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Identification of antitumor sulfonylurea binding proteins of HeLa plasma membranes

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Abstract

Plasma membranes of cultured HeLa S cells bound the tritiated antitumor sulfonylurea [^{3}H]LY181984 with high affinity (K_d of about 25 nM). The number of binding sites, estimated to represent 30 to 35 pmol/mg protein, would represent a low abundance protein of the total plasma membrane proteins. The binding proteins appeared to contain one or more thiols in the binding site as high affinity binding of [^{3}H]LY181984 was reduced by treatment with the covalent thiol blocking reagent, *N*-ethylmaleimide (NEM), or by oxidation with dilute hydrogen peroxide but was protected by glutathione or dithiothreitol. Elimination of binding of [^{3}H]LY181984 by NEM was prevented by excess unlabeled LY181984 (an active sulfonylurea) but less so by excess LY181985 (an inactive sulfonylurea). The binding proteins were specifically labeled with thiol reagents following reaction of unprotected thiols with unlabeled thiol reagents. Binding proteins at ca. 34 kDa were labeled. Plasma membrane proteins after solubilization with SDS under strongly reducing conditions still bound sulfonylurea. [^{3}H]LY181984 binding to plasma membrane proteins resolved on SDS-PAGE correlated as well with proteins in the 30–40 kDa range.

Keywords: Diarylsulfonylurea; Sulfonylurea; Antitumor drug; Binding protein; (HeLa cell)

1. Introduction

In a previous communication [1], the existence and subcellular location of binding sites for the antitumor sulfonylureas were established using equilibrium dialysis and a radiolabeled antitumor sulfonylurea, [^{3}H]LY181984, with plasma membrane vesicles of HeLa S cells. HeLa S cells were sulfonylurea-responsive, could be cultured readily and plasma membranes of good yield and fraction purity could be prepared.

The sulfonylureas as antitumor agents were identified initially from a program of screening against in vivo murine solid tumors that had been implanted subcutaneously [2–4]. Despite considerable clinical data, the site and mechanism of action of the antitumor sulfonylureas is unknown [4,5]. The drugs have been reported to be membrane active and weak uncouplers of mitochondrial oxidative phosphorylation [6]. The latter activity, however, is given as well by sulfonylureas inactive as antitumor agents and may be unrelated to their antitumor action [7]. There is no evidence for cell cycle specificity of the drug, no inhibition of DNA, RNA or protein synthesis and no mechanistic parallels to other known antitumor agents [3–7]. Yet the chemotherapeutic opportunities afforded by the sulfonylureas might be expected to be expanded were basic information available on their molecular mode of action. In this report, sulfonylurea protection of inactivation of sulfonylurea binding was used as an approach to identification of potential binding proteins of the HeLa plasma membrane.

Abbreviations: biotin maleimide, *N*-biotinoyl-*N'*-(6-maleimidohexanoyl)hydrazide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; HSA, human serum albumin; LY181984, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea; LY181985, *N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indoylphosphate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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2. Materials and methods

2.1. Equilibrium binding

Tritium-labeled *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea ([^{3}H]LY181984) (Lot 497-1-291 spe-

cific activity 2898 mCi/mg) was obtained from Lilly Research Laboratories, Indianapolis, IN and diluted with dimethylsulfoxide. For binding studies, a multi-cell rotating teflon cell equilibrium dialyzer (Spectrum Equilibrium Dialyzer, Spectrum Medical Instruments, Los Angeles, CA) with a dialyzing volume of 1 ml and a 47 mm diameter membrane (12 to 14 kDa molecular mass cut off) area was used. The dialysis membranes were prepared by soaking in water for 30 min followed by 30% ethanol for 30 min and several changes of distilled water. Both sides of the chamber were supplied with 0.5 ml final volume of a buffer solution containing 25 mM Tris, 100 mM potassium chloride, 5 mM magnesium chloride and 1 μM dithiothreitol, pH 7.4. To one side of each chamber was added the [^{3}H]LY181984 in DMSO and the fraction to be evaluated. An equivalent amount of DMSO was added to the opposite side of the chamber and equilibrium established by rotating the cells at 25° C overnight. A 100 μl sample was withdrawn from each chamber and radioactivity was determined by liquid scintillation spectrometry. Radioactivity was determined at the 99% confidence level. Determinations were in triplicate to increase the confidence of specific binding to about $\pm$5%.

