJ, Coy DH, Jensen RT. Elucidation of vasoactive intestinal peptide pharmacophore for VPAC1 receptors in human, rat and guinea pig. J Pharmacol Expt Ther 2002;301:37-50.
20. Igarashi I-I, Ito T, Mantey SA, Pradhan TK, Hou W, Coy DH, Jensen RT. Development ofsimplified vasoactive intestinal peptide analogs with receptor selectivity and stability for human vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide receptors. J Pharmacol Exp Ther 2005;315:370-381. [PubMed: 15994369]
21. Igarashi H, Ito T, Pradhan TK, Mantey SA, Hou W, Coy DH, Jensen RT. Elucidation ofthe vasoactive intestinal peptide pharmacophore for VPAC2 receptors in human and rat, and comparison to the pharmacophore for VPAC1 receptors. J Pharmacol Exp Ther 2002;302:445-460. [PubMed: 12388623]

22. Ishihara R, Shigemoto R, Mori K, Takahashi K, Nagata S. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. Neuron 1992;8:811-9. [PubMed: 13146251
23. Laburthe M, Couvineau A, Marie JC. VPAC receptors for VIP and PACAP. Receptors Channels 2002;8:137-153. [PubMed: 12529932]
24. Mahmoud 5, Staley J, TaylorJ, Bogden A, Moreau JP, Coy D, Avis I, Cuttitta F, Mulshine JL, Moody
TW. (psi 13’14)bombesin analogues inhibit the growth ofsmall cell lung cancer in vitro and in vivo.
Cancer Res 1991;51:1298-1302.
25. Moody TW, Chan D, Fahreenkrug J, Jensen RT. Neuropeptides as autocnine growth factors in cancer cells. Currant Pharmaceutical Design 2003;9:495-509. [PubMed: 125708131
26. Moody TW, Czerwinski G, Tarasova NI, Michejda CJ. VIP-ellipticine derivatives inhibit the growth ofbreast cancer cells. Life Sci 2002;71:1005-1014. [PubMed: 120887601

27. Moody TW, Fuselier J, Coy DH, Mantey S, Pradhan T, Nakagawa T, Jensen RT. Camptothecin-somatostatin conjugates inhibit the growth ofsmall cell lung cancer cells. Peptides 205(26):1560-6.
28. Moody TW, Hill JM, Jensen RT. VIP as atrophic factor in the CNS and cancer cells. Peptides 2003;24:163-177. [PubMed: 12576099]
29. Moody TW, Jensen RT. Breast cancer VPAC1 receptors. Ann NY Acad Sci 2006;1070:636-9.
30. Moody, TW.; Jensen, RT. VIP and PACAP as autocrine growth factors in breast and lung cancer. In: Kastin, A., editor. Handbook ofBiologically Active Peptides. London: Elsevier Science; 2006. p.
493-8.

31. Moody TW, Leyton J, Chan D, Brenneman DE, Fridkin M, Gelber E, Levy A, Gozes I. VIP receptor antagonists and chemotherapeutic drugs inhibit the growth ofbreast cancer cells. Breast Cancer Research and Treatment 2001;68:55-64. [PubMed: 11678309]
32. Moody TW, Sun LC, Mantey SA, Pradhan T, Mackey LV, Gonzales N, FuselierJA, Coy DH, Jensen RT. In vitro and in vivo antitumor effects of cytotoxic camptothecin-bombesin conjugates are mediated by specific interaction with cellular bombesin receptors. J Pharmacol Exp Ther 2006;318:1265-1272. [PubMed: 16766720]

Peptides. Author manuscript; available in PMC 2009 September 11.

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314

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33. Moody TW, Mantey SA, Pradhan TK, Schumann M, Nakagawa T, Martinez A, Fuselier J, Coy DH,
Jensen RT. Development ofhigh affinity camptothecin-bombesin conjugates which have targeted
cytotoxicity for bombesin receptor-containing cells. J Biol Chem 2004;279:23580-9. [PubMed:
Z 15016826]

34. Moody TW, Zia F, Draoui M, Brenneman DE, Fridkin M, Davidson A, Gozes I. A novel VIP
antagonist inhibits non-small cell lung cancer growth. Proc Nati Acad Sci USA 1993;90:4345-9. [PubMed: 8389448]
35. Nagy A, Schally AV. Targeting cytotoxic conjugates ofsomatostatin, luteinizing hormone-releasing hormone and bombesin to cancers expressing theirreceptors: a "smarter" chemotherapy. Curr Pharm Des 2005;11: 1167-1180. [PubMed: 158536641
36. Nicole P, Lins L, Rouyer-Fessard C, Bronuot C, Fulcrand P, Thomas A, Couvineau A, Martinez J, Brasseur R, Laburthe M. Identification ofkey residues for interaction ofvasoactive intestinal peptide with human VPAC1 and VPAC2 receptors and development ofhighly selective VPAC1 receptor agonist Alanine scanning and molecular modeling ofthe peptide. J Biel Chem 2000;275:24003-24012. [PubMed: 10801840]
37. Patterson LH, McKeown SR, Robson T, Gallagher R, Raleigh SM, On S. Antitumor prodrug development using cytochrome P450 (CYP) mediated activation. AnticancerDrug Des 1999;14:473-
4. [PubMed: 108342691

’:4

38. Pisegna JR, Wank SA. Multiple cloning and functional expression ofthe pituitary adenylate cyclase activating polypeptide type I receptor. Proc NatI Acad Sci USA 1993;90:6345-9. [PubMed: 8392197]
39. Reubi JC. In vitro identification ofvasoactive intestinal peptide receptors in human tumors: Implications for tumor imaging. J NucI Med 1995;36:1846-1853. [PubMed: 7562054]
40. ReubiJC, Laderach U, Waser B, GebbersJD, Robberecht P, Laissue JA. Vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptor subtypes in human tumors and their tisssues of origin. Cancer Res 2000;60:3105-3112. [PubMed: 10850463]
41. Said SI, Mutt V. Polypeptide with broad biological activity: Isolation from the small intestine. Science 1970;69:1217-8. [PubMed: 5450698]
42. Spengler D, Waeber C, Pantaloni C, flolsboer F, Bockaert J, Seeberg PH, Journot L. Differential signal transduction by five splice variants ofthe PACAP receptor. Nature 1993;365:170-5. [PubMed: 8396727]
43. Sun L, Fuselier JA, Coy DH. Effects of camptothecin conjugated to a somatostatin analog vector on growth oftumor cell lines in culture and related tumors in rodents. Drug Deliv 2004;l 1:231-8. [PubMed: 15371104]
44. Tripathy D, Slamon DJ, Cobleigh M, Arnold A, Saleh M, MortimerlE, Murphy M, Stewart SJ. Safety oftreatment ofmetastatic breast cancer with trastuzumab beyond disease progression. J Clin Oncol 2004;22:1063-1070. [PubMed: 150206071
45. WaschekiA, Richards MI, Bravo DT. Differential expression ofVIP/PACAP receptor genes in breast, intestinal and pancreatic cell lines. Cancer Res 1995;92:143-9.
46. Whitmarsh AJ, Davies ItT. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 1996;74:589-07. [PubMed: 89121801
47. Wood, WD.; Muss, HB.; Solin, LI.; Olopade, 0. Malignant tumors ofthe breast. In: DeVita, VT.; Hellman, S.; Rosenberg, SA., editors. Cancer: Principles and practice of oncology. Philadelphia: Lippincott, Williams and Wilkins; 2006. p. 1415-1477.
48. Zia H, Hida T, Jakowlew 5, Birrer M, Gozes Y, Reubi JC, Fridkin M, Gozes I, Moody TW. Breast cancer growth is inhibited by VlPhybrid, a synthetic VIP receptor antagonist Cancer Res 1996;56:3486-9. [PubMed: 8758916]

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Figure 1.

