increasing number of endogenous peptides that modulate physiological processes. The inherent lability of peptides and their poor oral absorption have made peptidomimetics attractive targets for drug development.

In this presentation I have discussed the design of a novel series of δ-selective opioid antagonists based on the message–address concept. The opioid peptides can be viewed to contain two elements: an essential message component that is recognized by the receptor subsite responsible for the signal transduction process and an address element that is recognized by a subsite that is unique to a single receptor type and functions to enhance binding to the site. Since the tyramine moiety in opiate structures is known to be important for activity, an identical element in Tyr[1] of the opioid peptides can be viewed as the message. A key moiety of the δ address was considered to be the phenyl group of Phe[4]. Combining the universal opioid antagonist naltrexone (5) with a strategically located address mimic afforded naltrindole (6, NTI), the first nonpeptide δ opioid receptor antagonist.

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Articles

Dexamethasone 21-(β-Isothiocyanatoethyl) Thioether: A New Affinity Label for Glucocorticoid Receptors

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The C-21 methanesulfonate ester of the synthetic glucocorticoid dexamethasone (Dex) is an efficient electrophilic affinity label of glucocorticoid receptors and exhibits irreversible antiglucocorticoid activity. In an effort to obtain other affinity labeling steroids with differing biological activities, several new derivatives of Dex were prepared which contained a reactive electrophilic substituent at various distances from the C-21 position. All compounds displayed relatively low affinity for rat glucocorticoid receptors (≤8% of that of Dex) in a cell-free competition assay. Nevertheless, one compound, dexamethasone 21-(β-isothiocyanatoethyl) thioether (Dex-NCS), appeared to be an affinity label by virtue of its ability to block the cell-free exchange binding of [^{3}H]Dex. [^{3}H]Dex-NCS was thus synthesized and reacted with cell-free receptors to give, after analysis on denaturing SDS-polyacrylamide gels, only one specifically labeled species at 98 kDa, which is the molecular weight of authentic rat glucocorticoid receptor. These data directly establish Dex-NCS as a new affinity label for glucocorticoid receptors. Data on the reactivity of Dex-NCS and the stability of [^{3}H]Dex-NCS-labeled receptors suggest that a cysteine SH group has been labeled.

Introduction

Affinity labeling of ligand-binding macromolecules gives covalent complexes which have numerous applications and can be studied under a greatly expanded variety of conditions. In the case of steroid receptor proteins, affinity labeling has been used to directly identify on denaturing SDS-polyacrylamide gels the native, mutant, and proteolyzed forms of receptor in various states of biological activity, purification, and chemical modification.[1-8] The covalent binding of the affinity label to the receptor protein is preserved during virtually all manipulations and facilitates the identification of molecules associated with the receptor (ref 7 and references therein). A classical use of affinity labels is to identify the amino acids involved in steroid binding.[8-12] A specialized use is to obtain irreversible agonists and antagonists.[13-17]

Despite the numerous applications of affinity labels for steroid receptors, the number of practical affinity labels is quite small.[18] This is not due to a paucity of methods for affinity labeling (for review, see ref 19). Rather, the discovery of new affinity labels has been hindered by the fact that most suitably modified ligands have such reduced

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binding affinities that there is negligible preferential labeling of the receptor. This problem is exacerbated by the absence of good structure–activity guidelines for the design of new affinity labels, and exceptions to the few guidelines have become common.[15,20-22] It has also proved difficult to predict whether a given derivative will be a reversible or irreversible agonist or antagonist.[14,16,21] Thus the only practical approach seems to be simply to synthesize and screen numerous chemically reactive steroidal derivatives. Using this approach, we have already synthesized several derivatives that would probe different areas of the steroid binding cavity of the receptor protein by virtue of containing reactive functional groups at various distances from the steroid nucleus.[23] Of these derivatives, only dexamethasone 21-mesylate (Dex-Mes) was particularly promising.[6,13,23] In this report, we describe the synthesis and screening of additional reactive derivatives that could react at other positions and/or with other nucleophiles. One such derivative, dexamethasone 21-(β-isothiocyanatoethyl) thioether (Dex-NCS) was found to be a new affinity label of glucocorticoid receptors.

Experimental Section

Unless otherwise indicated, all procedures were performed at 0 °C.

Chemical Materials and Methods. Instrumentation. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were not corrected. Beckman 4230 and Hewlett-Packard 8450 spectrophotometers were used to record IR and UV spectra, respectively. Low-resolution mass spectra were obtained on a V.G. Micromass 7070F [electron impact (EI) mode] or Finnigan 1015D [chemical ionization (CI) mode] spectrometer by Noel Whittaker and Wesley White of the Laboratory of Analytical Chemistry, NIDDK/NIH. Elemental analyses were performed by Atlantic Microlab., Inc. (C, N, H, Cl, Br, I) and Galbraith Laboratories, Inc. (F). The silica gel GF analytical thin layer (TLC) and preparative chromatography plates used throughout this work were purchased from Analtech, Inc.