2.2. Growth of HeLa cells

HeLa S cells were grown as described [1] on minimal essential medium (S-MEM) (Jolik modified) with glutamine (244 mg/l) and phosphate (1.3 g/l Na_2HPO_4) plus 5% donor horse serum but without $CaCl_2$. Gentamycin sulfate (50 mg/l) and sodium bicarbonate (2 g/l) were added.

2.3. Preparation of plasma membranes from HeLa cells

The procedure described for preparation of plasma membranes from HeLa cells has been described previously [8,9] and is repeated here. Cells were collected by centrifugation for about 12 min at 2000 rpm. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10 to 30 min to swell the cells. Homogenization was with a Polytron homogenizer for 30 to 40 s at 10500 rpm using a PT-PA 3012/23 or ST-probe and 7 to 8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1000 rpm ($175 \times g$) to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at $1.4 \cdot 10^6$ g min (e.g., 1 h at $23500 \times g$) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approx. 1 ml per pellet from $5 \cdot 10^8$ cells. The resuspended membranes were then loaded onto an aqueous two-phase partitioning system constituted on a weight basis.

The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) polyethylene glycol 3350 (Fisher), and 5 mM potassium phosphate, pH 7.2. The plasma membrane-enriched microsome fraction (1 g) was added to the two-phase system and the weight was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4° C). The phases were separated by centrifugation at 750 rpm ($150 \times g$) in a Sorvall HB 4 rotor for 5 min. The upper phases, enriched in plasma membranes, were carefully withdrawn with a pasteur pipette, diluted 5-fold with 1 mM sodium bicarbonate and the membrane were collected by centrifugation ($10000 \times g$ in a HB rotor for 30 min). The purity of the plasma membranes was determined to be 90% by electron microscopy and analysis of marker enzymes. The yield was 20 mg plasma membrane protein from 10^{10} cells.

2.4. PAGE analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was according to Laemmli [10] using a Bio-Rad Miniprotean II gel system. For binding of [^{3}H]LY181984, the minigels were loaded with 10 μg of HeLa plasma membrane protein per lane and were soaked overnight in 50 mM Tris-Mes, pH 7, containing 10 mM dithiothreitol and 0.3 μCi [^{3}H]LY181984 in a total volume of 5 ml contained in a Ziplock 1 pint (5 in $\times$ 7 in) bag with shaking. The gels were rinsed with water and sliced with a razor blade into 2.5 mm segments. Radioactivity was determined for each individual segment. Values for a control gel, without plasma membrane, were subtracted.

Two-dimensional gel electrophoresis was modified from O'Farrell [11]. In the first dimension, a 3.6% final acrylamide mixture (28.38% acrylamide: 1.62% *N,N'*-methylenebisacrylamide) containing 9.5 M urea, 2% (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate) (CHAPS), 2% ampholyte mixture (2 parts pH 3–10 to 1 part pH 5–7), 10% ammonium persulfate and TEMED was poured into 3 mm $\times$ 13 mm (i.d.) pyrex tubes and overlayed with water saturated isobutanol. The gels were allowed to polymerize for 1–2 h. The polymerized gels were rinsed with deionized water and placed in a Hoefer Scientific GT series tube gel electrophoresis unit and overlayed with 20 μl of urea extraction buffer containing 2.9 g ultra-pure urea, 2.0 ml 10% CHAPS, 0.25 ml ampholyte mixture, 0.1 ml 1 M dithiothreitol and 1.02 ml deionized water. The lower buffer chamber was filled with anolyte buffer consisting of 10 mM imino diacetic acid and the upper reservoir contained 10 mM ethylene diamine as the catholyte buffer. The gels were then prefocused at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min.