VIP-CPT conjugate binding. (Top) The ability of(A-NL-K)VIP (.), (A-NL-K)VIP-L2-CPT (.) and (A-NL-K)VIP-L2 (4) to inhibit specific 1251-VIP binding to T47D and 3T3 cells containing VPAC1-R was determined. (Bottom) The ability of(A-NL-K)VIP (.), (A-NL-K) VIP-L2-CPT (.) and (A-NL-K)VIP-L2 ()to inhibit specific 12511-Ro25-1 553 binding to SupTi and 3T3 cells containing VPAC2-R was determined. The mean value – S.D. of 3 determinations is indicated. This experiment is representative of4 others

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Figure 2.

MiTT assay. The ability of varying doses of(A-NL-K)VIP-L2-CPT (.) and (A-NL-K)VIP ()to inhibit MCF7 proliferation was determined. The mean value – S.D. of 8 determinations
is indicated; p <0.05; * using Student’s t-test. This experiment is representative of 2 others.

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Figure 3.

Clonogenic assay. The ability of varying doses of(A-NL-K) VIP-L2-CPT (.) and (A-NL-K) VIP (.)to inhibit MCF7 proliferation was determined. The mean value – S.D. of 3

determinations is indicated; p <0.01, **; p <0.05; * using Student’s t-test. This experiment is representative of 3 others.

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Figure 4.

Effect of(A-NL-K) VIP-L2-CPT on MCF7 cells. The ability of0 (A), 30 nM (B), 100 nM (C) and 300 nM (D) (A-NL-K) VIP-L2-CPT to inhibit MCF7 proliferation was determined. This experiment is representative of 3 others.

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Table 2
Ability of VIP-CPT conjugates to elevate cAMP in MCF7 cells.

Addition cAMP, fmol

None 7.0 2.0
VIP, 10 nM 52.2 –6.0
(A-NL-K)VIP, 1000 nM 70.5 7.2
(A-NL-K)VIP-L2, 1000 nM 58.3 4.4
(A-NL-K)VIP-L2-CPT, 1000 nM 60.8 7.3
Forskolin, 50 uM 76.3 3.1

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The agents were added to MCF7 cellsfor 5mm at 37°C. The mean value – S.D. of 4 determinations each repeated in duplicate isindicated. This experiment is representative of 2 others

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Abstract

The major problems of traditional chemotherapy are non-selectivity and non-specificity,

resulting in severe toxic side effects. Peptides are a new-generation of drug-delivery vector to

increase efficacy of this therapy and avoid the resulting damage. The cytotoxic somatostatin

(SST) conjugate JF-10-81 was developed by coupling camptothecin (CPT) to the N-terminus of

a SST analog (JF-07-69) using an activated carbamate linker. This conjugate selectively targets

somatostatin receptor subtype 2 (SSTR2) and also retains high binding affinity and rapid

internalization as well as anti-proliferative activity towards various tumor cells. JF-10-81 was

tested for its inhibitory activity against the growth of human tumors which included

neuroblastoma (1MR32), pancreatic cancer (CFPAC-1), leukemia (MOLT-4), pancreatic

carcinoid (BUN) and prostate cancer (PC-3). Both SSTR2 mRNAs and proteins were detected in

all these tumor cell lines. The conjugate displayed potent in vivo inhibitory activity, although

some of the potency measured in in vitro experiments was lost. JF- 10-81 was found to

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significantly inhibit growth ofthese SSTR-positive tumors, resulting in 87% tumor reduction in

neuroblastoma 1MR32 and 97% in leukemia MOLT-4 bearing animals, even inducing regression

of CFPAC-1 tumors. SSTR-over-expressing BON tumors were unfortunately relatively CPT-

insensitive in vitro, however, JF-10-81 again exhibited in vivo potency presumably by

specifically increasing CPT concentrations inside the tumor cells so that the inhibition rate for

JF-10-81 was 85%. Also, JF-10-81 was used to treat highly invasive PC-3 tumors where s.c.

injections inhibited both tumor growth (almost 60% reduction) and tumor metastasis (over 70%).

This conjugate demonstrated its broad and excellent anti-tumor activity by targeting SSTR2-

specific tumor tissues, supporting that short peptides and their analogs may be applied as ideal

drug-delivery carriers to improve the traditional chemotherapy.

Keywords: Somatostatin conjugate, somatostatin receptor, camptothecin, tumor growth,

metastasis.

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cells and non-specificity certainly rank among the likely potential causes of severe toxic side

effects. Also, the instability and/or insolubility of cytotoxic agents in aqueous media limit their

clinical application. Possible routes to overcome these problems and improve their therapeutic

properties are numerous and include various new drug delivery systems such as polymer [1,2],

nano-particles [3-6], antibody conjugates [7,8], and short peptide hormone conjugates [9-13].

Specific receptors for many ofthese peptides, the most investigated being somatostatin (SST)

and bombesin (BM), have been found to be expressed aberrantly in various tumors [14]. Taking

advantage of these observations, receptor-targeted cytotoxic peptide conjugates can be used to

target receptor-specific tumor tissues, increase efficacy of chemotherapeutic agents and decrease

severe toxic side effects as long as suitable cleavable linker groups can be synthesized. Hence,

several peptide conjugate candidates have been developed during recent years and short, easily

synthesized peptides [13] were used as vectors to couple with chemotherapeutic agents and

carry them to target receptor-specific tumor cells. In our laboratories, camptothecin (CPT), a

potent anti-tumor agent [15,16], has been successfully coupled to urotensin, BM, and SST

analogs. These new cytotoxic conjugates displayed relatively low toxic side effects and

significant inhibition to tumor cell proliferation and tumor growth [11, 12,13,17]. Also, cytotoxic

conjugates of SST [18], BM [19] and luteinizing hormone-releasing hormone (LI-IRH) [20]

coupled with 2-pyrrolinodoxorubicin, have been synthesized and investigated by others. These

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conjugates showed broad and excellent anti-tumor activities [18-21]. Peptide hormones as drug-

delivering vectors have their own specific advantages over other approaches such as rapid

internalization (via their respective GPCRs), better tumor-penetrating ability and quick

circulatory clearance [14].

