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Chemistry

Cytotoxic agents directed to peptide hormone receptors: Defining the requirements for a successful drug

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ABSTRACT **In principle, cell surface receptors that are overexpressed in tumor tissue could serve as targets for anticancer drugs attached to receptor ligands. The purpose of this paper is to identify the necessary elements for a successful receptor-targeted drug. We used the gastrin/cholecystokinin type B receptor as a model delivery system, and we report on the synthesis, trafficking, and *in vitro* and *in vivo* evaluation of heptagastrin, the C-terminal heptapeptide of gastrin, linked via an appropriate linker to a potently cytotoxic ellipticine derivative, 1-[3-[*N*-(3-aminopropyl)-*N*-methylamino]propyl]amino-9-methoxy-5,11-dimethyl-6H-pyrido[4,3-b]carbazole. These data, and previous work from our laboratory, show that the drug-complexed ligand is sorted to lysosomes whereas the receptor is recycled to the plasma membrane. The lysosomal processing of the ligand/drug construct depends on the linker between the ligand sequence and the cytotoxic moiety. We show that heptagastrin linked to ellipticine via a succinoyl-substituted pentapeptide, AlaLeuAlaLeuAla, is at least 10^3 more toxic to cholecystokinin type B receptor-positive NIH/3T3 cells than to isogenic NIH/3T3 cells lacking the receptor. The conjugated drug eradicated all receptor-positive tumor cells *in vivo* without producing any general toxicity. The data indicate that the density of the cell surface receptor, the properties of the cytotoxic moiety, and the correct processing of the drug-conjugated ligand in lysosomes are crucial to the effectiveness of a receptor-targeted drug.**

Systemic toxicity of drugs is one of the most serious problems of cancer chemotherapy and frequently is dose limiting. The appearance of the various classes of multiple drug resistance renders even good drugs ineffective by expelling them from tumor cells (1). Various strategies have been used to get around one or both of these difficulties, but they still are among the most intractable problems of cancer therapy. Targeting of drugs specifically to tumor cells has been the goal of many studies. Various protein toxins conjugated to mAbs directed to specific tumor antigens have shown some promise as drugs (2), but severe problems, such as the development of neutralizing antibodies (3), have limited the effectiveness of the method. Another promising approach is to use cellular receptors for growth factors (4–6), cytokines (7, 8), or steroid hormones (9, 10) as targets to deliver cytotoxic moieties to the receptor-bearing cells.

The utilization of receptors to target tumor cells is an attractive concept because it offers the possibility of minimizing nonselective toxic effects. Also, one would expect that multiple drug resistance would be less important in the case of cell surface receptor-mediated active transport of the drug across the cell membrane. However, there are also many problems associated with this approach. One is to identify receptors that are present predominantly on tumors cells and in sufficient density. Another is that, until recently, little was known about the mechanisms and dynamics of receptor trafficking after ligand binding. In most cases it was not known what modifications of ligands could be tolerated without affecting receptor binding. Furthermore, very little is known about the fate of the internalized ligand-receptor complex. It was suggested that some receptors recycle back to the cell surface (11), whereas others were shown to be partially or completely degraded (12–14). It is crucial for successful drug delivery by the receptor-mediated route that the drug moiety attached to the ligand does not dominate the transport properties of the complex. For example, the lack of success in targeting opioid receptor-positive cells by an enkephalin-ellipticine conjugate (15) was caused by the drug entering cells unspecifically. On the other hand, the melanotropin-daunomycin conjugate was toxic only to cells that expressed the melanotropin receptor (16).

The goal of the present study was to define the structural requirements for a receptor-targeted drug. We chose the gastrin/cholecystokinin (CCK) type B receptor (CCKBR) as a model system. Although peripheral to the principal theme of this paper, gastrin, a gastrointestinal (GI) hormone is known to stimulate the growth of certain GI cancers (17–19), and CCKBR was shown to be expressed in a number of established GI cancer cells (20–24). Circulating gastrin is a 17-residue and/or 35-residue peptide (25), but only the C-terminal tetrapeptide is absolutely required for binding to the receptor. CCKBR from several species have been cloned and their pharmacological properties have been determined (26). The CCKBR belongs to the G-protein-coupled superfamily of receptors, which is characterized by the presence of seven transmembrane domains.