To solubilized membrane proteins, 150 μl of a solution containing 9.5 M urea, 1% SDS and 1% β-mercaptoethanol were added followed by 50 μl of a solution

containing 10% CHAPS and 10% ampholyte mix and 200 μl of a solution containing 2% CHAPS, 0.5% ampholyte mix and 9.5 M urea to a final volume of 400 μl. The mixture was incubated for 1–2 h at room temperature and then centrifuged at 100000 × *g* for 30 min (SW 50.1 rotor, Beckman).

The solubilized sample was applied to the top of the gel and overlayed with 20 μl of urea extraction buffer. The sample was focused at 400 mV for 16 h and at 800 mV for an additional 4 h. The last 20 min of focusing was at 1600 mV.

For the second dimension, the tube gels were extruded and embedded [11] on a horizontal 10% SDS-PAGE gel and separated as described above [10].

2.5. Binding of N-[^{14}C]ethylmaleimide

To identify the proteins containing sulfonylurea protected thiol groups indicated from binding studies, HeLa plasma membranes (100 μg membrane protein) resuspended in 250 μl of buffer containing 50 mM Tris-Mes, pH 7, were incubated with 1 μM LY181984 (or 1 μM LY181985) in DMSO or DMSO alone (0.1% final concentration) for 15 min at 37° C followed by 500 μM unlabeled *N*-ethylmaleimide for 15 min at 37° C to react with all unprotected thiol groups. Human serum albumin (1% in 50 mM Tris-Mes, pH 7) was added to quench remaining unreacted *N*-ethylmaleimide and to facilitate release of sulfonylureas. The membranes were washed twice and then collected by microfuge centrifugation and resuspended a second time in buffered albumin and collected by centrifugation. Finally, the washed membranes were reacted with 5 μCi *N*-[^{14}C]ethylmaleimide to label any thiol groups that may have been protected by the sulfonylurea. The reaction was quenched after 15 min at 37° C with 0.5 mM DTT and the membranes were collected by centrifugation. Plasma membrane proteins were displayed on 9 cm 10% gels and analyzed by autoradiography with X-Omat S Kodak film.

2.6. Biotin maleimide labeling

Plasma membrane vesicles were treated for 15 min at 37° C in 20 mM Tris-Mes, pH 7.0, containing 0.2 mM NaOH and 2 mM KCN with or without sulfonylurea as described above. The plasma membrane vesicles were then reacted at 37° C for 15 min with 1 mM unlabeled NEM, transferred to 4° C, diluted with cold buffer containing 1% human serum albumin and collected by centrifugation. After pelleting, biotin maleimide (2 mM) was added to label thiols exposed following the sulfonylurea treatment. After an additional incubation for 15 min at 37° C, membranes were pelleted at 4° C, 0.5 mM dithiothreitol was added to quench unreacted biotin maleimide, washed and membrane proteins were displayed by SDS-PAGE on 10% gels. Biotinylated bands were identified after transfer to nitrocellulose by reaction with avidin-alkaline phosphatase (1 μg/ml) with visualization with NBT/BCIP.

3. Results

High affinity binding of [^{3}H]LY181984 was enhanced by reducing conditions during binding provided by 10 μM dithiothreitol compared to the oxidized conditions provided by 0.015% H_2O_2 (Fig. 1). The K_d for binding under reducing conditions was determined to be about 32 nanomolar and *n*, the number of specific binding sites, was determined to be between 1 and 2 pmol/50 μg protein (see also Table 1). With H_2O_2, the affinity of binding was reduced ($K_d = 0.1$ μM) but the number of sites appeared to be similar (Fig. 1).

Treatment with the thiol reagent, *N*-ethylmaleimide (NEM), also influenced [^{3}H]LY181984 binding. However, with NEM the number of sites exhibiting high affinity binding was reduced (Table 1). Sites with an average K_d of ca 30 nM were equivalent to 1.4 pmol/50 μg protein with untreated plasma membrane but were reduced to 0.3 pmol/50 μg protein following treatment with NEM.