Synthesis of Dexamethasone 21-Bromide (3). To a solution of Dex-Mes[24] (25 mg, 0.0542 mmol) in 1 mL of dried acetone was added 20 mg of KBr (0.168 mmol, 3 eq.) at room temperature. The reaction was completed after heating at 60 °C for 4 h, (TLC on silica gel with 2:1 benzene/ethyl acetate). Excess KBr was removed by filtration to give, after removal of solvent, 22.75 mg of a white solid (94% yield) which was recrystallized from MeOH to afford analytically pure solid (mp 235–237 °C). Mass spectrum, CI (NH_3), m/e: 457 (M + 2, 12), 455 (M, 10), 437 (M + 2 - HF, 100), 435 (M - HF, 93), 419 (36), 417 (29), 401 (14), 357 (39), 355 (33), 339 (26), 337 (18). Anal. ($C_{22}H_{28}O_4FBr \cdot H_2O$): C, H, Br.

Dex 21-OCO(CF_3)$_2$$C_6H_3$ (4). To a solution of dexamethasone 21-bromide (25 mg, 0.055 mmol) in 2 mL of DMF was added 2.2 equiv of KF (6.38 mg) with stirring for 5 min at room temperature. Then 2,6-bis(trifluoromethyl)benzoic acid (13.5 mg, 0.052 mmol) was added and the reaction was heated for 2 h at 50 °C, followed by stirring overnight at room temperature to complete the reaction, as determined by TLC on silica gel with 3:1 benzene–ethyl acetate. Diethyl ether (7 mL) was added and the mixture was extracted with water (3 × 5 mL) to remove most of the DMF. The ether extract was dried over Na_2SO_4 and flash evaporated to give 28.6 mg (82.5%) of a white solid which was recrystallized from MeOH to give analytically pure crystals (mp 227–229 °C).

Mass spectrum, CI (NH_3), m/e: 633 (M + 1, 42), 613 (M + 1 - HF, 100), 595 (M + 1 - HF - H_2O, 89), 577 (M - HF - 2H_2O, 17). IR ($CHCl_3$): 1725, 1660, 1600 cm^{-1}. Anal. ($C_{31}H_{31}F_7O_6$): C, H, F.

Synthesis of 2-[(*tert*-Butoxycarbonyl)amino]-1-ethanethiol. To cysteamine hydrochloride (61.2 mg, 0.539 mmol) in 450 μL of dioxane–water (80:20) under argon was added triethylamine (175 μL, 1.26 mmol) and di-*tert*-butyl dicarbonate (140 μL, 0.593 mmol) at room temperature to give a solid mass. After 10 min, 70 μL (0.5 mmol) more of triethylamine was added. The reaction was followed to completion (2.5 h) by TLC of the upper, nonaqueous layer (silica gel, $CHCl_3$–1% MeOH). The solid was dissolved by neutralization with 0.5 N HCl (1.7 mL) and extracted with ethyl acetate (3 × 2 mL). Additional 0.5 N HCl (0.5 mL, final pH = 2) was added and the organic material was extracted with ethyl acetate (3 × 2 mL). The combined extracts were dried over Na_2SO_4 and evaporated to give 83 mg (87%) of a TLC-pure, pale yellow oil with physical and spectroscopic properties identical with those in the literature.[25]

Dex 21-S(CH_2)$_2$$NHCO_2$-*t*-Bu. A solution of 2-[(*tert*-butoxycarbonyl)amino]-1-ethanethiol (2.65 equiv, 83 mg, 0.47 mmol), triethylamine (3 equiv, 75.1 μL, 0.539 mmol), and Dex-Mes (0.177 mmol) in dry acetone (5 mL) was reacted for 16 h at 0 °C and then 2 h at room temperature. The reaction solution was diluted 1:2 in acetone, and 0.5 N HCl (3 mL = 9 equiv, to pH = 4) and H_2O (1 mL) were added. The acetone was removed under reduced pressure, and 30 mL of H_2O at 0 °C was added to give a white precipitate. This precipitate was extracted with CH_2Cl_2 (3 × 17 mL) and ethyl acetate (3 × 10 mL). The extracts were combined, dried over Na_2SO_4, and reduced in volume. Purification by preparative TLC (silica gel, $CHCl_3$–5% MeOH) afforded 60 mg (62%) of a TLC-pure solid with physical and spectroscopic properties identical with those in the literature.[25]

Dex 21-S(CH_2)$_2$$NH_2$·HCl. Dex 21-S($CH_2$)$_2$$NHCO_2$-*t*-Bu (50 mg, 0.09 mmol) was reacted with 9.6 equiv (577 μL, 0.865 mmol) of 1.5 M HCl in AcOH for 15 min at room temperature (followed by TLC on silica gel with $CHCl_3$–5% MeOH). The hydrochloride was precipitated by diethyl ether (2.5 mL), collected by centrifugation, and washed with 2 × 3 mL of Et_2O to afford a white solid (31 mg, 70% yield) which was used immediately.

