

# Impermeant antitumor sulfonylurea conjugates that inhibit plasma membrane NADH oxidase and growth of HeLa cells in culture. Identification of binding proteins from sera of cancer patients

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## Abstract

The antitumor sulfonylurea LY237868 (*N*-(4-aminophenyl-sulfonyl)-*N'*-(4-chlorophenyl)urea) was conjugated through the A ring to  $\alpha$ -cyclodextrin or agarose bead material (Affigel 10) to prepare impermeant conjugates for activity measurements and affinity isolation of binding proteins from serum. When conjugated to  $\alpha$ -cyclodextrin, the resulting LY237868 conjugate inhibited both NADH oxidase activity and growth of HeLa cells in culture. The conjugate was at least one order of magnitude more potent as an inhibitor than the parent compound. These findings confirm previous results that demonstrate an antitumor sulfonylurea-binding protein with NADH oxidase activity at the external plasma membrane surface of HeLa cells that is shed into culture media conditioned by growth of HeLa cells. A comparable activity, responsive to sulfonylurea, was present in sera of cancer patients. LY237868 conjugated to agarose beads as the affinity support bound a large number of serum proteins. However, compared to serum from normal patients, the affinity support bound two proteins of  $M_r$  approx. 33.5 and 29.5 not found in sera of normal patients. The 33.5 kDa protein from human sera reacted with antisera to a 33.5 kDa protein from culture media conditioned by growth of HeLa cells that blocked and immunoprecipitated the sulfonylurea-responsive activity from HeLa cell plasma membranes. The results point to the 33.5 kDa protein from cancer patient sera that bound to the sulfonylurea affinity support as representing the circulating equivalent of the previously identified 34 kDa sulfonylurea-binding protein, with NADH oxidase activity at the external cell surface of cultured HeLa cells and a corresponding 33.5 kDa protein shed into culture media conditioned by growth of HeLa cells.

**Keywords:** Diarylsulfonylurea; Sulfonylurea; Antitumor drug; Drug conjugate; Cyclodextrin; HeLa

## 1. Introduction

Previous findings from our laboratories have described a high affinity ( $K_d = 30$  nM) sulfonylurea-binding activity of HeLa cells associated exclusively with the plasma membrane [1]. The binding activity was tentatively identified as a 34 kDa binding protein [2] with NADH oxidase activity inhibited by antitu-

Abbreviations: LY181984, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea; LY237868, *N*-(4-aminophenylsulfonyl)-*N'*-(4-chlorophenyl)urea;  $\alpha$ -CD,  $\alpha$ -cyclodextrin; DMSO, dimethylsulfoxide

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mor sulfonylurea [3]. The antitumor sulfonylurea-inhibited activity was associated with an NADH site at the external plasma membrane surface [4], and a comparable activity tentatively identified as a 33.5 kDa soluble form of the activity was identified from culture media conditioned by growth of HeLa cells [5]. An antitumor-responsive NADH oxidase activity subsequently was demonstrated in sera of cancer patients [6]. These studies were with *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea designated LY181984. The present study was with a similar compound designated LY237868 (*N*-(4-aminophenylsulfonyl)-*N'*-(4-chlorophenyl)urea) with antitumor activity [7] that could be readily conjugated to impermeant or affinity supports. The findings provide additional evidence using HeLa cells and plasma membranes isolated from HeLa cells for an external sulfonylurea-binding site at the HeLa cell plasma membrane surface. Additionally, when bound to an affinity support, the conjugated LY237868 bound two constituents of  $M_r$  33.5 and 29.5 kDa from sera of cancer patients not found in sera of normal individuals.

## 2. Materials and methods

### 2.1. Growth of cells

HeLa S cells were grown on Minimal Essential Medium (S-MEM) (Jolik modified) with glutamine (244 mg/l) and phosphate (1.3 g/l  $\text{Na}_2\text{HPO}_4$ ) and without  $\text{CaCl}_2$  plus 5% donor horse serum. Gentamycin sulfate (50 g/l) and sodium bicarbonate (2 g/l) were added.