The reduction of high affinity binding by *N*-ethylmaleimide was protected by first treating the membrane with LY181984 at a physiological concentration of 1 μM. Protection was evidenced by the observation that with sulfonylurea present during NEM treatment, there was only a 23% reduction in high affinity sites compared to 76% reduction by NEM in the absence of sulfonylurea. At 1 μM, the tumor-inactive sulfonylurea LY181985 was less

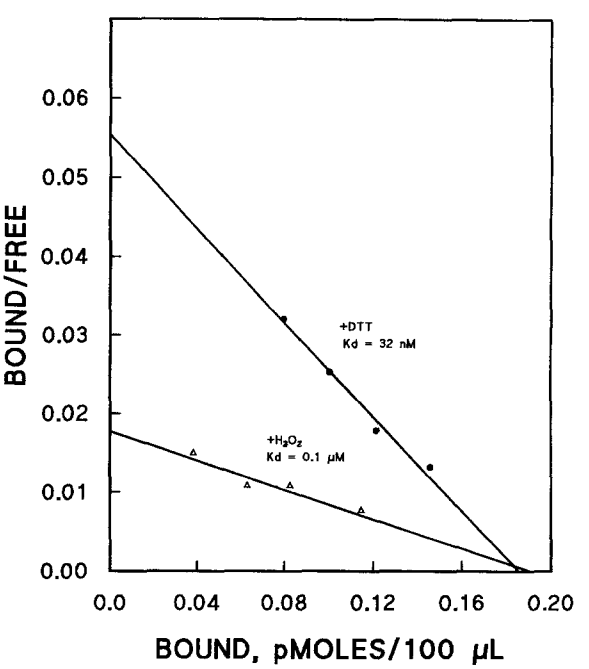

Fig. 1. Scatchard analysis of [^{3}H]LY181984 binding by purified plasma membranes of HeLa cells comparing reducing (10 μM dithiothreitol) ($r = 0.986$) and oxidizing (0.015% H_2O_2) ($r = 0.998$) conditions.

Table 1
High-affinity binding of [^{3}H]LY181984 by HeLa plasma membranes reduced by treatment with *N*-ethylmaleimide (0.1 mM) and protected from reduction by *N*-ethylmaleimide by pretreatment with unlabeled LY181984 (1 μM), an active anticancer sulfonylurea, but not by the inactive LY181985 (1 μM)

Treatment [a]	K_d (nM)	n (pmol/50 μg protein)
None	38 ± 16	1.44 ± 0.22
N-Ethylmaleimide	30 ± 9	0.34 ± 0.17
LY181984 + *N*-ethylmaleimide	38 ± 11	1.11 ± 0.19
LY181985 + *N*-ethylmaleimide	28 ± 6	0.64 ± 0.14
	(80 ± 22) [b]	(1.17 ± 0.19)

Binding was estimated by equilibrium dialysis at constant ligand (50 pmol) and membrane protein (50 μg) [1]. Values are averages of three (treatments) or six (none) determinations ± S.D..
[a] All membranes received an amount of DMSO equivalent to a final concentration of 0.1%.
[b] A population of binding sites with K_d between 60 and 100 nM did appear to be protected by LY181985 in the presence of *N*-ethylmaleimide (values in parentheses).