SSTRs, especially SSTR2, have been found to be highly expressed in various tumors

[14,22,23] and tumoral blood vessels [24]. The two naturally occurring compounds, SST-14 and

SST-28, bind with high affinity to all five receptor subtypes whereas certain clinically approved

SST analogs only selectively bind to SSTR2 while being much more stable in the circulation.

Such analogs are attractive starting points for use as vectors to precisely deliver non-selective

cytotoxic agents to receptor-selective target sites. For instance, CPT coupled to a SST analog

selectively bound to SSTR2 and was designed to retain high binding affinity even with the

attachment of large drug molecules. This CPT-SSA conjugate JF-10-81 has already shown

favorable anti-invasive and anti-angiogenic activities [25]. In the current study, we further extend

these studies to investigate the ability of JF- 10-81 to prevent the growth of several important

tumor types by sustained delivery formulations and metastasis of the highly invasive prostate

cancer PC-3 tumor by periodic injections.

Materials and Methods

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Materials

The cytotoxic CPT-SST conjugate JF- 10-81 was synthesized as described previously [9].

Continuous drug-releasing pellets containing IF- 10-81 (5 mg/pellet) formulated to release drug

for 60 days, were made by Innovative Research ofAmerica (Sarasota, FL).

Cell culture

The CHO-RI and CHO-R2 cell lines, which were transfected with rat SSTR1 and SSTR2,

respectively, were a kind gift from Dr. Agnes Schonbrunn (University of Texas - Houston,

Houston, TX). Cells were cultured in F-12 medium containing 10% fetal calf serum. All other

human tumor cell lines, including neuroblastoma 1MR32, leukemia MOLT-4, pancreatic cancer

CFPAC-1, prostate cancer PC-3 were purchased from ATCC (American Type Culture Collection,

Manassas, VA). Human pancreatic carcinoid BON cells were a kind gift from Dr. Courtney

Townsend (University ofTexas Galveston) and cultured as described previously [9,26].

Ligand-receptor binding and internalization assay

The assay was performed as a modification of the one previously described [27,28]. The

binding buffer contained Dulbecco’s Modified Eagle’s Medium (DMEM), 50 mM }{EPES, 2

mM glutamine, 11.5 mM glucose, 1% bovine serum albumin (BSA) and 5 mM sodium pyruvate.

The binding reactions took place in 1.5 ml eppendorftubes containing 70-100 pM of [1251]Tyr’1 -

SST-14 from PerkinElmer (Boston, MA 02118-2512), 1-1.5x105 cells and the tested SST

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analogs (10-5to 1 0’3M) for a total volume of 300 i These were incubated at room temperature for 50-60 minutes and centrifuged for 1-2 min at 9,000 g. The cell pellets were washed twice with phosphate-buffered saline (PBS) and counted for total radioactivity in a Titertek gamma counter (Huntsville, AL). The analyses were done using GraphPad Prism 4.0 (San Diego, LA). For further internalization [29], cells were then re-suspended and incubated in HBSS (pH4, acetic acid) on ice for 10 mm, washed with cold PBS and then re-counted for radioactivity. Due to the radioactivity ofmembrane-bound receptors being released by the acid wash, the remaining radioactivity represents the internalized peptide.
RT-PCR

RT-PCR reagents were purchased from Invitrogen (Carlsbad, CA). Total RNA from the 5 different tumor cells described above was isolated. The RT-PCR amplification ofthe mRNAs of all 5 SSTR subtypes was performed under the same conditions as described in the reference[30] with the following primers: SSTR1 -F: 5 ’-AAATGCGTCCCAGAACGGGACCT-3’, SSTR1 -R: 5 ’-CAGGTTCTCAGGTT’GGAAGTCTT-3’; SSTR2-F: 5 ’-GATGATCACCATGGCTGTG-3’, SSTR2-R: 5 ’-CAGGCATGATCCCTCTTC-3’; SSTR3-F: 5 ’-TCATCTGCCTCTGCTACCTG-3’, SSTR3-R: 5’-GAGCCCAAAGAAGGCAGGCT-3’; SSTR4-F: 5’-CATGGTCGCTATC-CAGTGCA-3’, SSTR4-R: 5 ’-GTGAGACAGAAGACGCTGGTGAACAT-3’; SSTR5-F: 5’- GCTCTTGGTGTTCGCGGACGT-3’, SSTR5-R: 5’-CAGGTFGACGATGTFGACGGTGAAG-8

3’ [30]. The predicted RT-PCR product sizes are 993bp to SSTR1, 892bp to SSTR2, 221bp to

SSTR3, 276bp to SSTR4 and 298bp to SSTR5, respectively [30]. Experiments were done in

triplicate.

Western blot

The protocol was employed as described (Santa Cruz, CA). Briefly, cells were harvested,

resuspended in RIPA buffer with cocktail inhibitors, homogenized by passing through a 21

gauge needle, mixed with loading buffer containing fresh DTT and heated for 5 min at 95°C.

Supernatants were loaded to run on 8-16% Tris-glycine gel after centrifugation at 10,000g.

Protein was transferred from gel to nitrocellulose membrane which was then blocked with 5%

fat-free milk, washed and incubated with SSTR2 antibody (Santa Cruz). The membrane was

washed again and incubated with second antibody (Santa Cruz). Eventually, films were

developed according to the ECL system protocol (Amersham Biosciences, England).

Cellproliferation assay

The kits for the cell proliferation assay (MTT Assay) were purchased from Promega

(Madison, WI) and the protocol was the same as described previously [26].

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In vivo experiments

Male nude mice, 5-7 weeks of age upon arrival, were purchased from the National Cancer

Institute (NCI, Frederik, MD). The animal study was approved by the Tulane University Health

Sciences Center Institutional Animal Care and Use Committee (IACUC).

Tumor growth: The tumor tissue segments, grown from CFPAC-1, 1M1R32, MOLT-4 and

BON cells injected into nude mice, were s.c. implanted into the flanks of nude mice as described

previously [9]. All the tumors were passaged from mouse to mouse. Tumor-carrying mice then

were implanted with continuous drug-releasing pellets containing 5 mg ofJF- 10-81.

For treatments of pancreatic cancer CFPAC- 1 tumors, mice were separated into 2 groups.

Each mouse in the control group was implanted with a placebo pellet. Each mouse in the test

group was implanted with a pellet containing iF-i 0-81 (5 mg/pellet) with a drug-release time of

60 days. Tumor volumes were measured and bodyweights were taken once a week. The

treatment for all other tumors (BON, 1MR32 and MOLT-4) was the same as above.

Tumor metastasis: The experiment was performed as described [31] with modification.

Human prostate cancer PC-3 cells were re-suspended in a PBS solution and further mixed with

matrigel at a ratio of 1:3. The mixtures were implanted via i.p. injection at a volume of 200 ul

(lx106cell/ml). One week later, mice were divided into 3 groups of 8-9 mice each. The control

group was treated with PBS. The test groups were treated with the cytotoxic conjugate JF-10-81

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(3.5mg/kg) by i.p. or s.c. injection, 3 times a week, for a total of 12 injections. Mice were

monitored and checked each week. Eventually, mice were killed by over-anaesthetization and

autopsies were performed and tumor metastasis investigated.