Attached HeLa cells (ATCC CCL2), were grown in 150-cm flasks in Minimal Essential Medium (Gibco) (pH 7), at 37°C with 10% bovine calf serum (heat-inactivated), plus 50 mg/l gentamycin sulfate (Sigma). Cells were trypsinized with Sigma IX trypsin for 1 to 2 min and harvested by scraping and taken up in TD-Tris buffer (140 mM NaCl, 5 mM KCl, 0.7 mM  $\text{Na}_2\text{HPO}_4$  and 25 mM Tris (pH 7.4)) to a final cell concentration of 0.1 g wet weight (gww) per ml. Cell survival was determined by Eosin Y exclusion.

### 2.2. Growth measurements

Attached HeLa cells were treated in  $35 \times 10$  mm plastic dishes in 2.5 ml culture medium. Sulfonyl-

ureas were dissolved in DMSO and added in 2.5  $\mu\text{l}$  of DMSO to yield a final DMSO concentration of 0.1%. Controls received 2.5  $\mu\text{l}$  of DMSO. Growth inhibitions were determined from cell numbers estimated after 24, 48, 72 and 96 h of treatment.

### 2.3. Purification of plasma membranes from HeLa cells

HeLa cells grown as suspension cultures were collected by centrifugation for 6 min at 3000 rpm or 15 min at 1000 rpm. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM  $\text{NaHCO}_3$  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 7–8 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 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  $23\,500 \times g$ ) 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 approximately 1 ml per pellet from  $5 \cdot 10^8$  cells. The resuspended membranes are then loaded onto the two-phase system constituted on a weight basis as described [8]. The upper phase, enriched in plasma membranes, was diluted 5-fold with 1 mM sodium bicarbonate and the membranes were collected by centrifugation. The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry [8]. The yield was 20 mg plasma membrane protein from  $10^{10}$  cells.

### 2.4. Spectrophotometric assay of NADH oxidase activity

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mix containing 25 mM Tris-MES buffer (pH 7.2), 1 mM KCN to inhibit any potential mitochondrial oxidase activity, and 150  $\mu\text{M}$  NADH at 37°C with constant stirring. Activity was measured using a Hitachi U3210 with continuous recording over 5–10-

min intervals. A millimolar extinction coefficient of 6.22 was used to calculate NADH disappearance.

## 2.5. Conjugation of LY237868 with $\alpha$ -cyclodextrin ( $\alpha$ -CD)

The overall conjugation strategy of LY237868 with  $\alpha$ -CD is shown in Fig. 1.

### 2.5.1. Reaction of $\alpha$ -CD with succinic anhydride

$\alpha$ -CD was dried at 105°C overnight. From the dry  $\alpha$ -CD, 2.7 g of  $\alpha$ -CD (2.8 mol) was dissolved in 50 ml of pyridine (dried over NaOH). To this solution, 25 g of succinic anhydride (250 mmol) in 30 ml of pyridine was added. The reaction was heated overnight at 80°C with stirring. After reaction, 50 ml distilled water was added and the insoluble residue was washed with excess dichloromethane and dried under vacuum. Persuccinylated  $\alpha$ -CD (5.07 g, 2.2 mmol, m.p. = 125–130°C uncorrected) was purified by reversed-phase chromatography (Sephasil C18, 2.5  $\times$  18 cm, Pharmacia Biotech) with a solvent system composed of 5% methanol in dichloromethane. The  $R_f$  of the product on silica gel TLC was 0.28

while that of the  $\alpha$ -CD was 0.54 (n-butanol/ethanol/water = 4:3:2). The degree of succinylation of the  $\alpha$ -CD was 14.1 as determined by plasma desorption mass spectrometry.

### 2.5.2. Conjugation of persuccinylated $\alpha$ -CD with LY237868

For conjugation of the persuccinylated  $\alpha$ -CD with LY237868, 0.14 g persuccinylated  $\alpha$ -CD (0.059 mmol) and 0.29 g of LY237868 (0.90 mmol) were dissolved in 20 ml of dry pyridine. To this solution, 0.26 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (1.36 mmol) was added. The reaction was stirred overnight at 25°C. The  $\alpha$ -CD-LY237868 conjugate (0.24 g, 0.036 mmol, m.p. 194–196, uncorrected) was purified by precipitation with ethyl acetate. The product (expected average molecular mass of 6723) was immobile on silica gel TLC. Neither FAB mass spectrometry nor plasma desorption mass spectrometry was useful in the analysis of the molecular mass of the conjugate. Formation of the conjugate was confirmed by  $^1\text{H-NMR}$  spectrometry and FT-IR spectrometry (Section 2.11 and Section 2.12).