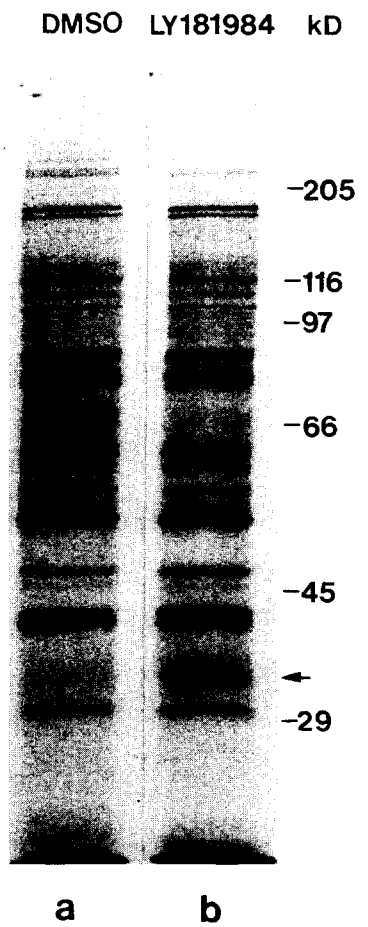

Fig. 2. SDS-PAGE analysis of *N*-[^{14}C]ethylmaleimide-labeling of HeLa plasma membrane peptide with sulfonylurea-protected thiols. HeLa plasma membranes were first reacted with unlabeled *N*-ethylmaleimide (NEM) in the presence of excess LY181984 (active), and the NEM and LY181984 removed by washing with albumin. Protein bands protected by LY181984 against reactions with NEM, and subsequently labeled with [^{14}C]NEM appeared consistently within the apparent molecular mass range 32 to 36 kDa (arrow). Lane a, DMSO alone; lane b, DMSO plus 1 μM LY181984.

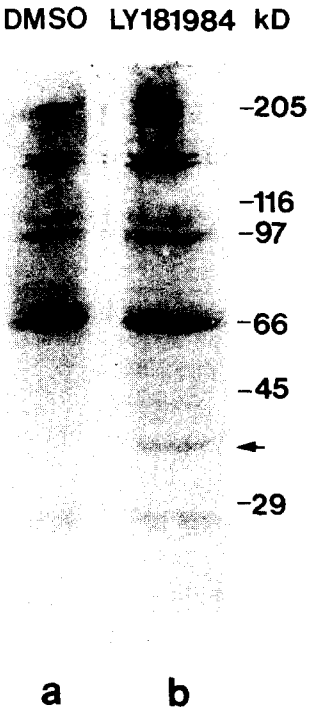

Fig. 3. SDS-PAGE analysis of biotin maleimide-labeling of HeLa membrane peptides with sulfonylurea-protected thiols. Conditions were as described in Fig. 2 except that *N*-[^{14}C]ethylmaleimide was replaced by biotin-maleimide and visualization was by reaction with avidin-conjugated alkaline phosphatase rather than autoradiography. Lane a, DMSO alone; lane b, DMSO plus 1 μM LY181984.

effective in protecting high affinity sites although a binding component with a somewhat higher kDa was observed (values in parentheses) (Table 1).

The response of [^{3}H]LY181984 to reducing and oxidizing conditions (Fig. 1) and to *N*-ethylmaleimide treatment (Table 1) suggested an involvement of one or more sulfonylurea-protected thiols in the binding site. This possibility was investigated using both *N*-[^{14}C]ethyl- and biotin-maleimide.

Using *N*-[^{14}C]ethylmaleimide, the binding proteins protected by sulfonylurea were labeled following reaction of unprotected thiols with unlabeled *N*-ethylmaleimide. When displayed on SDS-PAGE, minor protein components in the range of 32 to 36 kDa were consistently influenced by sulfonylurea presence (Fig. 2).

Bands of the apparent doublet at 34 to 36 kDa were observed when LY181984 (active) was present during the initial treatment with unlabeled NEM (Fig. 2, lane b) but not with DMSO alone. Other radiolabeled components appeared not to be influenced by LY181984.