We had reported earlier (27) that an acyltriazene linked to pentagastrin (the C-terminal pentapeptide of gastrin) bound readily to the CCKBR and was selectively toxic for CCKBR-positive AR42J cells, a rat acinar cell pancreatic carcinoma line. Subsequent studies in our laboratory, which used fluorescent-labeled heptagastrin and CCK-8 (11, 28, 29), showed that both CCKBR and CCK-A receptor agonists undergo internalization by endocytosis upon binding to the receptors. The receptors and ligands are sorted in endosomes (11). Both CCK-A and CCK-B receptors are recycled to the cell surface with very high efficiency, whereas the peptide ligands end up in lysosomes, where presumably they are degraded (11, 29). This paper reports on the synthesis, processing, and selective toxicity of drugs directed to CCKBR-positive cells. The

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Abbreviations: CCK, cholecystokinin; CCKBR, CCK type B receptor; WT, wild type; HG, human gastrin I (2–17)(15-Nle); 7G, heptagastrin (5-Nle); Ell, 1-[3-[*N*-(3-aminopropyl)-*N*-methylamino]propyl]amino-9-methoxy-5,11-dimethyl-6H-pyrido[4,3-b]carbazole; AL5, Ala-Leu-Ala-Leu-Ala; EAG, Ell-AL5-Su-7G; MALDI-TOF MS, matrix-assisted laser desorption time of flight mass spectra; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl.

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present data define the parameters required for successful receptor-targeted drugs.

MATERIALS AND METHODS

Materials. Human gastrin I (2–17) (15-Norleucine) (HG) was purchased from Research Plus (Bayonne, NJ). Rink amide resin was from NovaBiochem, and sasrin resin was from Bachem. Human liver cathepsin D and cathepsin B were purchased from Calbiochem.

Cells. NIH/3T3 cells were obtained from the American Type Culture Collection. NIH/3T3 cells stably expressing the human CCKBR ($B_{max} = 1.2 \pm 0.29 \times 10^5$ receptors/cell) were prepared and maintained as described (29).

Instruments. NMR spectroscopy was performed on a Varian VXR-500 spectrometer with tetramethylsilane as the internal standard. The matrix-assisted laser desorption time of flight mass spectra (MALDI-TOF MS) were generated on a Bruker Reflex II mass spectrometer as described (30).

Synthesis of Ellipticine Derivative. 9-Methoxy-1-chloro-5,11-dimethyl-6H-pyrido[4,3-b]carbazole was prepared according to a previously published procedure in 7% yield (31). This compound was treated with 10-fold excess of boiling 3,3′-diamino-*N*-methyldipropylamine under nitrogen until the starting compound disappeared on TLC (1.5–2 h). Excess of amine was evaporated under reduced pressure, and the residue was separated by chromatography on Silica gel 230–400 mesh by using methylene chloride with 0–15% of methanol as the eluent. The purified product was crystallized from hexane/ethyl ether mixture (2:1) providing 1-[3-[*N*-(3-aminopropyl)-*N*-methylamino]propyl]amino-9-methoxy-5,11-dimethyl-6H-pyrido]4,3-b]carbazole (Ell). Yield 55%. Anal. ($C_{25}H_{33}N_5O$) C, H,N. NMR (Me_2SO-d_6): 10.96 (s), 7.75 (d, 5.95), 7.75 (d, 2.48), 7.42 (d, 8.63), 7.12 (dd, 8.63, 2.48), 6. 98 (d, 5.95), 6.47 (broad), 3.88 (s, 3H), 3.49 (broad dd, 11.1, 6.5, 2H), 3.33 (s, 3H, overlapped with H_2O), 2.63 (s, 3H), 2.51 (m, 2H, overlapped with dimethyl sulfoxide (DMSO)-d6), 2.46 (t, 6.84, 2H), 2.34 (t, 7.2, 2H), 2.18 (s, 3H), 1.85 (qui, 6.84, 2H), 1.49 (qui, 7.0, 2H).

Synthesis of Heptagastrin, H-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2 (7G). Rink amide resin was used as a solid support. The 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were incorporated successively by executing the steps of the standard batch solid-phase peptide synthesis protocol (32). Product was cleaved from the resin and deprotected by 70% trifluoroacetic acid (TFA) in methylene chloride. Solvent was evaporated under reduced pressure at 20°C. The synthesized peptide was precipitated by adding peroxide-free ether. The crude product was purified by preparative HPLC on a C18 column (eluent: 10–70% acetonitrile/water/0.05% TFA). Product was lyophilized and characterized by MALDI-TOF MS and NMR. MALDI-TOF MS: $C_{44}H_{55}N_9O_{10}$ calculated 869.975, found 870.4.

Synthesis of Ell-CO-7G and Ell-CO-HG. The solution of *p*-nitrophenyl chloroformate (202 mg, 1 mmol) in chloroform (3 ml) and the solution of 2,6-lutidine (116 μl, 1 mmol) in chloroform (3 ml) were added simultaneously dropwise to the solution of Ell (420 mg, 1 mmol) in chloroform (5 ml) at 0°C. After 30 min the precipitate of the *p*-nitrophenyl carbamate of Ell, which formed during the reaction, was washed with chloroform, dried, and used without additional purification.