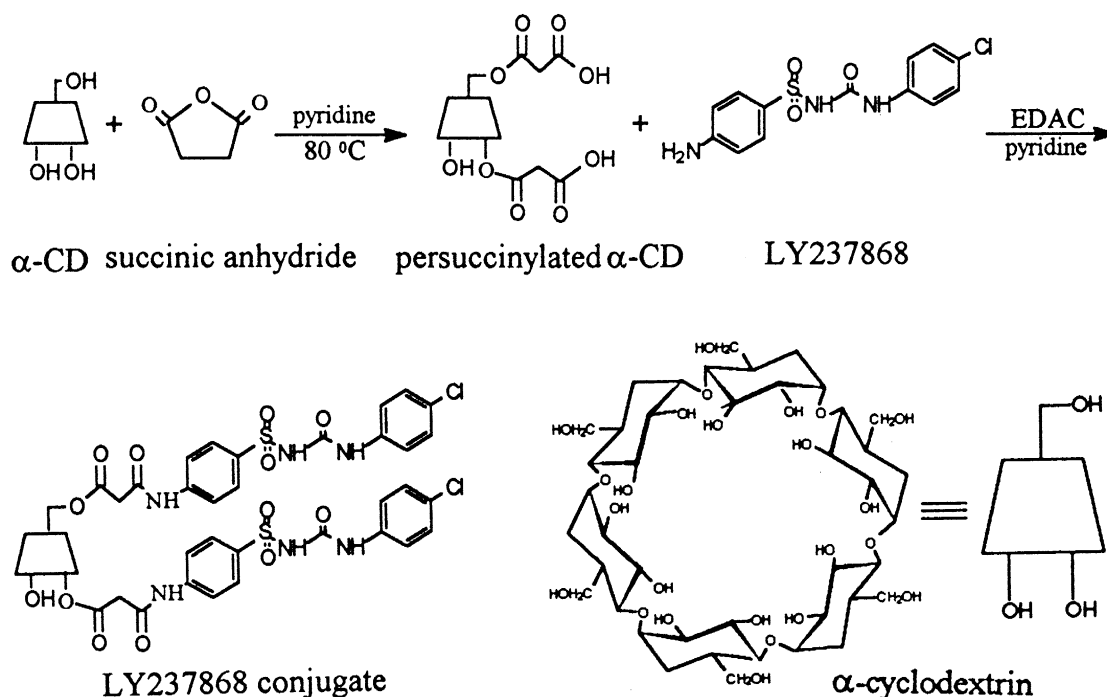


Fig. 1. Scheme showing the overall strategy of conjugation of LY237868 with  $\alpha$ -cyclodextrin ( $\alpha$ -CD).

## 2.6. Synthesis of sulfonylurea–acetic acid conjugate

Acetic acid *N*-hydroxy succinimide ester (0.037 g) (0.23 mmol) and LY237868 (0.045 g) (0.14 mmol) were dissolved in 2 ml of dry methanol (dried over molecular sieve). The reaction was for 5 h at room temperature with stirring. The conjugate was purified by silica gel chromatography (50 ml bed volume, Iatrobeds, Iatron Laboratories, Japan) (MeOH/CH<sub>2</sub>Cl<sub>2</sub> = 10:1). Fractions containing the conjugate (26 mg, 0.07 mmol, *R<sub>f</sub>* value = 0.79) were pooled and dried.

## 2.7. Preparation of sulfonylurea affinity support

For preparation of the affinity support, 0.5 g of LY237868 (1.5 mmol) was dissolved in methanol. To this solution, 5.0 ml of Affigel 10 resin (BioRad) in methanol suspension was added. The reaction was for 5 h at room temperature with stirring and the remaining reactive groups of the resin were blocked by excess ethyl amine for an additional 2 h. The resin was washed with methanol and then 20 mM Tris-HCl (pH 7.2), containing 5% methanol and used directly as the sulfonylurea affinity support.

## 2.8. Affinity purification of serum proteins

For affinity purification, pooled sera from cancer patients (breast, prostate, lung, colon, ovarian, etc.) or from normal individuals was loaded on the affinity support in the presence of 1  $\mu$ M reduced glutathione. Elution was with increasing concentrations of the active antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(phenyl)urea (LY181984) of 1, 10 and 100/ $\mu$ M prepared in 20 mM Tris-HCl, pH 7.2, containing 5% methanol. Eluted proteins were analyzed by SDS-PAGE (9.5%) with detection using silver staining [9].