Similar results were obtained using detection with biotin maleimide in place of *N*-[^{14}C]ethylmaleimide with detection using avidin-labeled alkaline phosphatase (Fig. 3). A band reactive with biotin maleimide at about 32 to 34 kDa was observed in lane b where the membrane vesicles were initially treated with unlabeled NEM in the presence of LY181984 (active). With LY181984 present

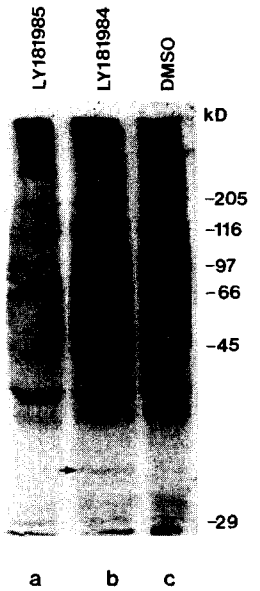

Fig. 4. As in Fig. 3 except comparing LY181985 (inactive) and DMSO with LY181984 (active). This experiment was repeated several times with consistent results.

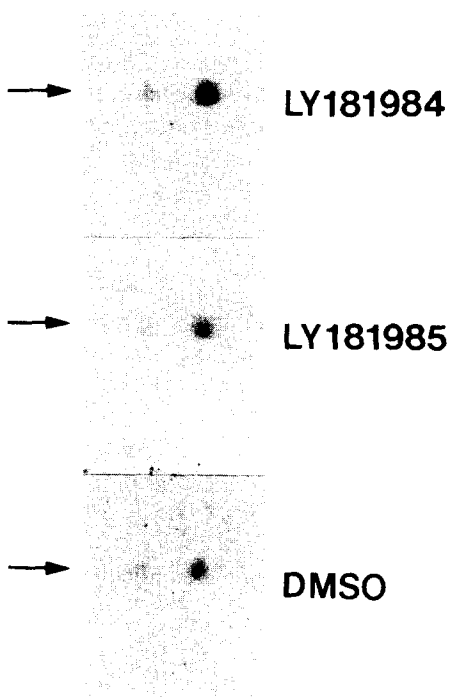

Fig. 5. Two-dimensional gel electrophoretic analysis of the 34 kDa region of HeLa plasma membranes protected from reaction with *N*-ethylmaleimide (NEM) by incubation with sulfonylurea. The membranes were first reacted with unlabeled NEM in the presence of active (LY181984) and inactive (LY181985) or no (DMSO) sulfonylurea. The sulfonylurea was removed by centrifugation through sulfonylurea-free medium. Membranes then were subsequently reacted with [^{14}C]NEM to label any sulfhydryl groups protected by the sulfonylurea. Both the active (LY181984) and the inactive (LY181985) sulfonylureas protected although the degree of protection was considerably greater with the active LY181984. The isoelectric point of the major protected radioactive component was estimated to be about 4.5.

Table 2
Binding of [^{3}H]LY181984 by HeLa plasma membranes after denaturation in the presence of sodium dodecylsulfate.

Fraction	pmol bound
Sample buffer (SDS)	0.3
HeLa plasma membrane	4.0
HeLa PM boiled in sample buffer	3.9

Binding was estimated by equilibrium dialysis at constant ligand (50 pmol) and membrane protein (100 μg).

during the treatment with unlabeled NEM, the protected component was at about 34 kDa (arrow). In these experiments, the inactive LY181985 was ineffective in thiol protection (Fig. 4). To confirm the presence of a radiolabeled band protected by active antitumor sulfonylurea, two-dimensionional gel analysis was employed (Fig. 5). The experiment was designed as described for Fig. 2 (lane b) and the major [^{14}C]NEM-labeled protein at 34 kDa was resolved as shown in Fig. 5. Compared were LY181985 (inactive), DMSO and LY181984 (active). The isoelectric point estimated from mobility in the first dimension was about 4.5.