7G or HG (1 μ mol) was dissolved in a mixture of acetonitrile (150 μl) and an aqueous solution of sodium bicarbonate (0.2 M, pH 9.0, 150 μl). A solution of Ell-*p*-nitrophenyl carbamate (1.3 μmol) in the same solvent mixture was added. The reaction mixture was stirred at room temperature for 3 h. Products were separated by HPLC under reverse-phase conditions using C18 column (eluent: 30–70% acetonitrile/water/0.05% TFA). Yields were 65–93%. MALDI-TOF MS: Ell-CO-7G ($C_{70}H_{86}N_{14}O_{12}$) calculated 1315.54, found 1315.5; Ell-CO-HG ($C_{119}H_{152}N_{24}O_{31}$) calculated 2414.7, found 2415.8.

Synthesis of H-Ala-Leu-Ala-Leu-Ala-Ell. Fmoc-Ala-Leu-Ala-Leu-Ala-OH was synthesized on sasrin resin. The peptide was cleaved from the resin by 30% TFA in methylene chloride, containing 5% ethanedithiol and 3% phenol (3 × 20 min) without deprotection of the N terminus. The product was purified by HPLC. Fmoc-Ala-Leu-Ala-Leu-Ala-OH (0.3 mmol), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (0.33 mmol), 1-hydroxybenzotriazole(0.33 mmol), and diisopropylethylamine (0.3 mmol) were dissolved in dimethylformamide (DMF) (5 ml). Solution of Ell (0.3 mmol) in DMF (2 ml) was added at room temperature, and the reaction mixture was stirred 2 h. The product was purified by HPLC. The N terminus was deprotected with 20% piperidine in DMF just before the coupling reaction described below. Solvents were evaporated in vacuum, and the solid residue was washed twice with ethyl ether. This residue was H-Ala-Leu-Ala-Leu-Ala-Ell of sufficient purity for further coupling.

Synthesis of Ell-Ala-Leu-Ala-Leu-Ala-Su-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2 (EAG). The N terminus of 7G loaded on the rink amide resin was deprotected and reacted for 1 h with succinic anhydride (3 molar equivalents) dissolved in methylene chloride. The resin was washed with methylene chloride. Two-fold excess of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, 1-hydroxybenzotriazole, and diisopropylethylamine in DMF was added. After 2 min a solution of 1.5 molar equivalents of H-Ala-Leu-Ala-Leu-Ala-Ell in DMF was added. After 1 h of gentle agitation, the resin was washed and dried. The product was deprotected, cleaved, and purified as described above (synthesis of 7G). MALDI-TOF MS: calculated for $C_{94}H_{127}N_{19}O_{18}$ 1811.16, found 1811.3.

All peptide conjugates were further characterized by 1H and double-quantum filtered COSY/NMR spectra and showed all the expected peaks and cross peaks for backbone, side chains, and Ell protons.

Measurement of Intracellular $[Ca^{2+}]_i$. Cells grown on Nunc cover glass chamber slides were incubated with 1 μM Fura-2/AM for 20 min in a CO_2 incubator, rinsed with PBS, and mounted on the stage of a Zeiss Axiovert inverted microscope. $[Ca^{2+}]_i$ measurements were performed by using an Attofluor digital imaging system (Atto Instruments, Rockville, MD). Fluorescence of Fura was excited at alternating wavelengths of 340 and 380 nm. Fluorescence was monitored by an intensified charge-coupled device camera using 505 cutoff filter. Calibrations of $[Ca^{2+}]_i$ signals were performed by using Ca^{2+} standards containing 1 μM Fura.

Confocal Laser Scanning Microscopy. Cells were grown in coated 50-mm cover glass bottom dishes (MatTek, Ashland, MA) in medium without phenol red. After incubation with the drugs, the cells were rinsed and observed on a Zeiss inverted LSM 410 confocal laser scanning microscope. Fluorescence of ellipticine was excited by using a 488-nm argon/krypton laser, and emitted fluorescence was detected through a 505-nm cutoff filter. Fluorescence of rhodamine green was excited by using 488-nm argon/krypton laser and detected through a 515- to 540-nm band pass filter.

Toxicity Assays. The cells were seeded on 24-well plates (Nunc) a day before the assay and were cultured in the presence of various concentrations of the compounds for 4 days under 5% CO_2 at 37°C. The cultures were fixed for 1 h by addition of ice-cold 50% TFA to give a final concentration of 10%. Fixed cells were rinsed with water and stained for 20 min with 0.4% sulforhodamine B in 0.1% acetic acid. The wells were washed with 0.1% acetic acid and left to dry overnight. The absorbed sulforhodamine B was dissolved in unbuffered 1% Tris solution in water (pH 9.5–10). The absorbency of extracted sulforhodamine at 540 nm was measured on a MR7000 plate reader (Dynatech) (33).