## 2.9. Western blot analysis of immunoprecipitates with antisera to a 33.5 kDa protein with sulfonylurea-inhibited NADH oxidase activity from culture media conditioned by growth of HeLa cells

For immunoprecipitation, NP-40 at a final concentration of 0.1% was added to 100  $\mu$ l of conditioned, defined culture medium from HeLa cells or 100  $\mu$ g HeLa S plasma membranes. Protein A Sepharose (PAS) was added (3.6 mg in 50  $\mu$ l) and the mixture was incubated at 4°C for 4 h with shaking. The PAS

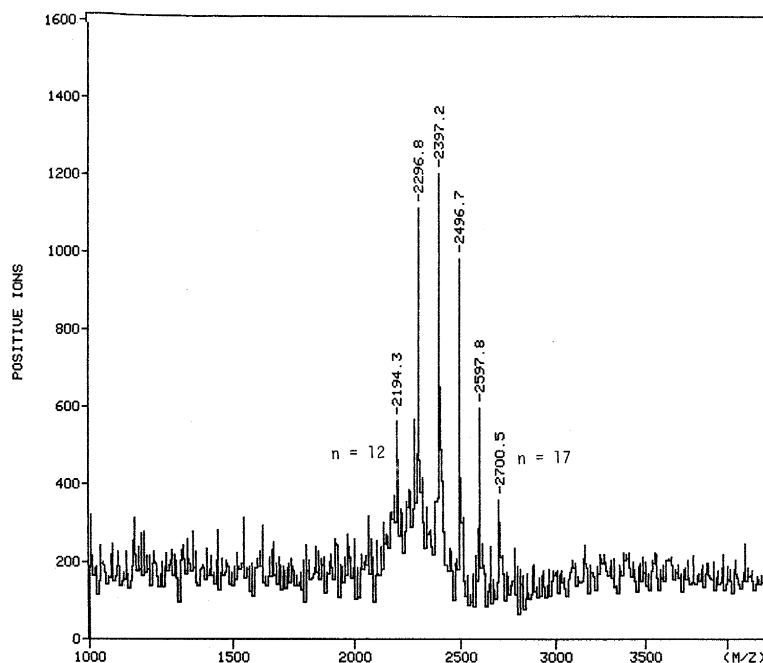


Fig. 2. Plasma desorption mass spectrum of persuccinylated  $\alpha$ -cyclodextrin. The degree of substitution is designated by *n*.

was removed by centrifugation (Microfuge, 1 min) and 2.5  $\mu$ l of antisera or preimmune sera were added and incubated overnight at 4°C with shaking. PAS again was added and the mixture incubated for 2 h. The PAS was collected by centrifugation and the supernatant assayed for NADH oxidase activity. The pellets were washed twice with 0.1% ND-40 and once each with PBS and water and analyzed by 10% PAGE and Western blot.

For Western blot analysis, immunoprecipitates from sera were separated on 10% SDS-PAGE and then transferred by electroblotting onto nitrocellulose. To block unspecific antibody binding sites, the blot was placed in a solution of 1% bovine serum albumin, 10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20 (TBS-T) for 15 min. The blot was placed in the primary antibody solution (1:1000) in TBS-T overnight at 4°C with shaking. The blots were washed with TBS-T four times for 15 min each after which the blots were placed into secondary antibody solution (Goat anti-rabbit linked to alkaline phosphatase, Jackson ImmunoResearch Laboratories, West Grove, PA, 1:25,000 in TBS-T) for 30 min at room temperature with shaking. The blot was washed with TBS-T three times for 15 min each and placed in a mixture of 0.33/mg/ml nitro blue tetrazolium and 0.16 mg/ml of 5-bromo-1-chloro-3-indolyl phosphate prepared in 100 mM Tris (pH 9.5), containing 100 mM NaCl and 5 mM  $MgCl_2$  and incubated with shaking until the purple color of positive bands appeared. The color development reaction was stopped by placing the blots in 20 mM Tris (pH 8), containing 5 mM EDTA.

#### 2.10. Plasma desorption mass spectrometry

The plasma desorption mass spectra were obtained with a Bioion 20R mass spectrometer (Uppsala, Sweden). Samples were applied to a nitrocellulose-coated mylar target and data were collected at an accelerating voltage of 17 kV.