Results

High binding affinity ofthe CPT-SST conjugate to SSTR subtype 2

Both native SST-14 (DC-44-76) acting as the control peptide and the CPT-SST conjugate

JF-10-81 were tested. As expected in CHO-RI and CHO-112 cells, synthetic SST-14 exhibited

high affinity for SSTR1 (EC50: 1.85 nM) and SSTR2 (1.45 nM) (Fig.1), no affinity to the non-

transfected CHO cells themselves. The vector JF-07-69 (2.35 nM) and its cytotoxic conjugate

JF-10-81 (2.46 nM) displayed similar high affinity binding to SSTR2. However, both of them

had relatively weak binding affinity to SSTR1, the EC50 values being 100 fold less than for

SSTR2 (Fig. 1). The further internalization assay showed that the conjugate JF-1 0-81 could

quickly internalize into the SSTR2-specific cells (data not shown). Therefore, this conjugate

could potentially be applied for receptor-targeted chemotherapy.

Expression ofSSTR2 in cultured tumor cells

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It has been demonstrated that SSTRs, especially SSTR2, are aberrantly expressed in many

tumors [14]. Herein, the expression of mRNAs of all 5 SSTRs and protein of SSTR2 in 5 tested

tumor cells were investigated by RT-PCR and western blot.

As shown in Fig.2, the results of RT-PCR demonstrated that SSTR2 and SSTR5 mRNAs

existed abundantly in all 5 tumor cell lines, except for a lack of SSTR5 in MOLT-4 cells. SSTR1

was highly expressed in BON and CFPAC-1 cells. CFPAC-1 cells showed the existence of

mRNAs of all 5 SSTR subtypes, with traces of one or more subtypes detectable in the other cells

(Fig.2). Further, SSTR2 proteins (’64 kD) were detected in all 5 tumor cells by western blot

(Fig.3). The expression of SSTR2 in all tested tumor cells was identical to previous reports that

SSTR2 is abberantly expressed in these tumor tissues, thus confirming that SSTR2 could be used

as a specific target for JF-10-81 to treat these receptor-positive tumors.

Although not easily detected in many in vitro cultured tumor cells, binding affinities in 5

tumor cells were further investigated with SST-14 which has high affinity to all 5 SSTR subtypes.

We found that BON and 1MR32 cells have higher binding affinity, with other 3 tumor cells

showing weak binding to SST-14. The EC50 values were 1.45 nM to BON cells, 28.38 nM to

1MR32, 195.36 nM to MOLT-4, 782.35 nM to CFPAC-1 and over 1000 nM to PC-3,

respectively.

Inhibition ofthe CPT-SSA conjugate to in vitro tumor cellproliferation

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The CPT-SSA conjugate (JF-10-81) has been shown to have anti-angiogenic activity via a

series ofin vitro and in vivo experiments [25]. In the present study, it was tested for its inhibitory

activity against five chosen SSTR-abundant human tumor cell lines, including CFPAC-1, 1MR32,

MOLT-4, BON and PC-3 by using the MTF assay. The EC50 values found were: 2.8 nM (CPT)

and 46.1 nM (JF-10-81) in 1MR32 cells, 2.0 nM (CPT) and 27.6 nM (JF-10-81) in MOLT-4 cells,

25.5 nM (CPT) and 196.8 nM (JF-10-81) in CFPAC-1 cells, 170.8 nM (CPT) and 1233.4 nM

(JF-10-81) in BON cells, 20.8 nM (CPT) and 453.6 nM (JF-10-81) in PC-3 cells (Fig.4 and

Table 1). All the tumor cells were relatively CPT-sensitive compared to CPT-insensitive BON

cells. Also, JF- 10-81 exhibited the same trends as CPT itself. However, generally the conjugate

partly lost its cytotoxic activity, with its EC50 being almost 10-20 fold less when compared to

CPT itself. As expected, the vector peptide JF-07-69 did not work even at high concentrations

(Fig.4 and Table 1).

Inhibition ofthe CPT-SSA conjugate to tumor growth and metastasis

JF- 10-81 was further evaluated for its ability to inhibit tumor growth and metastasis in

these SSTR2-positive tumor models mentioned above. PC-3 tumors were treated with single

injections ofthe conjugate. The others were implanted with continuous drug-releasing pellets (5

mg/pellet). As shown in Table 2 and Figs. 5 and 6, JF-10-81 (5 mg/pellet) displayed dramatic

inhibitory activity in all tested tumors.

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In the experiment using the pancreatic cancer (CFPAC- 1) tumor cells and a treatment of 5

mg/pellet/mouse, tumor volume in the control group increased from 168 – 52 mm3 to 2196 – 832

mm (up 1207%) after 4 weeks. Tumor volume in JF-10-81-treated group (5 mg/pellet)

significantly decreased from 197 – 68 mm3 to 44 – 14 mm (down 78%) after 4 weeks, the

inhibition rate is -92%. Tumor volumes decreased further to 8 – 8 mm (down 96%) 7 weeks

later (Fig.5 and Table 2), with tumor disappearance in 4 out of 9 mice. Tumors were regressed

with JF-10-81 treatment.

Inhibitory activity was also demonstrated in JF- 10-81 -treated neuroblastoma (1MR32)

tumors. After 6 weeks, tumor volume sharply increased from 758 – 292 mm to 9098 – 4929

mm (up 1100%) in the control group, but only from 763 – 218 mm3 to 1854 – 816 mm3 (up

143%) in the JF-10-8 1 -treated group (5 mg/pellet). The inhibitory rate was 87% (Table 2).

In the treatment ofleukemia (MOLT-4) tumors, after 5 weeks tumor volume went from 393

– 142 mm3 to 5615 – 3143 mm3 (up 1328%) in the control group, but just 270 – 89 mm3 to 430 –

330 mm (up 59%) in the conjugate-treated group (5 mg/pellet). The inhibitory rate was 97%

(Table 2).

Notably, in the SSTR-over-expressing, but CPT-resistant pancreatic BON tumors, iF- 10-81

(5 mg/pellet) appeared to display a much more potent inhibitory activity than did CPT alone (5

mg/pellet). The percentage increase of tumor volumes was 755% in the control group, 71% in

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JF- 10-81 -treated group (5 mg/pellet) (Table 2) and 278% in the CPT-treated group (5 mg/pellet)

(data not shown). The inhibitory rates for JF-10-81 and CPT treatment are 85% and 42%,

respectively.

On average, iF- 10-81 clearly displayed potent anti-tumor activity (depending on cell type).

This could perhaps be because this conjugate concentrated CPT inside tumor cells by targeting

receptors on cell surfaces, although favorable changes in the metabolism and clearance of

biologically active, conjugated CPT cannot be ruled out. Even at a lower dose (1 mg/pellet), iF-

10-81 was efficacious against pancreatic cancer CFPAC- 1 tumor growth with the inhibitory rate

reaching 56%. However, JF-10-81 at the same dose (1mg/pellet) did not work well in 1MR32,

MOLT-4 and BON tumor treatments (data not shown). These results also indicate that the

inhibitory ability ofJF-10-81 was dose-dependent.