Dex 21-S(CH_2)$_2$NCS (6). A mixture of Dex 21-S-(CH_2)$_2$$NH_2$·HCl (31 mg, 63.6 μmol), 1.3 mL of water, 3.2 mL of $CHCl_3$, and 4 mL of saturated Na_2CO_3 was vigorously stirred for 15 min, and then treated with 7 μL (10.56 mg, 92 μmol) of redistilled thiophosgene. After vigorous stirring for 30 min, the $CHCl_3$ layer was separated, washed with water (3 × 7 mL), dried over Na_2SO_4, and evaporated to give, after preparative TLC (silica gel, diethyl ether), 23 mg of a white solid (73.2 % yield). This material was crystallized from MeOH to give analytically pure material (mp 174–176 °C). Mass spectrum, CI (NH_3), m/e: 494 (M + 1, 56), 474 (M + 1 - HF, 74), 456 (M + 1 - HF - H_2O, 100), 438 (36), 428 (34), 420 (46). IR ($CHCl_3$): 2075 (NCS), 1715, 1650 cm^{-1}. Anal. ($C_{25}H_{32}O_4FNS_2$): C, H, N, F, S.

Synthesis of [^{3}H]Dex 21-S(CH_2)$_2$NCS. [^{3}H]Dex 21-S-(CH_2)$_2$$NHCO_2$-*t*-Bu. [^{3}H]Dexamethasone 21-mesylate (44.7 Ci/mmol, Du Pont–New England Nuclear, 1 mL = 1 mCi = 2.23 × 10^{-5} mmol) in ethanol was concentrated to dryness under a

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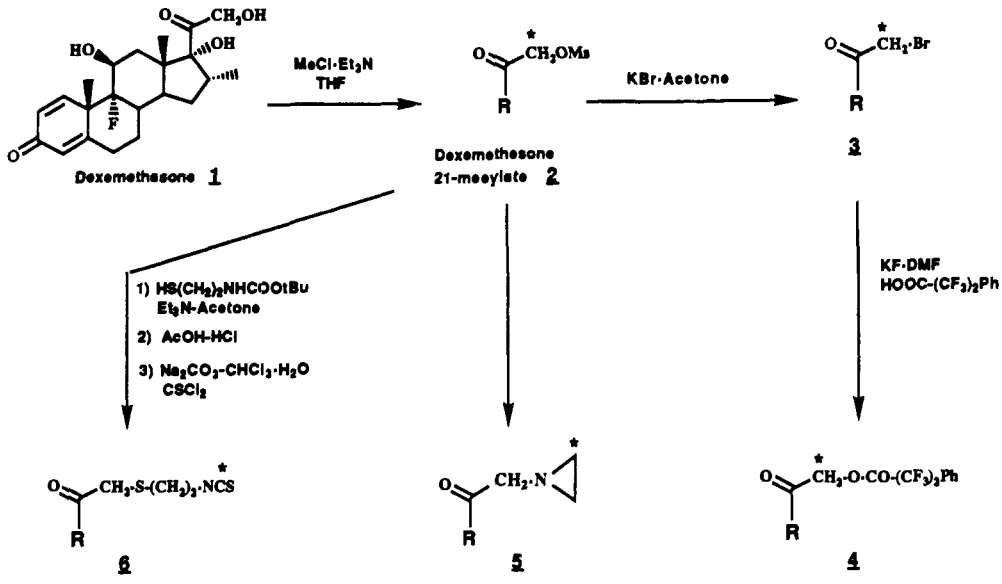
Scheme I.[a] Synthesis and Structure of Derivatives of Dexamethasone (R = tetracyclic nucleus of dexamethasone)[a]



[a] The asterisk (*) indicates the reactive position of the molecule.

stream of argon and redissolved in 1 mL of acetone at 0 °C. A solution of $HS(CH_2)_2NHCOO$-*t*-Bu (0.1 mg, 5.65×10^{-4} mmol = 25 equiv) in acetone (0.5 mL) containing 0.2 μL of Et_3N (1.43×10^{-3} mmol = 64 equiv) was added and the reaction solution stirred at 0 °C for 2 h and then at room temperature for 1 h. The product was separated by preparative TLC (silica gel, $CHCl_3$–5% MeOH), extracted with EtOH, and concentrated under a stream of argon. This solution (95% of the initial radioactivity = 2.12×10^{-5} mmol) was stored at −20 °C.