#### 2.11. $^1H$ -NMR spectrometry

$^1H$ -NMR spectra were recorded at 20°C on a Varian VXR-500 spectrometer operating at 500 MHz. All compounds were dissolved in  $DMSO-d_6$  and  $^1H$

chemical shifts were referenced to  $DMSO-d_6$  (2.58 ppm).

#### 2.12. Fourier transform-infrared (FT-IR) spectrometry

IR spectra were obtained with a Perkin-Elmer Model 1600 FT-IR spectrometer with 16 kilobytes of

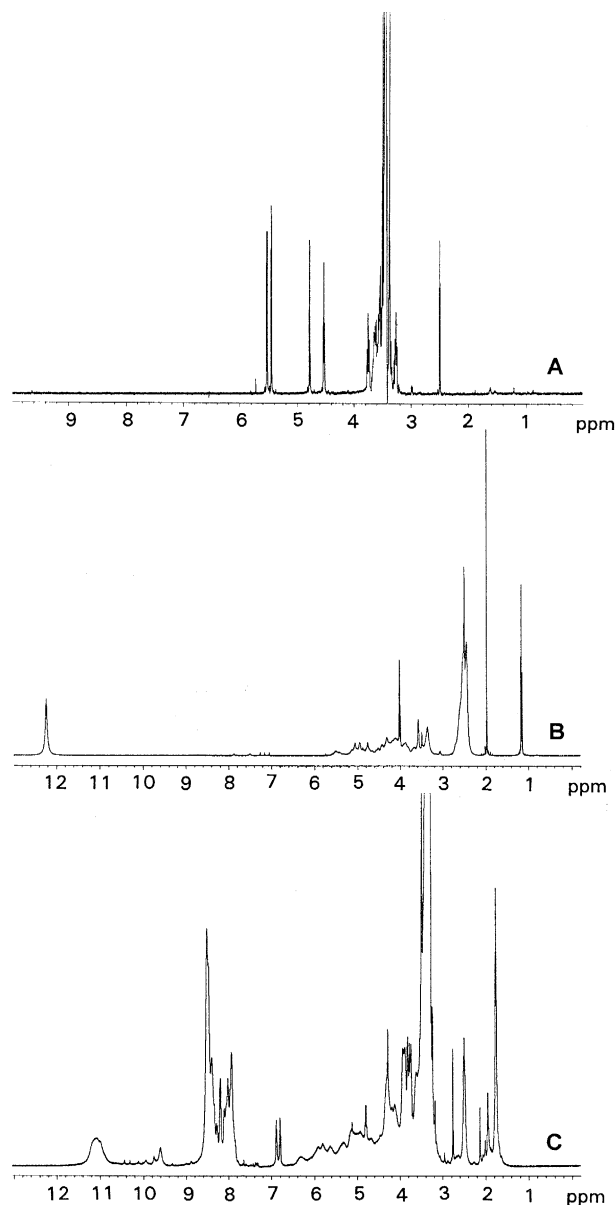


Fig. 3.  $^1H$ -NMR spectra. (A)  $\alpha$ -Cyclodextrin. (B) Persuccinylated  $\alpha$ -cyclodextrin. (C)  $\alpha$ -Cyclodextrin conjugated to LY237868. Each compound was dissolved in  $DMSO-d_6$  and  $^1H$ -NMR spectra were recorded at 500 MHz.