The highly invasive PC-3 cells were chosen for anti-tumor metastasis experiments. PC-3

tumors in the control groups were found to metastasize to the gut, liver, chest, skin etc. of nude

mice. However, in treated groups, we found that there was an obvious decrease in overall tumor

metastasis, with no metastasis taking place in some mice. JF-10-81 at 3.5mg/kg blocked almost

73% oftumor metastasis using s.c. injections and 60% by i.p. injections (Fig.6). Meanwhile, IF-

10-81 was observed to result in an almost 60% reduction of primary tumor weights using s.c.

administration and 40% by i.p. injections.

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Discussion

Peptide-based receptor-targeted chemotherapy is one of a new generation of promising

approaches for increasing the specificity of cancer-cytotoxin treatments. Peptides might be

optimal drug-delivery vectors due to their unique advantages which have been discussed [14].

Certain peptides such as SST, BN, LHRH or their analogs have been used to deliver cytotoxic

compounds by taking advantage of the peptide receptors being aberrantly expressed and

sometimes over-expressed in many human tumors and in the blood vessels around tumors

[13,14,22,23,24,32]. Furthermore, the structure-activity relationships of these peptides are well

investigated and allow for attachment of large drug molecules using a large variety of cleavable

linker chemistries with little or no loss ofreceptor affinity and retention ofsignificant anti-tumor

activities [9,10,20,21]. For example, AN-238, which was made by linking 2-pyrrolino-DOX to

the SST analog RC-121, exhibited potent anti-tumor activity by targeting tumor cells [33,34] or

tumoral blood vessels [10]. In our previous studies, cytotoxic conjugates of SST and BN analogs

have revealed some utility against tumor growth and angiogenesis [9,25,35]. The CPT-SST

conjugate JF- 10-81 was found to be stable and readily water-soluble [9,25,36] and is predicted to

display less systemic toxicity than the naked cytotoxic agent [36]. Also, IF- 10-81 inhibited

growth of SSTR-expressing tumors [9,11] and blocked development of angiogenesis [25]. In

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addition, a number of other peptide conjugates have been shown to have more specificity to

tumor cells with less toxicity to normal cells [12,13,37,3 8].

In the present work, our most widely studied conjugate (JF- 10-81) was further tested for its

potentially broad anti-tumor activity using human tumors ranging through neuroblastoma,,

pancreatic cancer, pancreatic carcinoid, leukemia and prostate cancer. With all these tumors, as

expected, RT-PCR and western blot revealed the presence of SSTR2. The internalization assay

demonstrated that this SSTR2-specific JF-1 0-81 could quickly be taken up in SSTR2-transfected

cells. In our experience, a frequent low dose was found to work better than a less frequent high

dose. In a previous study, JF-1 0-81 did not affect human prostate cancer PC-3 tumor growth

using a less frequent low dose protocol (0.73 mg/kg, twice a week) [9]. However, current results

indicate that JF-10-81 obviously inhibited PC-3 tumor growth at the more frequent higher dose

(3.5 mg/kg, 3 times a week), resulting in an almost 60 % decrease in volume (Fig.5). Taking

these observations into account and the expectation that the targeted conjugate would have an

improved therapeutic/toxicity ratio, an approach using a continuous drug-releasing pellet of 60

days was implemented. The results show that this new continuous drug-releasing approach

results in significantly improved tumor responses and with no evidence of animal body weight

losses. JF- 10-81 resulted in an over 85% reduction in tumors of neuroblastoma 1MR32 and

leukemia MOLT-4 with solid tumor regression and even disappearance of some CFPAC- 1

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tumors occurring. As for S STR-overexpressing, but CPT-insensitive BON tumors, JF- 10-81

exhibited much more potent inhibitory effects with over double the inhibitory rate seen with the

CPT formulation perhaps by increasing the CPT concentration within the vascularized solid

tumor or possibly inside the tumor cells themselves (data not shown). And previously IF- 10-81

also displayed anti-angiogenic activity in vitro [25], and now additionally showed its ability to

inhibit the metastasis of the highly invasive PC-3 tumors (Fig.5). Therefore, this conjugate IF-

10-81 with optimal drug-releasing approach could be a new potential chemotherapeutics.

Reportedly, the cultured tumor cells 1MR32 and CFPAC-1 highly express some SSTRs

[33,39,40], with BON cells natively expressing high levels of SSTR1, SSTR2 and SSTR5

[41,42]. Our results are similar to the previous reports. Abundant SSTR2 mRNAs were detected

in all 5 cultured tumor cells and SSTR2 proteins were detected as well. However, except BON

and 1M1R32 cells, we observed little or very weak binding affinities oftotal 5 SSTRs in CFPAC-

1, MOLT-4, PC-3 and many other tumor cells. Somatostatin binding has been demonstrated in

tumor tissues taken from various primary tumors including neuroblastoma, pancreatic cancer,

carcinoid and leukemia and prostate cancer [14]. Part of this problem may lie in the over

passaging of commercially available cell lines and we have observed that native tumor cells and

even receptor-transfected cells with initially high binding affinity can gradually lose their

receptor density and binding specificity and rise non-specific binding after multiple passages.

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There is the possibility that cultured cells lose their natural in vivo micro-environments that can

stimulate expression of SSTRs and their functional appearance on cell surfaces. This and

additional interactions with other cells or growth factors could be part of several possible

explanations for why JF-10-81 had greater in vivo but less in vitro effects than naked CPT.

Additionally, in animals the conjugate would have the ability to target and kill SSTR-positive

tumor blood vessels thus providing an additional mechanism for inhibition oftumor growth [10].

In conclusion, this CPT-SST conjugate has been shown to effectively inhibit angiogenesis

and the progression of various SSTR-positive tumors, with low toxicity. Apparently, through

targeting receptor-specific tumor sites, this kind of novel cytotoxic peptide conjugates can

increase cancer cell selectivity and cytotoxicity and reduce toxic side effects relative to the

traditional chemotherapy.

Acknowledgement

The authors thank Dr. Agnes Schonbrunn for her gift of SSTR-transfecting cell lines and

Dr. Courtney Townsend for his gift ofBON tumor cells.

References

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1 Huh KM, Min HS, Lee SC, Lee HG, Kim 5, Park K. A new hydrotropic block copolymer
micelle system for aqueous solubilization of paclitaxel. Journal ofControlled Release 2008; 126:122-9.

2 Bencini M, Ranucci E, Ferruti P, Trotta F, Donalisio M, Comaglia M, et al. Preparation and
in vitro evaluation of the antiviral activity of the Acyclovir complex of a beta-cyclodextrinlpoly(amidoamine) copolymer. Journal ofControlledRelease 2008; 126:17-25.
3 Lee LY, Wang CH and Smith KA. Supercritical antisolvent production of biodegradable
micro- and nanoparticles for controlled delivery of paclitaxel. Journal ofControlled Release

2008; 125:96-106.
4 Haley B, Frenkel E. Nanoparticles for drug delivery in cancer treatment. Urol Oncol. 2008;
26:57-64.