[^{3}H]Dex 21-S$(CH_2)_2$NCS. Crude [^{3}H]Dex 21-S-$(CH_2)_2NHCO_2$-*t*-Bu in EtOH was evaporated to dryness under argon and treated with 1 μL of 1.5 M HCl in AcOH (1.5×10^{-3} mmol) at room temperature for 10 min, and then at 0 °C for 5 min. Two drops of saturated Na_2CO_3 solution were added to raise the pH to 8–9 followed by 0.5 mL of water and 1.5 mL of $CHCl_3$ and vigorous stirring for 30 min. Thiophosgene (0.01 μL, 1.3×10^{-4} mmol) dissolved in 2 mL of $CHCl_3$ was added and the two-phase reaction was stirred vigorously for 1 h at room temperature. The organic phase (~5 mL) was separated, washed with water (3 × 1 mL), and concentrated. The product was separated by preparative TLC (silica gel, diethyl ether) and extracted with EtOH. The solvent was removed under a stream of argon and the steroid was redissolved in 2.5 mL of toluene (36.6% of the initial radioactivity recovered = 8.18×10^{-6} mmol). A second preparative TLC yielded an 87% yield of the applied radioactivity in 3 mL of toluene. The steroidal isothiocyanate was ~90% pure by TLC (three developments in diethyl ether; material was visualized by fluorography) and was stored at −20 °C.

Biochemical Materials and Methods. Materials. [^{1}H]Dex and cortisol (Sigma) and [^{3}H]Dex (40–47 Ci/mmol, Amersham Corp.) were commerciallly available. TAPS (Ultrol grade) was purchased from Behring Diagnostics; HEPES was from Calbiochem. Reagents for SDS-polyacrylamide gel electrophoresis including Coomassie Blue R-250 were from Bio-Rad. The molecular weight standards (obtained from Pharmacia P-L Biochemicals) were as follows: P, phosphorylase b, M_r 97 400; B, albumin, M_r 66 300; O, ovalbumin, M_r 45 000; C, carbonic anhydrase, M_r 30 600; S, soybean trypsin inhibitor, M_r 21 500; and L, α-lactalbumin, M_r 14 400. Fluorescent Ult-Emit autoradiography marker was from Du Pont–New England Nuclear.

All ^{3}H-labeled samples were counted in Hydrofluor (National Diagnostics) at 40–55% counting efficiency in a Beckman 5801 liquid scintillation counter with automatic cpm-to-dpm conversion.

Buffers and Solutions. TAPS buffer (pH 8.8 or 9.5) was composed of 25 mM TAPS, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% glycerol. HEPES buffer (pH 7.5) was composed of 25 mM HEPES, 1 mM EDTA, and 10% glycerol. The pH of each buffer was adjusted at 0 °C with sodium hydroxide. Two-fold concentrated SDS sampler buffer contained 0.6 M Tris (pH 8.85), 2% SDS, 0.2 M dithiothreitol, 20% glycerol, and bromphenol blue.

Cells and Preparation and Labeling of Receptors. The growth of HTC cells in spinner and monolayer cultures of S77 medium supplemented with 5% fetal and 5% newborn bovine serum (Biofluids) and 0.03% glutamine has been described.[26] HTC cell cytosol containing the steroid-free receptors was prepared, stored in liquid N_2, and labeled at 0 °C with [^{3}H]Dex ± excess [^{1}H]Dex ± 20 mM Na_2MoO_4.[3,11] Free steroid was removed by adding a 10% dextran-coated charcoal suspension in buffer (added volume = 20% of reaction volume). Nonspecific binding/labeling equaled that seen with excess [^{1}H]Dex.

Polyacrylamide Gel Electrophoresis. The preparation of samples for gels and the procedures for electrophoresis are as described.[27] Constant-percentage acrylamide gels (10.5–11% with a 1:40 ratio of bisacrylamide to acrylamide) were run in a water-cooled (15 °C) Protean II slab gel apparatus (Bio-Rad) at 30 mA/gel. Gels were fixed and stained in 50% methanol, 7.5% acetic acid containing 0.01% Coomassie Blue R-250, destained in 10% methanol, 7.5% acetic acid, incubated for 1 h in Enhance (Du Pont–New England Nuclear) and 30–60 min in 10% Carbowax PEG 8000 (Fisher) with constant shaking at room temperature, dried on a Bio-Rad Model 443 slab gel drier at 60 °C with a sheet of dialysis membrane backing (Bio-Rad) directly over the gel to prevent cracking, and fluorographed for 7–12 days with Kodak X-OMAT XAR-5 film.