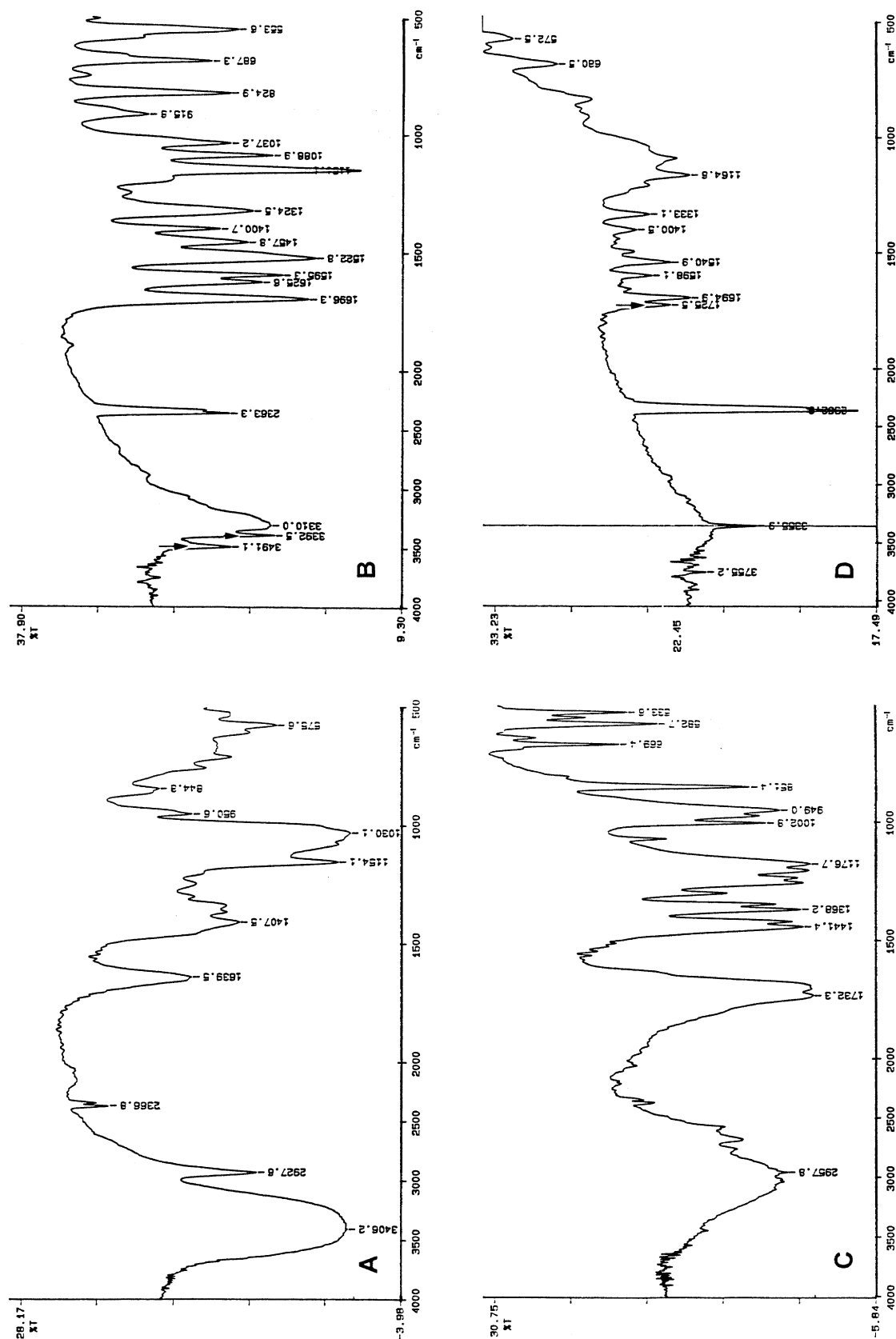


Fig. 4. Infrared spectra of LY237868,  $\alpha$ -cyclodextrin ( $\alpha$ -CD) and the LY237868 conjugate. Upon conjugate preparation, the N–H stretching vibrations of the primary amino group of LY181984 at 3491.1 and 3392.5  $\text{cm}^{-1}$  (arrows in B) were replaced by a C=O stretching vibration at 1725.5  $\text{cm}^{-1}$  showing the formation of an amide bond (arrow in D). (A)  $\alpha$ -Cyclodextrin. (B) LY237868. (C) Persuccinylated  $\alpha$ -cyclodextrin. (D) LY237868-conjugated  $\alpha$ -cyclodextrin.

battery-backed memory, software with extensive graphics and data processing capability based on Perkin-Elmer's CD-3 infrared data system. Samples for IR spectral analysis were prepared by the KBr disc method at room temperature. Spectra were obtained from 4000 to 600  $\text{cm}^{-1}$ .

### 3. Results

To test the concept that the NADH oxidase inhibited by sulfonylurea was accessible to NADH supplied from the external surface, we utilized an impermeant conjugate of an LY181984 analog substituted with an amino group in position 3 of the A ring (LY237868). This amino group was then used to link the sulfonylurea to an impermeant cyclodextrin.