5 Wissing SA, Kayser 0, Muller RH. Solid lipid nanoparticles for parenteral drug delivery.
Adv Drug Deliv Rev. 2004; 56: 1257-72.
6 Sengupta 5, Eavarone D, Capila I, Zhao G, Watson N, Kiziltepe T, et al. Temporal targeting
of tumour cells and neovasculature with a nanoscale delivery system. Nature 2005; 436: 568-72.

7 Guillemard V and Saragovi HU. Taxane-Antibody Conjugates Afford Potent Cytotoxicity,
Enhanced Solubility, and Tumor Target Selectivity 1. Cancer Res. 2001; 61:694-9.
20

8 Hsieh FY, Tengstrand E, Lee JW, Li LY, Silverman L, Riordan B, et al. Drug safety

evaluation through biomarker analysisA toxicity study in the cynomolgus monkey using

an antibody-cytotoxic conjugate against ovarian cancer. Toxicol Appi Pharmacol. 2007;

224:12-8.

9 Sun LC, Fuselier JA, Coy DH. Effects of camptothecin conjugated to a somatostatin analog

vector on growth of tumor cell lines in culture and related tumors in rodents. Drug Deliv.

2004; 11: 231-8.

10 Kiaris H, Schally AV, Nagy A, Szepeshazi K, Hebert F, Halmos G. A targeted cytotoxic

somatostatin (SST) analogue, AN-238, inhibits the growth ofH-69 small-cell lung carcinoma

(SCLC) and H-157 non-SCLC in nude mice. EurJCancer 2001; 37: 620-8.

11 Moody TW, Fuselier JA, Coy DH, Mantey S, Pradhan T, Nakagawa T, et al. Camptothecin-

somatostatin conjugates inhibit the growth ofsmall cell lung cancer cells. Peptides 2005; 26:

1560-6.

12 Moody TW, Mantey SA, Pradhan TK, Schumann M, Nakagawa T, Martinez A, et al.

Development of high affinity camptothecin-bombesin conjugates that have targeted

cytotoxicity for bombesin receptor-containing tumor cells. JBiol Chem 2004; 279: 23580-9.

21

13 Sun LC, Coy DH. (2008) Cytotoxic conjugates of peptide hormones for cancer chemotherapy.

Drugs ofthe Future 2008; 33: 217-23.

14 Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. Endocr

Rev 2003; 24:389-427.

15 Kohn KW, Pommier Y. Molecular and biological determinants of the cytotoxic actions of

camptothecins. Perspective for the development of new topoisomerase I inhibitors. Ann N Y

AcadSci 2000; 922:11-26.

16 O’Leary J, Muggia FM. Camptothecins: a review of their development and schedules of

administration. EurJCancer 1998; 34:1500-8.

17 Moody TW, Sun LC, Mantey SA, Pradhan T, Gonzales N, Coy DH, et al. In vitro and in vivo

antitumor effects of cytotoxic camptothecin-bombesin conjugates are mediated by specific

interaction with cellular bombesin receptors. JPharmacol Exp Ther 2006; 318: 1265-72.

18 Engel JB, Schally AV, Halmos G, Baker B, Nagy A, Keller G. Targeted therapy with a

cytotoxic somatostatin analog, AN-238, inhibits growth of human experimental endometrial

carcinomas expressing multidrug resistance protein MDR-1. Cancer 2005; 104: 1312-21.

22

7:Ø

19 Engel JB, Schally AV, Halmos G, Baker B, Nagy A, Keller G. Targeted cytotoxic

bombesin analog AN-215 effectively inhibits experimental human breast cancers with a low

induction ofmulti-drug resistance proteins. Endocr Relat Cancer. 2005; 12: 999-1009.

20 Keller G, Schally AV, Gaiser T, Nagy A, Baker B, Halmos G, et al. Receptors for

luteinizing hormone releasing hormone (LHRH) expressed in human non-Hodgkins

lymphomas can be targeted for therapy with the cytotoxic LI-IRH analogue AN-207. Eur J

Cancer. 2005; 41: 2196-202

23

21 Buchholz S, Keller G, Schally AV, Halmos G, Flohia F, Heinrich E, et al. Therapy of ovarian

cancers with targeted cytotoxic analogs of bombesin, somatostatin, and luteinizing hormone-

releasing hormone and their combinations. PNAS 2006; 103: 10403-7.

22 Fujita T, Yamaji Y, Sato M, Murao K, Takahara J. Gene expression ofsomatostatin receptor

subtypes, SSTR1 and SSTR2, in human lung cancer cell lines. Life Sci 1994; 55:1797-806.

23 Taylor JE, Theveniau MA, Bashirzadeh R, Reisine T, Eden PA. Detection of somatostatin

receptor subtype 2 (SSTR2) in established tumors and tumor cell lines: evidence for SSTR2

heterogeneity. Peptides 1994; 15:1229-36.

24 Denzler B, Reubi JC. Expression of somatostatin receptors in peritumoral veins of human

tumors. Cancer 1999; 85: 188-98.

25 Sun LC, Luo J, Mackey LV, Fuselier JA, Coy DH. A conjugate of camptothecin and a

somatostatin analog against prostate cancer cell invasion via a possible signaling pathway

involving PI3KIAkt, alphaVbeta3!alphaVbeta5 and MMP-2/-9. Cancer Let. 2007; 246: 157-

66.

26 Sun LC, Vasilevich NI, Fuselier JA, Hocart SJ, Coy DH. Examination ofthe 1,4-disubstituted

azetidinone ring system as a template for combretastatin A-4 conformationally restricted

analogue design. Bioorg Med Chem Lett 2004; 14: 2041-6.

24

W5i

27 Pradhan TK, Katsuno T, Taylor JE, Kim SH, Ryan RR, Mantey SA, et al. Identification of a

unique ligand which has high affinity for all four bombesin receptor subtypes. Eur J

Pharinacol 1998; 343: 275-87.

28 Liu C, Chen J, Kuei C, Sutton S, Nepomuceno D, Bonaventure P. et al. Relaxin-3/insulin-like

peptide 5 chimeric peptide, a selective ligand for G protein-coupled receptor (GPCR) 135 and

GPCR142 over leucine-rich repeat-containing G protein-coupled receptor 7. Mol Pharmacol

2005; 67: 231-40.

29 Anthony CT, Hughey S, Lyons J, Weiss S, Hornick CA, Drouant GJ, et al. The effect of drug

dose and drug exposure time on the binding, internalization, and cytotoxicity ofradiolabeled

somatostatin analogs. JSurg Res 2004; 119:1-13.

30 Panetta R, Patel YC. Expression of mRNA for all five human somatostatin receptors

(hSSTR1-5) in pituitary tumors. Life Sciences 1995; 56: 333-42.