Hydrolysis of Drug-Peptide Conjugates by Lysosomal Endopeptidases. Solutions (1 mM) of the drug-peptide conjugates in 0.1 M sodium acetate buffer (pH 4.5) were incubated with the human liver cathepsin D or cathepsin B at 1:4,000 molar ratio at 37°C for 2 h. The mixtures were frozen in liquid nitrogen and kept at −70°C before analysis by MALDI-TOF MS. The identity of the major digestion fragments, which contained ellipticine, was confirmed by HPLC separation of the hydrolysates under reverse-phase conditions (Vydac 218TP C18 column, eluent: 10–60% acetonitrile/water/0.05% TFA, photodiode array detector) and MALDI-TOF MS spectra of separated products. The details of this analytical procedure are presented elsewhere (30).

Testing of the Anti-Tumor Activity of Ellipticine Derivatives in Athymic Nude Mice. Male athymic mice (27–31 g weight) were injected s.c. with a suspension of 10^7 NIH/3T3 cells stably transfected with human CCKBR, in 0.5 ml of PBS. The animals were divided into groups of 10, and each group was injected i.p. with 100 μl of 0.1 mM solution of a compound in DMSO or pure DMSO in the case of the control group. The administration of the compounds was started 40 h after injection of the cells and was continued daily for 12 days. The mice were weighed and the tumors were measured twice a week. The animals were sacrificed on the 21st day after injection of the cells. The tumors were removed and divided into two pieces. One piece was fixed in 4% formaldehyde for 16 h and processed for immunohistochemical analysis. The second portion was homogenized and processed for ligand binding studies.

Characterization of Ligand Binding Properties of Tumor-Derived Cells. Tumors removed from animals were homogenized with razor blades and treated with 1% collagenase for 20 min in a 5% CO_2 incubator. The suspension was passed several times through a pipette. Cells were peletted, resuspended in DMEM, and plated on coated 50-mm cover glass bottom dishes (MatTek). Fourteen hours later, attached cells were incubated with 10 nM rhodamine green-7G for 30 min, rinsed with medium and observed under a confocal microscope as described (29).

Immunohistochemical Detection of CCKBR in Tumors. Tumors grown in athymic mice were trimmed to pieces not more than 0.5 cm in each dimension and fixed in 4% formaldehyde in PBS for 16 h at 4°C. Fixed specimens were processed to sections and stained with rabbit polyclonal antibodies to CCKBR as described (34).

RESULTS

Synthesis of Drug-Peptide Conjugates. A series of ellipticine-hormone conjugates was prepared. An ellipticine derivative, 9-methoxy-1-chloro-5,11-dimethyl-6H-pyrido[4,3-b]carbazole (31), was condensed with 3,3′-diamino-*N*-methyldipropylamine, and the product of this reaction, Ell, was used for the preparation of all Ell-hormone conjugates. Although CCKBR recognizes peptide sequences as short as the C-terminal tetrapeptide, our previous experience (27) showed that tetrapeptide conjugates were poorly soluble. 7G and HG conjugates of Ell behaved almost identically in terms of receptor binding and signaling when tested on CCKBR NIH/3T3 cells, so consequently all of our subsequent studies were performed with the more economical 7G derivative. In this paper we contrast the behavior of 7G linked directly to ellipticine by a urea link (Ell-CO-7G) with a construct consisting of Ell linked to the alanyl-leucyl pentapeptide, AlaLeuAlaLeuAla (AL5), attached via a succinoyl moiety to N terminus of 7G (EAG). It had been shown that AL5 is sensitive to lysosomal hydrolases (35, 36), which we confirmed in our studies. The succinoyl spacer was necessary to invert the sense of the peptide linkages, because both 7G and AL5-Ell have exposed N termini. The synthetic scheme is shown on Fig. 1. An important advantage of this construct is that after enzymatic hydrolysis it provides an ellipticine derivative bearing an amino group on the end of the side chain. This amino group is important for enhanced binding of Ell to DNA (37, 38).