Persuccinylated  $\alpha$ -CD was identified by plasma desorption mass spectrometry (Fig. 2) and  $^1\text{H}$ -NMR spectrometry (Fig. 3B). Degree of substitution of  $\alpha$ -CD by succinyl groups was 14.1 as determined

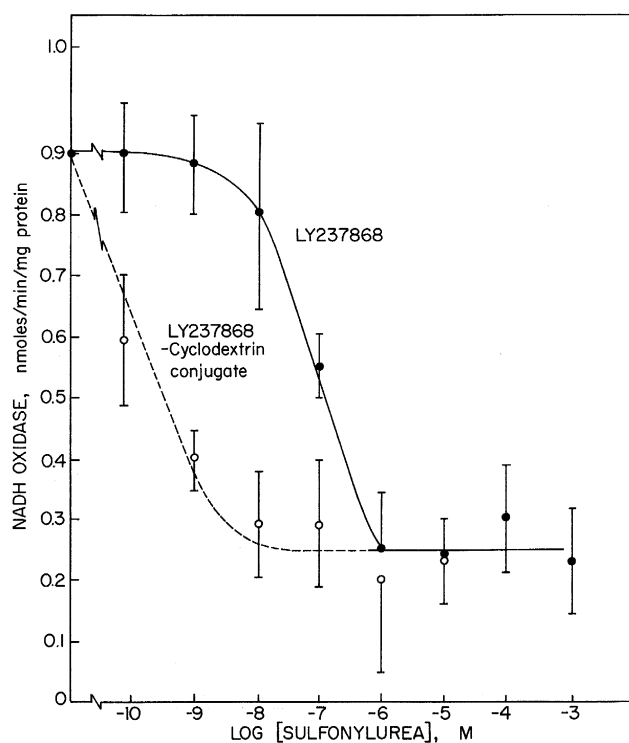


Fig. 5. Inhibition of NADH oxidase of sealed right-side-out vesicles of HeLa plasma membrane by the  $\alpha$ -cyclodextrin conjugate of LY237868 as a function of concentration compared to the unconjugated LY237868. Both were inhibitory.

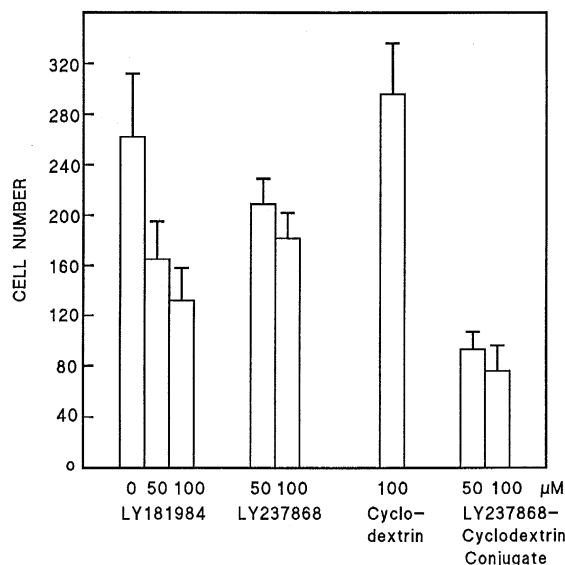


Fig. 6. Cytotoxicity of the  $\alpha$ -cyclodextrin conjugate of LY237868 at two concentrations compared to  $\alpha$ -cyclodextrin and LY181984 or LY237868 based on growth over 72 h of attached HeLa cells. The O is for the DMSO control. All drugs were dissolved in DMSO. Results are averages of three experiments  $\pm$  standard deviations among experiments.

from the intensity of the major peaks of the plasma desorption mass spectrum.

The molecular mass of the  $\alpha$ -CD conjugated to LY237868 could not be obtained by plasma desorption mass spectrometry, due to the high expected average molecular mass of 6723. However, its cross-linking was verified by  $^1\text{H}$ -NMR spectrometry (Fig. 3C) and its immobility upon silica gel thin layer chromatography.

Cross-linking also was verified from the infrared spectra (Fig. 4) that showed the disappearance of N–H stretching vibrations of the primary amino group of LY237868 (arrows in Fig. 4B) in a CD-LY237868 conjugate (Fig. 4D), and its replacement by an amide bond as shown by C=O stretching vibrations at 1725.5  $\text{cm}^{-1}$  (arrow in Fig. 4D).