31 Kuperwasser C, Dessain S, Bierbaum BE, Garnet D, Sperandio K, Gauvin GP, et al. A Mouse

Model ofHuman Breast Cancer Metastasis to Human Bone. Cancer Res 2005; 65: 6130-8.

32 Watson JC, Balster DA, Gebhardt MB. Growing vascular endothelial cells express

somatostatin subtype 2 receptors. BrJCancer 2001; 85: 266-72.

25

33 Szepeshazi K, Schally AV, Halmos G, Sun B, Hebert F, Csernus B, et al. Targeting of

cytotoxic somatostatin analog AN-238 to somatostatin receptor subtypes 5 and/or 3 in

experimental pancreatic cancers. Gun Cancer Res 2001; 7: 2854-61.

34 Nagy A, Schally AV, Halmos G. Synthesis and biological evaluation of cytotoxic analogs of

somatostatin containing doxorubicin or its intensely potent derivative, 2-

pyrrolinodoxorubicin. PNAS 1998; 95:1794-9.

35 Sun LC, Luo J, Mackey LV, Fuselier JA, Coy DH. Effects of camptothecin on tumor cell

proliferation and angiogenesis when coupled to a bombesin analog used as a targeted

delivery vector. Anticancer Drugs 2007, 18: 341-8.

36 Bezerra Y, Fuselier JA, Peyman GA, Oner H, Drouant G, Coy DH. Study ofinhibitory effects

of an antiangiogenic somatostatin-camptothecin conjugate on laser-induced choroidal

neovascularization in rats. Retina 2005; 25: 345-54.

37 Moody TW, Mantey SA, Fuselier JA, Coy DH, Jensen RT. Vasoactive intestinal peptide-

camptothecin conjugates inhibit the proliferation of breast cancer cells. Peptides 2007;

28:1883-90.

38 Langer M, Kratz F, Rothen-Rutishauser B, Wunderli-Allenspach H, Beck-Sickinger AG.

Novel peptide conjugates for tumor-specific chemotherapy. JMed Chem 2001; 44:1341-8.

26

3St

39 O’Dorisio MS, Chen F, O’Dorisio TM, Wray D, Qualman SJ. Characterization ofsomatostatin

receptors on human neuroblastoma tumors. Cell Growth Differ. 1994; 5: 1-8

40 Qin Y, Ertl T, Groot K, Horvath J, Cai RZ, Schally AV. Somatostatin analog RC-160 inhibits

growth of CFPAC-1 human pancreatic cancer cells in vitro and intracellular production of

cyclic adenosine monophosphate. mtJCancer 1995; 60:694-700.

41 Evers BM, Townsend Jr CM, Upp JR, Allen E, Hurlbut SC, Kim SW, et al. Establishment

and characterization of a human carcinoid in nude mice and effect of various agents on tumor

growth. Gastroenterology 1991; 101: 303-11.

42 Ludvigsen E, Stridsberg M, Taylor JE, Culler MD, Oberg K, Janson ET. Subtype selective

interactions ofsomatostatin and somatostatin analogs with sstl, sst2, and sst5 in BON-1 cells.

Med Oncol 2004; 21:285-95.

27

Table 1 Effects ofJF-1O-81 on human tumor cell proliferation by MIT assay

Cell Name Cancer type EC50 (nM)

CPT JF-10-81 JF-07-69

1MR32 Neuroblastoma 2.82 0.14 (3)a 46.06 8.47 (5) >10,000

CFPAC-1 Pancreatic cancer 25.53 5.6(6) 196.87 37 (4) >10,000
BON Pancreatic carcinoid 170.86 14.62 (3) 1248.7 114.20(3) >10,000
PC-3 Prostate cancer 20.76 2.82 (5) 453.55 52.36 (3) >10,000
MOLT-4 Leukemia 1.96 0.32 (5) 27.63 0.88 (6) >10,000
Notes: a. Mean – SEM (n) and the experiments were separately repeated n times

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Table 2 Effects ofiF- 10-81 on growth of different kinds ofhuman tumors by treatmentwith continuous drug-releasing pellets.

Tumor Group Dose Time Group
Type (mg/ea) (day) (no./ea)
Final

Tumor Volume (mm3) Inhibition
Initial (%)C

Pancreatic placebo 0 28/60 10 168.12 –52.30"
CFPAC-1 tumor JF-10-81 5 28/60 10 196.90 –67.96
49/60

Neuoroblastoma placebo 0 42/60 6 758.12–291.9
lMR32tumor JF-10-81 5 42/60 6 763.14–217.75

Leukemia placebo 0 35/60 6 393.35 141.92
MOLT-4 tumor JF-1 0-81 5 35/60 6 270.27 88.65

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2195.62 – 83262b

43.58 –14.13 -92.44%
8.30 – 7.89

9097.9 –4928.52
1853.92 – 815.96 86.92%

5615.32 – 3143.98
429.76 – 329.76 96.94%

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values are 1.85 (DC-44-76), 192.3 (JF-07-69) and 866.3 nM (JF-10-81), and to SSTR2,
1.45 (DC-44-76), 2.35 (JF-07-69) and 2.46 nM (JF-10-81), respectively.
Fig.2 Expression of mRNAs of 5 SSTR subtypes. Total RNAs were isolated for RT-PCR
amplification. The PCR products are 993 bp for SSTR1, 892 bp for SSTR2, 221 bp for SSTR3, 276 bp for SSTR4 and 298 bp for SSTR5. SSTR2 mRNA was detected in all 5 of
the following human tumor cells: PC-3, 1MR32, BUN, CFPAC-1 and MOLT-4. The other
four subtypes were undetectable in one or more tumor cell lines.
Fig.3 Expression of SSTR2 protein (- 64 KD) detected in 5 tumor cells by western blot.
Fig.4 Cell proliferation assay using the CPT-SSA conjugate JF-1O-81, vector JF-07-69 and CPT with human pancreatic cancer CFPAC- 1 cells.
Fig.5 Human pancreatic cancer CFPAC-1 tumors treated with continuous drug-releasing
pellets (5 mg/each) containing the conjugate iF- 10-81.
Fig.6 Inhibition of JF- 10-81 to the highly invasive prostate cancer PC-3 tumor growth and
metastasis.

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Abogado
LUZ CLEMENCIA DE PAEZ

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Fecha de Presentación Internal. 03 de marzode 2003

Como resultado del examen de fondo, realizado con fundamento en el articulo 45 de la Decision 486 de la ComisiOn de la Comunidad Andina, se le comunica:

1. Documentos estudiados

DescripciOn: Folios: 73a 201

Reivindicaciones: Folios: 202 a 229
Folios: 263
Folios: 287 a 88F

Figuras: Folios: 231 a 232

Prioridad:
Fecha: 0.1/0.3/2002
Pals: US
Nümero 60/360,831

Rta. del solicitante: Folios: 281 a 284

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Anexos: Folios: 289 a 361 coma se radicO el 21/05/10

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2. Objeto de la invención

Titulo modificado de la solicitud (folio 238): "Conjugados unidos a carbamato
de agentes terapØuticos o citotóxicos y pØptidos biologicamente activos".