Further definition of the diarylsulfonylurea binding was facilitated by the unexpected observation that binding to plasma membranes solubilized in the presence of SDS and dithiothreitol was comparable to that of native membranes (Table 2). When resolved on SDS-PAGE gels, the major binding component localized to the molecular mass region between 30 and 38 kDa (Fig. 6). Additional binding was seen in the 50 to 60 kDa range and occasionally at about 100 kDa and 150 kDa. These findings are of importance in

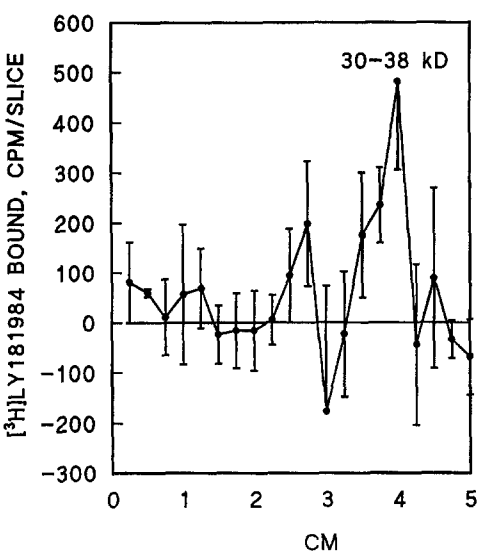

Fig. 6. Binding of [^{3}H]LY181984 to SDS-PAGE gel segments of HeLa plasma membranes compared to radioactivity in a blank gel. The gel regions in the 30 to 38 kDa range bound radioactivity significantly above that of a blank gel. Binding was determined in the presence of SDS-PAGE sample buffer which did not markedly affect sulfonylurea binding. Values are means of two gels analyzed in parallel ± mean average deviations as a function of distance migrated in centimeters (cm). The experiment was repeated several times with consistent results.

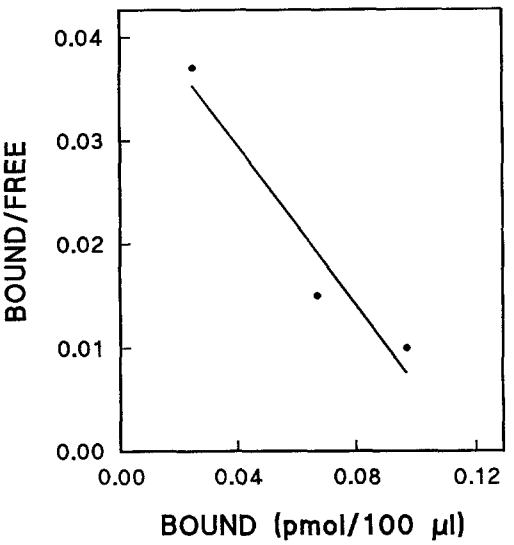

Fig. 7. Scatchard analysis ($r = 0.968$) of HeLa plasma membrane fractions from a preparative SDS-PAGE gel corresponding to a molecular mass of ca. 34 kDa. The fractions prepared under reducing conditions and corresponding to ca 100 μg starting plasma membrane protein bound with high affinity ($K_d = 30$ nM).

that they provide direct confirmation of constituents binding [^{3}H]LY181984 in the same range of molecular masses as those binding NEM following LY181984 protection.

Binding to proteins of the molecular mass range 30 to 38 kDa was confirmed by preparative SDS-PAGE where solubilization, electrophoresis and binding were carried out in the presence of dithiothreitol. Shown (Fig. 7) is a Scatchard analysis of binding of [^{3}H]LY181984 to fractions 23 and 24 of the separation corresponding to a molecular mass of about 34 kDa did bind sulfonylurea with an apparent high affinity similar to that of the starting plasma membranes analyzed in parallel ($K_d = 30$ nM). In addition, the purified protein fractions exhibited approximately the same number of high affinity binding sites as the equivalent amount of starting plasma membrane loaded. Other fractions containing similar amounts of protein of relative molecular mass greater or less than 34 kDa did not bind [^{3}H]LY181984 with high affinity.

4. Discussion

For the studies described, the diarylsulfonylureas were numbered experimental compounds, provided from the synthetic activities of Lilly Research Laboratories, Indianapolis, IN (Fig. 8). The compound designated LY181984 was active against solid tumors. The structurally related compound designated LY181985 was inactive. Activity was defined on the basis of assays using murine solid tumors grown subcutaneously in an auxiliary site with treatment initiated the day following implantation [4].