Results

Selection and Synthesis of Potential Affinity Labeling Steroids. Previous studies indicated that C-21 derivatives of dexamethasone (1, Dex; see Scheme I) containing a reactive electrophilic substituent can afford both high yields of affinity-labeled glucocorticoid receptor and relatively high specificity for the labeling of receptor.[13,15,28] In our search for new affinity labels, we decided to further modify the C-21 position of Dex since it can accommodate substituents with relatively little effect on steroid binding affinity to the receptor.[25] Four derivatives were chosen to probe for different amino acids at various distances from the C-21 position. They were α-keto bromide **3** and bis-[(trifluoromethyl)phenyl] ester[29] **4**, both of which should

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be displaced by nucleophilic attack at the C-21 carbon but with different selectivities from that seen for the known affinity label Dex-Mes[6,7,13] (2) due to differences in hard and soft acid–base reactivity,[30] aziridine 5, which would react at a position that is two bonds removed from the C-21 carbon, and isothiocyanate 6, which would react at a position that is five bonds removed from the C-21 carbon. The synthesis of 3, 4, and 6 proceeded uneventfully (Scheme I). Unfortunately, several approaches (reaction of aziridine with 2 in DMF,[31] 2-chloramine reaction with 2 followed by base-catalyzed cyclization, and reaction of 2 with ethanolamine followed by tosyl chloride and base) failed to yield 5.

Chemical Reactivity of Potential Affinity Labels. Dex-Br (3) was slightly more reactive with simple nucleophiles in organic solutions than was Dex-Mes (2). Thus both derivatives displayed essentially no reaction at 0 °C with simple thiols in acetone solutions but reacted within seconds in solutions containing triethylamine. The reaction of 3 was four times faster than 2 with acetate anion (reaction in acetone solutions at 0 °C was completed in ~22 vs ~93 h) and twice as fast with *n*-propylamine in DMF at 64 °C (~1 vs ~2 h). As with 2,[24] no reaction of 3 was observed with ethanol, phenol, imidazole, or guanidine (data not shown). Thus 3 should preferentially react with the SH group of cysteines when bound to the receptor.

The esters of 2,6-bis(trifluoromethyl)benzoic acid are reported to be quite reactive in a reaction that involves S_N2 displacement at the alcoholic carbon as opposed to addition/elimination at the carbonyl carbon.[29] It was therefore surprising to find that the steroidal ester 4 was completely unreactive toward thiols, ethanol, phenol, *n*-propylamine, imidazole, guanidine, or acetic acid under neutral or basic conditions at 0 °C or at room temperature (data not shown). Thus 4 may not react with any amino acids of the receptor unless local differences in pH, polarity, and strain render 4 more reactive.

Isothiocyanate 6 in acetone at 0 °C reacts rapidly (10 min) with *n*-propylamine, slowly (24 hr) with β-mercaptoethanol, and not at all with ethanethiol, ethanol, phenol, imidazole, guanidine, or acetic acid. However, an extremely slow reaction of the isothiocyanate with EtOH was seen upon storage at 0 °C. Under basic conditions, 6 reacts with thiols in a matter of seconds at 0 °C (data not shown). Thus the preferred reacting group in the receptor would be the SH group of cysteines and possibly the NH_2 group of lysines.

Properties of Potential Affinity Labels in Intact Cells. We have previously found that a combination of several assays are required to identify potential affinity labels for the glucocorticoid receptor.[13,19,32] The first two that we used were both in intact rat hepatoma tissue culture (HTC) cells: (1) quantitation of the affinity for whole cell receptors and (2) determination of the dose–response curve for the induction of tyrosine aminotransferase (TAT) enzyme activity. A left shift in the dose–response curve, for either agonist or antagonist activity, relative to the whole-cell receptor occupancy curve is consistent with the affinity labeling of receptors. In our whole-cell affinity assay, conducted for 30 min at 37 °C, the order of apparent affinities was Dex > Dex-NCS (6) > Dex-Br (3) > Dex ester (4) (Table I). In TAT induction experiments, Dex ester was a full antagonist while Dex-Br and Dex-NCS were partial agonists with the same EC_{50} (i.e., concentration of steroid required for 50% of agonist activity) (Table I). For both Dex-Br and Dex ester, there was good agreement between the fold excess required to cause 50% inhibition of Dex induction of TAT (i.e., IC_{50}) and the fold excess required to cause 50% inhibition of Dex binding to whole cell receptors (=1/relative affinity)(Table I). This is consistent with the absence of any irreversible antagonist activity and of any covalent labeling of the receptors.[13] The reasonable agreement of the EC_{50} and IC_{50} values for Dex-Br (3) argue that this steroid also

Table I. Biological Properties of Synthetic Steroids[a]

steroid	relative affinity for receptors: in whole cells	relative affinity for receptors: in cell extracts	biological activity re induction of TAT: type	EC_{50}	IC_{50}
Dex-Br (3)	0.063	0.021	partial agonist (20%)	8.3	13
Dex ester (4)	0.0087	0.079	full antagonist		77
Dex-NCS (6)	0.31	0.028	partial agonist (48%)	9.1	44

[a] The values for the relative affinity [$K_a^{steroid}/K_a^{Dex}$] of each steroid for glucocorticoid receptors in cell-free and whole-cell competition assays[20,23] were calculated from the ratios of steroid concentrations (Dex and steroid) giving 50% inhibition of [^{3}H]Dex binding and then corrected by the method of Rodbard[33] to account for the partial occupancy of receptors by the uncompeted [^{3}H]Dex. The reciprocal (1/relative affinity) gives the fold excess of steroid that is required to give the identical levels of bound receptor as seen with a given concentration of Dex. The biological activity of each steroid was determined as described in the legend for Figure 1. The amount of agonist activity (in parentheses) is the percent of full TAT induction (by 10^{-5} M Dex) that is seen with 10^{-5} M steroid. The EC_{50} is the concentration of steroid required for 50% of TAT induction by that steroid; the IC_{50} is the fold excess of steroid that is required for half-maximal reduction of TAT induction by Dex (usually 10^{-7} M). The data given are the average values from two to four experiments.