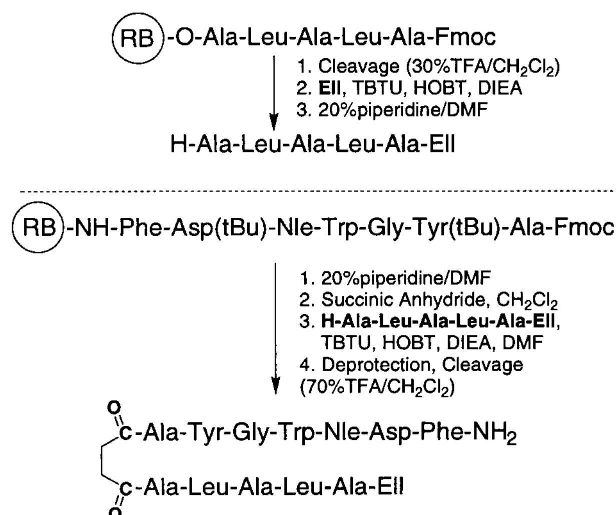

FIG. 1. Procedure for the solid-phase peptide synthesis of EAG. RB is the resin bead. TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBT, 1-hydroxybenzotriazole; DIEA, diisopropylethylamine.

Properties of Ell-Peptide Conjugates. All ellipticine-hormone conjugates induced intracellular Ca^{2+} release in CCKBR NIH/3T3 cells at concentrations as low as 10 nM, which was identical to that observed for the parent gastrin (data not shown). We had shown previously (29) that rhodamine green-modified 7G separated from CCKBR after internalization and was sorted to lysosomes. Because of the weak fluorescence of Ell and the overlap of its emission spectrum with the emission spectra of organelle markers, it was impossible to perform colocalization experiment of Ell-hormone conjugates with lysosomal markers. However, confocal laser scanning microscopy images (Fig. 2*A*) of the CCKBR NIH/3T3 cells exposed to Ell-peptide conjugates showed that Ell fluorescence exhibited a very similar pattern of localization to that of the rhodamine green-7G (29). This finding suggests that the Ell-peptide molecules also were delivered to lysosomes. Free ellipticine, on the other hand, was concentrated almost exclusively in the nucleus (Fig. 2*B*).

Enzymatic Cleavage of Ellipticine-Peptide Conjugates. Studies of hydrolysis of Ell-CO-7G by the lysosomal endopeptidases cathepsin B and D indicated that the compound was not cleaved appreciably by either enzyme, with better then 80% of the substrate recovered after a 2-h incubation. Similar experiments with EAG, however, indicated that although cathepsin B cleaved the substrate mainly in the 7G moiety (Gly 5–Trp 4 bond) in approximately 50% yield, cathepsin D digested the molecule almost completely (*ca.* 90%), with most of the cleavage occurring in the AL5 linker (products: Ala-Ell 26%, Ala-Leu-Ala-Ell 29%, 7G moiety cleavage products ≈30%).

Cytotoxicity of Ellipticine-Peptide Conjugates *in Vitro*. The cytotoxicity of ellipticine and its conjugates was tested on wild-type (WT) NIH/3T3 cells and CCKBR NIH/3T3 cells. The CCKBR NIH/3T3 cells also expressed a neomycin resistance marker. The CCKBR NIH/3T3 cells incubation mixtures also contained neomycin to maintain the CCKBR(+) phenotype. The unconjugated ellipticine exhibited toxicity at a subnanomolar level in gastrin receptor positive and negative cells. The cytotoxicity of Ell-CO-7G (Fig. 3*A*) and Ell-CO-HG on CCKBR NIH/3T3 cells and WT NIH/3T3 cells was almost identical ($IC_{50} \approx 1$ μM). Confocal microscopy experiments

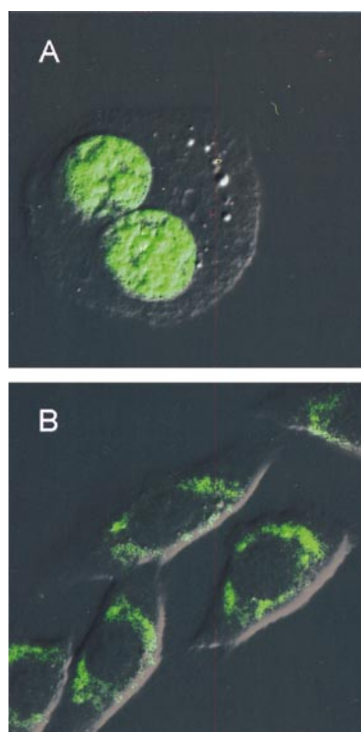

FIG. 2. Confocal laser scanning microscopy image of CCKBR NIH/3T3 cells exposed to Ell (*A*) and Ell-peptide conjugate (*B*). The presence of the compound is indicated by the green fluorescence. Ell is concentrated mainly in the nucleus, whereas Ell-peptide conjugate is present in perinuclear organelles, which are believed to be lysosomes. Magnification: ×2016.

showed that Ell-CO-7G was internalized selectively by receptor-positive cells. This finding suggests that the reason for the lack of selective toxicity of this compound is inefficient intracellular processing. The toxicity of EAG is shown in Fig. 3*B*. Remarkably, this compound had an IC_{50} in the nanomolar range when tested on CCKBR NIH/3T3 cells, whereas the receptor negative WT NIH/3T3 cells were affected only at micromolar concentrations.