A plasma membrane location for a site of action of the diarylsulfonylureas in HeLa cells was indicated from binding studies with [^{3}H]LY181984. Using equilibrium dialysis and [^{3}H]LY181984, the bulk of the specific binding of HeLa homogenates was localized to the plasma membrane [1]. Nuclei, mitochondria- or endoplasmic reticulum-enriched fractions showed little or no specific binding. With freshly harvested cells, only minor binding was observed with the soluble fraction.

Binding to plasma membranes was of high affinity with a K_d of about 30 nM. The number of binding sites, estimated to represent 30 to 35 pmol/mg protein, would correspond to a low abundance protein representing a purification of about 1000-fold from the plasma membrane and about 6000-fold from the total homogenate assuming a molecular mass of 34 kDa as indicated by the present findings.

Preliminary indications of involvement of one or more thiols in sulfonylurea binding to the plasma membrane vesicles of HeLa cells came from findings where binding affinity, but not necessarily the number of sites, was dependent upon reducing conditions during binding. The covalently-binding thiol reagent *N*-ethylmaleimide also adversely affected high affinity binding but by reducing the number of high affinity sites. Similar results were obtained with the impermeant thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB/Ellman's Reagent) (not shown).

To attempt to label specifically a potential sulfonylurea-binding protein, HeLa S plasma membranes were incubated with LY181984 followed by a brief exposure to unlabeled *N*-ethylmaleimide to react with all unprotected thiol groups. The membranes were then centrifuged through buffer to release the bound sulfonylurea and ultimately reacted with either *N*-[^{14}-C]ethylmaleimide, biotin maleimide or coumarin maleimide with detection either by autoradiography, by blotting onto nitrocellulose and incubation with avidin-linked alkaline phosphatase or with detection directly on the gels based on fluorescence (not shown). With all three methods, the active sulfonylurea LY181984 protected reactive functional groups, presumably thiols, associated with a 36 kDa peptide and, to a lesser extent, with a 57 kDa peptide. Other proteins were labeled by the *N*-[^{14}C]ethylmaleimide presumably due to unmasking of cryptic thiols and/or other reactive groups by washing the membranes following the initial reaction with unlabeled *N*-ethylmaleimide. However, only enhanced labeling of the 34 kDa component was observed to result from protection with active sulfonylurea during the initial incubation with unlabeled *N*-ethylmaleimide. The inactive sulfonylurea LY181985 was partially effective in

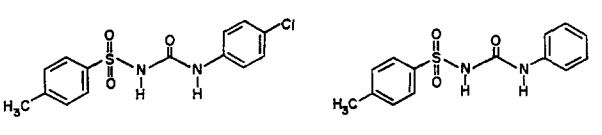

Fig. 8. Structure of diarylsulfonylurea antitumor agents.

protection of binding sites in the experiments of Table 2 in that a normal number of sites, but having decreased affinity, were present compared to DMSO alone. However, in the labeling experiments of Figs. 2 to 5, the LY181985 was ineffective in eliciting the markedly-enhanced labeling of a 34 kDa membrane component seen with LY181984. When resolved by two-dimensional electrophoresis, the sulfonylurea-protected component exhibited an acidic p*I* of about 4.5.

An unexpected observation was the preservation of high affinity binding sites in HeLa plasma membranes after solubilization in detergent plus dithiothreitol. Binding was preserved as long as the mixture was maintained fully reduced.

To further correlate the presence of a binding protein with material in the molecular weight range of 34 kDa, SDS-PAGE gels were incubated with [^{3}H]LY181984 in the presence of dithiothreitol overnight. The gel was rinsed briefly to remove excess radioactivity and sliced. Analysis of gel slices compared to a blank gel loaded with sample buffer but without plasma membranes revealed specific binding principally in the 30 to 34 kDa portion of the gel. Binding in the molecular mass range 50 to 65 kDa and occasionally at higher molecular weights sometimes was observed as well but was more variable than that seen in the 32 to 36 kDa molecular mass range.

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