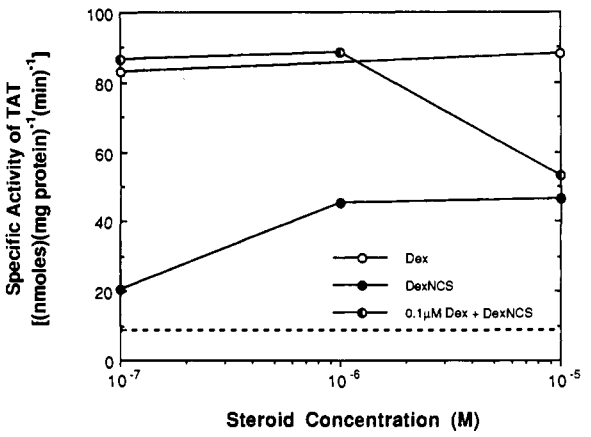

Figure 1. Biological activity of Dex-NCS in HTC cells. Duplicate monolayer cultures (60-mm dishes) were treated with Dex (○), freshly dissolved Dex-NCS (●), or 1×10^{-7} M Dex plus varying concentrations of Dex-NCS (◐) for 17 h at 37 °C. All steroid solutions were prepared in absolute EtOH and diluted 1:100 to give a final EtOH concentration of 1%. Basal activity was determined by treating cells with medium containing 1% EtOH (---). The cells were harvested and ruptured and the specific TAT enzyme activity was determined as described.[13] The average of duplicate plates was then plotted against the concentration of that steroid present in varying amounts. Similar results were obtained in a second experiment.

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Table II. Effect of pH during Dex-NCS Incubation on the Subsequent Inhibition of Exchange Binding with [^{3}H]Dex[a]

	% inhibition of exchange binding seen after [^{3}H]Dex incubation for			
pH	1 h	3 h	5 h	20 h
8.8	43.8 (17.7, n = 4)	37.0 (14.6, n = 4)	34.1 (11.2, n = 4)	
7.6	63.2 (18.7, n = 9)	60.5 (18.3, n = 9)	56.6 (19.1, n = 9)	45.7 (17.9, n = 7)

[a] Exchange binding assays were conducted in duplicate as described in the legend of Figure 2 with final solution pHs of 8.8 and 7.6 at 0 °C. The amount of exchangable binding in the assay was determined from preincubations with 60 or 120 nM cortisol; Dex-NCS concentrations were 290 or 580 nM. The data listed are the average values, with the standard deviation (SD) and the number experiments (n) being given in parentheses.

does not exhibit any irreversible agonist activity. With Dex-NCS, there is a fair correlation between the fold excess of steroid that is required for 50% inhibition of Dex binding to whole cell receptors (=1/relative affinity) (Table I) and for half-maximal induction of TAT (i.e., EC_{50}) (Table I and Figure 1). However, both concentrations are significantly lower than that which is required to give half-maximal antagonist activity (Table I and Figure 1). This result suggests that the agonist activity of Dex-NCS may involve irreversible binding of the steroid to whole-cell receptors.

Properties of Potential Affinity Labels in Cell-Free Extracts. Comparisons of the relative affinity of steroids for whole-cell receptors at 37 °C and for cell-free receptors at 0 °C can be very informative concerning the potential affinity labeling properties of a steroid. While there are several exceptions,[20,21] a higher affinity for whole-cell receptors than for cell-free receptors would be expected for an affinity-labeling steroid since the very slow rate of dissociation of Dex at 0 °C in cell-free systems can mask the irreversible effects of affinity labeling. An affinity in the whole-cell assay which is lower than in the cell-free assay is usually indicative of metabolism of the steroid to a less active steroid.[22] By these criteria, the data of Table I suggest that bromide **3** is not metabolized in whole cells and is not an affinity label, thus confirming our above conclusions. These data also suggest that ester **4** is metabolized to a lower affinity steroid, and that isothiocyanate **6** is covalently labeling the receptors of intact cells.

Strong, but not conclusive,[34] evidence of an affinity label can be obtained in a cell-free wash-out assay by determining whether the binding of the prospective compound can block the subsequent exchange binding to receptors.[13,32] In such an assay, all of the prebound ester **4**, and most if not all of bromide **3**, is exchangable (data not shown). With the isothiocyanate **6**, however, there is appreciable inhibition of exchange binding (Figure 2) with less nonexchangable binding occurring after incubations of the isothiocyanate and receptor at higher pHs (Table II). Further support for the affinity labeling of receptors by the isothiocyanate comes from the observation that this inhibition of exchange binding is blocked by excess cortisol (Figure 2).