***In Vivo* Activity of Ellipticine-Hormone Conjugates.** The drug in DMSO solution was administered to 10 animals, which 40 h earlier had been injected s.c. with CCKBR NIH/3T3 cells. Groups of 10 animals each, after receiving the tumor cells, also were treated with DMSO alone, 7G in DMSO, Ell-CO-7G, and Ell in DMSO at the same molar concentration as EAG. Treated animals did not exhibit symptoms of drug-related toxicity and did not lose weight, and histological analysis failed to reveal any pathological changes in internal organs. Tumors appeared in all groups of animals although the number of animals that developed tumors and the size of the tumors appeared to be smaller in the group of animals treated with EAG than in the control groups. Also, a delay of 4–6 days in the appearance of the tumors was observed. However, a statistically significant difference could not be established, because of the large individual variation. The tumors and cells derived from them, obtained from animals treated with the DMSO, free ellipticine, free 7G, and Ell-CO-7G all expressed CCKBR on the level comparable to the original cells, as revealed by immunohistochemical detection of the receptor (data not shown) and binding studies with rhodamine green-7G (Fig. 4*B*). On the other hand, cells derived from tumors where the animals had been treated with EAG were receptor negative according to both tests (Fig. 4*A*).

DISCUSSION

The foundation for this paper was our previous studies of trafficking of ligand-bound gastrin/CCK-B and CCK-A recep-

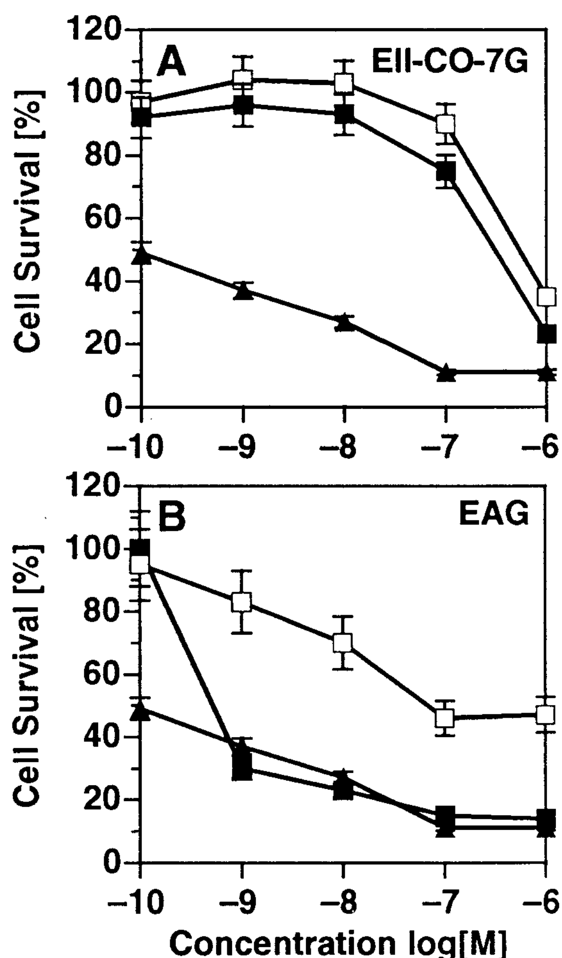

FIG. 3. Activity of Ell-CO-7G (*A*) and EAG (*B*) in cell toxicity assay against receptor-positive CCKBR NIH/3T3 cells (■) and receptor-negative WT NIH/3T3 cells (□). Ell (▲) was equally cytotoxic to both cell lines.

tors. Thus, the synthesis of fluorescent-labeled peptide ligands for these receptors (28) allowed us to probe the trafficking of the ligands in live cells in real time (11, 29). It was shown that these constructs behaved very similarly to natural ligands in terms of initiation of signal transduction, and their ultimate localization in lysosomes, where the peptides were degraded. Because the fluorescent dye-labeled ligands could be regarded as models for ligand/cytotoxin conjugates, the findings on ligand trafficking were directly relevant to the design of receptor-targeted drugs. Additionally, it became clear that the fluorescent dye-labeled ligands could be used as analytical tools to detect the delivery of labeled ligands (and presumably also drugs) to tumor tissue, and to receptor-positive normal tissue (25). Studies on the trafficking of CCKAR fused to the green fluorescent protein (11) demonstrated that although this receptor underwent spontaneous endocytosis, independent of the presence of ligand, agonists induced more efficient endocytosis. On the other hand, a number of antagonists inhibited the process. Once inside the cell, the receptor-ligand complex was found to be sorted in endosomes, with the ligand being delivered to lysosomes, whereas the receptor was recycled back to the cell surface with very high efficiency, with a period of 60–90 min.