La presente solicitud se refiere a conjugados de agentes terapØuticos o
citotôxicos y pØptidos biologicamente activos unidos a carbamato.

El problema planteado en esta solicitud es la necesidad de medios efectivos para Ia union de agentes citotOxicos y molØculas de interØs, como pØptidos biolOgicamente activos o anticuerpos, a la vez que se mantenga la actMdad de cada componente para maximizar el efecto terapØutico deseado y se minimice la toxicidad de los mismos.

Para solucionar este proble,ma tØcnico la solicitud en estudlo proporciona conjugados de agentes terapØuticós o citotOxicos y una molØcula blanco. Dichos conjugados presentan la siguientØ fOrmula general.

La ClasificaciOn lnternaciofläl
A61 K 47/42 y 31/27.

3. De la solicitud de Patente

Reivindicaciones (artIculo 30 D 486)

de PâtØntes (IPC) es: C07 K, A61 K 38/08,

El capitulo reivindicatorio no fue corregido apropiadamente y continua presentando los siguientes defectos:

Posee una amplitud que excede lo desarrollado por el solicitante, al incluir
una estructura de uniOn a tres compuestos que pueden presentar exagerado nümero de variacionesque no se encuentran definidos inequlvocamente y no presentan sustento en Ia descripciOn ni pueden ser tornados como equivalentes biolOgicos. P.r eJenplo, es de notar que los Unicos agentes citotOxicos empleados fueron,camp.toteçina y combretastatina, los cuales ademÆs, en la demostraciOn:de tas@,.de IiberaciOn de citotoxicidad presentaron una marcada variabilidadincluyendo que la combretastatina no superara los efectos de lamolØcuIano conjugada como se evidencia en la Tabla 2. Igualmente los sustituyentes R que vienen de la figura de la Tabla I desde R2, son apenas 5 y los pØptidos porciOn objetivo comprenden ü nicamente pØptidos de somatostatina o bombesina por lo que el nuevo capitulo reivindicatorio no cumple con los requisitos presentados por este despacho en el anterior concepto tØcnico.

La mUltiple cantidad de compuestos posibles, no permiten determinar el alcance de Ia invenciOn ni los compuestos que realmente tienen acciOn terapØutica. Por ejemplo los sustituyentes en Y pueden ser una cantidad ilimitada de pØptidos o polImeros, que no tienen relaciôn alguna entre si ni estructural ni funcionalmente por lo tanto no es aceptable una definiciOn tan amplia para una secuencia espaciadora. AdemØs de esto, no existe sustento de la mejor forma de realizar la invnciOn con dichas secuencias.

La expresiOn "pØptido inteatiria1 vasoactivo" no corresponde a una caracteristica tØcnica del compuesto que se pretende reivindicar, es importante resaltar que las caracterlstipastØcnicas esenciales de la invenciOn

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deben ser definidas par su estructura o su secuencia y, como ya se ha indicado, no cualquier pØptido presentarla el efecto descrito.

Por lo anterior, el capitulo reivindicatorio NO cumple con el requisito de definiciôn, claridad, concision o sustento por la descripciOn de acuerdo al art. 30
de la DecisiOn 486.y/o art.22 Tratado PCT.

4. Determinación del Estado de la TØcnica

La fecha que se ha tenido en cuenta para determinar el Estado de la TØcnica es:

Fecha de presentaciOn de la solicitud prioritaria: 01 de marzo de 2002.

Teniendo en cuenta la fecha indicada se realizO la bUsqueda en el estado de Ia tØcnica de documentos relacionados con la solicitud encontrÆndose los sig u ientes documentos mÆsrelevantes:

NO Documento Titulo Fecha de
patente

Conjugates useful in the 7 septiembre de Dl US 5.948.750 treatment of prostate cancer 1999

5. AnÆlisisde patentabilidad (artIculo 14 D486)

A pesar de las objeciones presentadas al capitulo reivindicatorlo, se efectuO el anÆlisisde patentabilidad segün la estructura general presentada.

5.1.Nivel_inventivo(artIcuIol8D486)

La reivindicaciOn I y sus dependl t’dM.ilgan un compuesto representado
par la siguiente formula:

0

x

NN0 N

- 00?y

N ’-N
y 11-10,

z

NNQ

(CH2)n 0
R

El documento del Estado de la TØcnica rnÆsprOximo es Dl (ver folios: 270-273) que enseæaun conjugados que comprenden agentes citotôxicos (X) unidos a pØptidos como las posibles sustituciones Q, mediante un compuesto de uniOn que presenta una estructura similarala reivindicada (ver figura 3, columna 12), igualmente con un radical R. AdemÆsen Dl las caracteristicas de Y y Z coma molØculas espaciadoras y pØpti’dós ligädores yase habla reconocido como se observa en la descripciOn de la inver?ciOn1(columnas 2 en adelante).

Al no precisarse el efecto tØônico que presenta la uniOn del carbamato a los agentes activos descritos, dicha molØcula de uniOn puede considerarse una alternativa evidente de Ia descrito por Dl para los agentes activos.

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La Unica diferencia con DI es que esta solicitud presenta la estructura de union
con un sustituyente en el grupo amino del carbamato. Puesto que dicha diferencia no produce un efecto sorprendente dada la amplitud de las reivindicaciones, no existe razOn para considerar que exista altura inventiva en Ia solicitud. .

El problema tØcnico planteado..enestaScitUd que era de proporcionar un medio efectivo para la union de. agentes ciotOxicbs y molØculas de interØs, como pØptidos biolOgicamente activos o anticuerpos, se habla resuelto previamente mediante el mismo tipo de compuestos ya conocidos. Por esta razOn se considera que las sustituciones de los grupos citotOxicos, y los radicales R y Y, Z y Q como molØculas espaciadoras, pØptidos ligadores y pØptidos biolOgicamente activos respectivamente, son evidentes para una persona medianamente versada en Ia materia.

De esta forma se establece que las reivindicaciones I a 13 no cumplen con el requisito de Nivel Inventivo.

6. Conclusion:

Los requisitos de patentabilidad de presente solicitud se encuentran afectados as!:

No Docurnento NO Renindicackmes Requisito que afecta
Dl US 5.948.750 Nivelinventivo

En cumplimiento del arUculo 45 de la Decision 486 de la ComisiOn de la Comunidad Andina, se le indica que debe dar respuesta dentro del tØrmino de 60 dias contados a partir de la fecha de notificaciOn de la presente comunicaciOn. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesiOn, se denegara la patente.

Notifiquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capItulo quinto del titulo primero de la Circular Unica NO 10 de 2001.

ALIX CARMENZ*IEDES DE VERGEL
Directora de Nu as Creaciones(C)

El anterior requerimiento. füe riôtifiâd pbr fijaciOn en lista, de fecha 2 7.46O. 2fl10

CONCEPTO TECNICO EXAMINADOR TECNICO
No. 2760 COdigo No. 202089