Cell-Free Labeling of Receptors with [^{3}H]Dex-NCS. In order to obtain definitive proof that the isothiocyanate does affinity label glucocorticoid receptors, the ^{3}H-labeled form of the steroid was synthesized in ≥90% purity. With this [^{3}H]Dex-NCS, a labeled species of 98 000 molecular weight was observed (Figure 3). The facts that this is the size of HTC cell glucocorticoid receptors on these gels[3,7,27] and that the covalent labeling of only this band is inhibited by coincubation with excess nonradioactive Dex argue that [^{3}H]Dex-NCS is an affinity label of the receptor.

The labeling of the receptor, and of all the other nonreceptor molecules in Figure 3, by [^{3}H]Dex-NCS entails a relatively labile chemical linkage. Heating of the samples

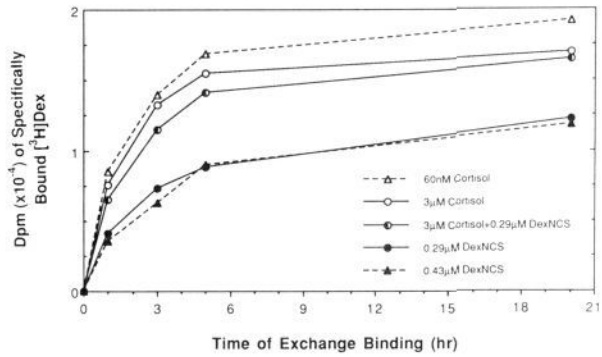

Figure 2. Inhibition of [^{3}H]Dex exchange binding to cell-free receptors prebound with Dex-NCS. As in the previously described exchange assay,[23] crude HTC receptors in TAPS buffer (final pH = 7.6) were preincubated for 3 h at 0 °C with the indicated concentrations of cortisol (△, ○), Dex-NCS (●, ▲), or cortisol plus Dex-NCS (◐). After addition of activated charcoal to remove free steroid, and to inactivate steroid-free receptors, followed by centrifugation to remove the activated charcoal, the preincubated cytosols were adjusted to 1.9×10^{-8} M [^{3}H]Dex ± 575-fold excess unlabeled Dex. After subsequent incubation for the indicated time, activated charcoal was again added to remove free steroid and the amount of specifically bound [^{3}H]Dex (=total − binding in presence of unlabeled Dex) that was formed by exchange binding was plotted versus the time of exchange.

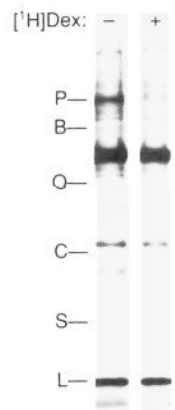

Figure 3. Fluorograph of [^{3}H]Dex-NCS-labeled HTC cell receptors. A 50% solution of HTC cell cytosol in pH 7.5 HEPES buffer was incubated with 3.1×10^{-7} M [^{3}H]Dex-NCS ± 30-fold excess unlabeled Dex for 2.5 h at 0 °C. An aliquot was mixed with an equal volume of 2× SDS sample buffer at room temperature and then electrophoresed on an SDS-polyacrylamide gel (10.5%). The above fluorograph of the dried gel was obtained after a 13 day exposure of the film.

in SDS sample buffer for 5 min at 100 °C causes a 94% reduction in the radioactivity recovered from gels, compared to unheated samples. No such an effect of temperature was observed for the recovery of Dex-Mes labeling (data not shown). The exchange binding assay above suggested that the isothiocyanate gave more covalently labeled receptor as the pH of the reaction was decreased from 8.8. Similarly, the amount of specifically ^{3}H-labeled,

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98 kDa receptors is increased at pHs lower than 8.8 (data not shown).

Discussion

Currently there exists only one electrophilic affinity label for glucocorticoid receptors. This is Dex-Mes, which displays irreversible antiglucocorticoid activity.[1,9,13] We have now prepared several new potential electrophilic affinity labels which could have irreversible agonist or antagonist activity. One of these, Dex-NCS (6 in (Scheme I) fulfills all of the requirements for being a new affinity label. (1) Dex-NCS has reasonable affinity for glucocorticoid receptors in cell-free extracts and in whole cells (Table I). (2) It is biologically active for the induction of the glucocorticoid-inducible enzyme TAT (Table I and Figure 1). (3) The apparent whole cell affinity of Dex-NCS for receptors is higher than the cell-free affinity (Table I), in keeping with the differences in off-rates of covalently vs noncovalently bound steroid being more noticeable at the higher temperatures of the whole-cell assay.[20] (4) Receptors prebound with Dex-NCS have a markedly reduced capacity to exchange–bind added [^{3}H]Dex (Figure 2). (5) The labeling of a species with the same molecular weight as the receptor (i.e., 98 kDa) by [^{3}H]Dex-NCS is covalent in that it is not dissociated on a denaturing SDS-polyacrylamide gel (Figure 3). (6) The covalent labeling by [^{3}H]Dex-NCS of only this 98 kDa species is inhibited by excess nonradioactive glucocorticoid steroid (Figure 3).