We had made numerous previous attempts to prepare drug-ligand conjugates for specific delivery into receptor-positive cells. These included a number of alkylating agents, anthracycline and methotrexate derivatives attached to peptides recognized by CCKAR and CCKBR. In most cases,

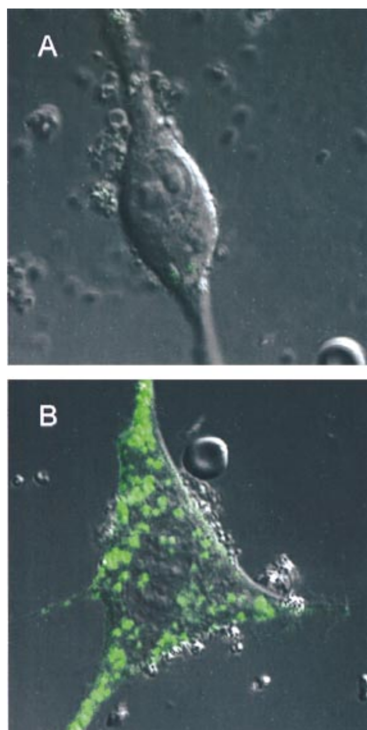

FIG. 4. Confocal laser scanning microscopy images of NIH/3T3 cells derived from tumors treated with EAG (*A*) and a control group (*B*). The presence of the receptor is indicated by the fluorescence of internalized rhodamine green-7G. Magnification: ×2016.

despite the selective delivery to the receptor expressing cells, these attempts did not lead to selectively cytotoxic materials of sufficient potency (data not shown). Other workers also have noted disappointing results with some receptor-targeted drugs (15, 39). Our hypothesis was that a successful drug must meet certain minimal criteria. Clearly the drug must be targeted to a receptor that is expressed more abundantly in tumor tissue relative to normal tissue. Moreover, the receptor should recycle back to the cell surface, because in principle, it then could act as a pump to bring more drug into the cell. This pumping is particularly important when one considers that the number of receptor molecules on a given cell rarely exceeds 10^6 (40). The method of attachment of the cytotoxic moiety to the peptide ligand is critical. The linker between these moieties must be stable in circulation, but must be hydrolyzed readily by lysosomal hydrolases. Finally, because of the relatively small number of receptors per cell, the intrinsic toxicity of the toxic moiety must be high, with an IC_{50} in the nanomolar range.

The equilibrium constant, K_d, for ligand/receptor binding determines in large measure the minimum concentration of the ligand required to produce a biological effect. We estimated that K_d for the fluorescent gastrin derivatives was in the nanomolar range (e.g., 2 nM for rhodamine green-7G conjugate; ref. 28). Therefore, we would predict that the resultant cytotoxic effect of a drug conjugated with a peptide ligand would become apparent only close to the concentration defined by K_d. In the case of CCKBR we would not expect to observe toxicity at lower than nanomolar concentration, irrespective of the intrinsic toxicity of the bound toxin. In fact, it can be argued that the utilization of even more toxic agents is not an advantage, because the additional toxicity would not enhance conjugated drug activity, but could give rise to the possibility of nonspecific release of the bound drug at concentrations where it may cause harm.

After the minimum concentration of the conjugated drug required for internalization is reached ($>K_d$), the level of receptor expression on the cell surface is the most important determinant of the amount of prodrug delivered to the cell. The cells that we have used in the study expressed CCKBR at a density ($B_{max} = 1.2 \pm 2.9 \times 10^5$ receptors/cell) that is not higher than in the naturally occurring tumor cells. Assuming a nanomolar concentration of the extracellular drug, a receptor number of 10^5 molecules/cell ($1.7 \cdot 10^{-19}$ mol), and a median cell volume of 2,200 μ_3 (41), the concentration of a drug-conjugate inside the cell, after one cycle of internalization, can be estimated to be $\approx 10^{-7}$ M. If the intrinsic cytotoxicity of the drug is high (e.g., $IC_{50} \approx 1$ nM), and assuming that the intracellular processing of the conjugate is fast, one cycle of internalization should produce the desired toxic effect. Thus, the receptor-mediated transfer of the drug produces a 100-fold increase of the drug within the affected cell. Clearly, cells that express even higher amounts of receptor will be able to deliver more drug to the intracellular compartment. Additionally, receptor recycling may boost that concentration even higher.