The labeling efficiency of the receptor by [^{3}H]Dex-NCS is currently low (7–16% at pH 7.5). However, it is also not clear that we have maximized the conditions for labeling. The results of the exchange binding assay suggest that ~45% of the receptors are being covalently labeled (Table II). Thus it is possible that the instability of covalent adduct under the conditions of analysis by SDS-PAGE (see above) may artifactually lower the yield of the covalently labeled receptors.

This same instability, however, provides a clue as to the functional group that is labeled by Dex-NCS. In particular, it would be predicted that, under weakly basic conditions, the instability of the reaction products with various functional groups would follow an order of instability of thiol ≈ acid > alcohol > amine.[35] Our observed order of chemical reactivity of various functional groups was thiol > amine ≫ alcohol ≥ acid. These two lines of evidence suggest that the amino acid of the receptor which most likely has been labeled by Dex-NCS (Figure 3) is a cysteine. Two lines of evidence argue that Cys-656, which is labeled by Dex-Mes,[9] is not being labeled by Dex-NCS. First, both Dex-Mes and Dex-NCS react essentially with only ionized thiols but the labeling by Dex-NCS increases at pH < 8.8 (Table II) while Dex-Mes labeling was maximal at pH 8.8.[2] Second, the reactive position of Dex-NCS is further away from the C-21 of Dex than is the reactive position of Dex-Mes. Thus it would appear that Dex-NCS is labeling a thiol group other than Cys-656. An attractive candidate is the second thiol that can be linked with Cys-656 with either MMTS[11] or arsenite.[36-38] Further work is required to confirm this hypothesis. Thus studies with Dex-NCS promise to expand our knowledge of the steroid binding cavity of glucocorticoid receptors.

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Registry No. 1, 50-02-2; 1 21-S$(CH_2)_2$NHCO$_2$Bu-*t*, 73816-22-5; 1 21-S$(CH_2)_2$NH$_2$·HCl, 131567-17-4; 3, 131567-18-5; 4, 131567-19-6; 6, 131567-20-9; [^{3}H]dexamethasone 21-mesylate, 131567-21-0; [^{3}H]dexamethasone 21-S$(CH_2)_2$NHCO$_2$Bu-*t*, 131567-22-1; [^{3}H]-dexamethasone 21-S$(CH_2)_2$NCS, 131567-23-2; 2,6-bis(trifluoromethyl)benzoic acid, 24821-22-5; cysteamine hydrochloride, 156-57-0; 2-[(*tert*-butoxycarbonyl)amino]-1-ethanethiol, 67385-09-5.

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Synthesis and Antiviral Activity of 5-Heteroaryl-Substituted 2′-Deoxyuridines

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The synthesis of 5-heteroaryl-substituted 2′-deoxyuridines is described. The heteroaromatics were obtained from three different 5-substituted 2′-deoxyuridines. Cycloaddition reaction of nitrile oxides on the 5-ethynyl derivative 1 gave the isoxazoles **4a–e**. The thiazole derivatives **14a–c** were obtained from the 5-thiocarboxamide **11**, while 5-pyrrol-1-yl-2′-deoxyuridine (**17**) could be synthesized directly from 5-amino-2′-deoxyuridine. The compounds were evaluated for antiviral activity. Selective activity against herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV) was noted for 5-(3-bromoisoxazol-5-yl)-2′-deoxyuridine (**4c**). The compound was inactive against herpes simplex virus type 2, cytomegalovirus, and thymidine kinase (TK)-deficient mutants of HSV-1 and VZV, which indicates that, most likely, its antiviral activity depends on phosphorylation by the virus-specified TK.

Introduction

Substitution of the 5-methyl group of thymidine by other groups has led to a multitude of compounds with either cytostatic or antiviral properties,[1] i.e. 5-fluoro-2′-deoxyuridine, 5-ethyl-2′-deoxyuridine, 5-iodo-2′-deoxyuridine, and 5-(*E*)-(2-bromovinyl)-2′-deoxyuridine. The biological activity of these compounds is dependent on their conversion to the 5′-O-phosphorylated metabolites. The enzymes that convert these compounds to their 5′-*O*-monophosphates may be of either cellular or viral origin. 5′-O-Phosphorylation of 5-fluoro-2′-deoxyuridine affords the 5-fluoro-2′-deoxyuridine 5′-monophosphate, a potent inhibitor of thymidylate synthetase and a widely used

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