Sensitivity of drug-hormone conjugates to lysosomal peptidases is a crucial feature of any prodrugs directed to peptide hormone receptors. The toxic moiety can express its cytotoxic properties only when it is freed from the hormone and is able to diffuse out of lysosomes. Products of the enzymatic digestion have to have similar toxic properties to the parent drug. EAG, in which an inverted peptide linker was used, showed good sensitivity to lysosomal proteases and also very good cytotoxicity. Small fragments such as Ala-Ell or Ala-Leu-Ala-Ell liberated by the initial hydrolysis can be further cleaved by aminopeptidases or may be toxic themselves (38).

EAG was prepared to demonstrate the desirable properties of a receptor-targeted drug. Ellipticines are highly cytotoxic but rather nonspecific (37). We had shown that the ellipticine derivative Ell was very toxic to NIH/3T3 cells in culture ($IC_{50} < 10^{-10}$ M, Fig. 3). Linking Ell directly to 7G via a urea linker (Ell-CO-7G), resulted in an agent that was only weakly toxic to CCKBR NIH/3T3 cells (Fig. 3*A*), even though Ell was highly toxic to both WT NIH/3T3 cells and to the receptor-transfected counterparts. We ascribe the poor toxicity of Ell-CO-7G to very slow processing of the conjugate in lysosomes. EAG, on the other hand, presents an entirely different picture (Fig. 3*B*). The inclusion of the lysosome-sensitive linker (35, 36) into the construct produced a highly selective cytotoxic agent. This drug was toxic in CCKBR NIH/3T3 cells in the nanomolar range, or close to the equilibrium constant for binding of the ligand to the gastrin receptor. The construct was much less toxic to WT NIH/3T3. The observed micromolar toxicity of EAG to the receptor-negative cells may be caused by the presence of a low affinity receptor in WT NIH/3T3 cells (42), which recognizes 7G, or perhaps could be caused by a premature hydrolytic breakdown of the construct in the medium. However, *in vitro* experiments indicated that EAG was stable in the medium for at least 24 h.

The *in vivo* experiments with EAG were also very instructive. As mentioned above, ellipticine-hormone conjugates bind to their cognate receptors with high avidity, close to that of the natural hormone ($K_D \approx 1$ nM (43). We administered sufficient EAG to the animals to achieve an initial body fluid concentration of 3 μM. Histological examination indicated no toxic side effects, even in organs that express CCKBR (stomach, kidneys, and brain) (25). Although the tumors in animals treated with EAG appeared 4–6 days later than in the control groups and appeared to be smaller, the differences were not statistically significant, because of the large individual variation and the small sample size. Although we failed to irradicate the tumor, the immunohistochemical analysis showed that the tumors from animals that had been treated with EAG no longer expressed the receptor. In contrast, tumors from animals that had been treated with DMSO, ellipticine, Ell-CO-7G, or 7G all showed receptor levels similar to the initial CCKBR NIH/3T3 cells.

Thus, the drug killed the receptor-positive cells in the tumor. The selection of the resistant, receptor-negative phenotype is a consequence of our model, where the cells are heterogeneous with respect to the level of CCKBR expression, although they are monoclonal. We attribute the heterogeneity to the properties of the expression system. *In vitro* the receptor-negative phenotype was suppressed by neomycin in the culture medium. This suppression was not possible *in vivo*.

Another conclusion derived from the *in vivo* experiment is that the drug was not destroyed in circulation and that it apparently was processed properly in lysosomes. Finally, the drug apparently was concentrated in cytotoxic concentrations in the receptor-positive cells, unlike free ellipticine, which failed to affect the tumors at an equimolar dose. These data validated some of our assumptions.

The *in vitro* and *in vivo* results indicate that a receptor-targeted drug such as EAG contains the correct elements to be a highly potent but very selective anti-tumor agent. The elements that have paramount importance in determining this activity are: the type of toxic moiety, which must be potent but stable enough to survive the harsh environment of lysosomes; the linker, which must be stable in circulation but readily and correctly processed in lysosomes to release the toxic moiety; and the ligand, which must bind to a receptor expressed abundantly on tumor cells, and which is processed after endocytosis so that it delivers the toxin to lysosomes, whereas the receptor recycles to the cell surface. These conclusions should be generally applicable to any receptor-targeted drug. However, the question of whether a receptor-targeted drug could select for a less differentiated, receptor-negative subpopulation of cells in a given tumor is not answered by the present study. Ongoing research in our laboratory is directed to resolving this issue and to the application of our findings to clinically relevant tumors.

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