On a sulfonylurea basis, the cross linked compound was as active or even more active than the parent compound in inhibiting the NADH oxidase of HeLa plasma membranes (Fig. 5). Inhibition by 50% of the activity was observed at a conjugate concentration equivalent to 1 nM LY237868. When tested for inhibition of growth of HeLa cells, the conjugate was considerably more inhibitory than the parent com-

pound (Fig. 6) and even more active than the active cancer sulfonylurea LY181984.  $\alpha$ -Cyclodextrin by itself was without effect.

In subsequent experiments, the LY237868 was conjugated to agarose beads (Affigel 10, BioRad) as an affinity support. In a model study, we have linked LY237868 to acetic acid *N*-hydroxysuccinimide ester using the same active ester chemistry as used in preparing sulfonylurea affinity support. In this procedure, acetic acid *N*-hydroxysuccinimide ester was linked to the LY237868 to form the 4'-acylamine derivative. The product was conclusively identified by determining its exact mass by mass spectrometry: Found, 367.03767; Calcd., 367.03936 for  $C_{15}H_{14}N_3O_4S$ .

To test the affinity support, sera pooled from cancer patients was passed over the column. Elution of bound proteins was with the active antitumor sulfonylurea LY181984 in DMSO. A number of pro-

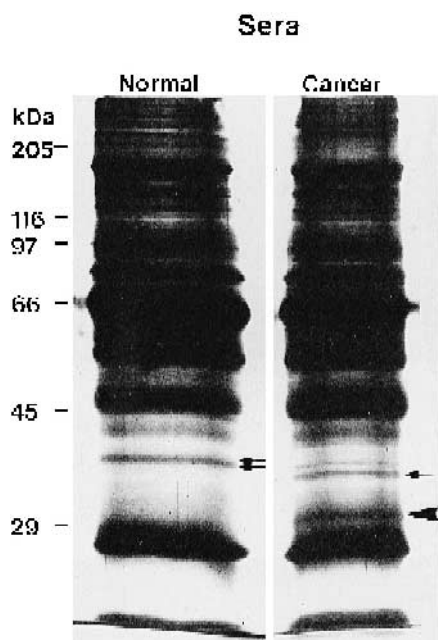


Fig. 7. Proteins eluting from a sulfonylurea affinity column (which binds albumin among a variety of other serum proteins) with 1  $\mu$ M LY181984. Of interest was a band at about 33.5 kDa in sera from cancer patients (small arrow) not found in sera of normal individuals. Also seen was a band at about 29.5 kDa (large arrow) also found in sera from cancer patients and not found in sera of normal individuals. A band at 36 kDa was found in both normal and cancer but was more prominent in sera of normal individuals (double arrows).

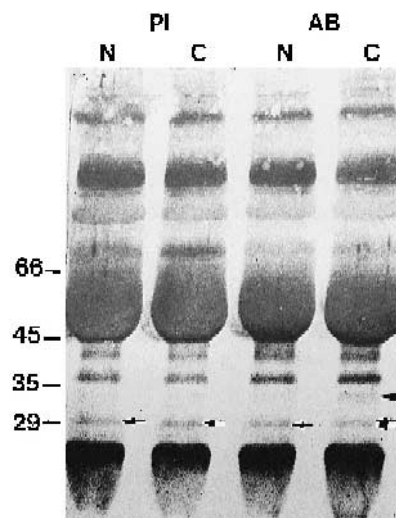


Fig. 8. Western blot of immunoprecipitates. Rabbit polyclonal antisera to tNOX isolated from culture media conditioned by growth of HeLa cells (AB) were used for detection. Lanes were loaded with immunoprecipitates from 50  $\mu$ l of pooled sera from normal individuals (N) or pooled sera of cancer patients (C). The two lanes on the left were reacted with preimmune sera (PI). The two lanes on the right were reacted with antisera (AB). The antisera reacted specifically with a 33.5 kDa component (arrowhead) obvious only with the pooled sera from cancer patients. The small arrow indicates the position of the 29.5 kDa component as part of a poorly resolved doublet (double arrows) partly obscured by material present in both sera of cancer patients and of normal volunteers and reactive as well with the preimmune sera (single arrows).

teins, including serum albumin, bound to the column. However, comparing pooled sera from cancer patients to pooled sera from normal individuals several important differences were noted (Fig. 7). Bands with a  $M_r$  of 33.5 and 29.5 (29–30.5) kDa, respectively, were eluted from the affinity support with 1  $\mu$ M LY181984 with pooled sera from cancer patients and were absent with sera pooled from normal individuals. A protein band at 36 kDa was somewhat more evident with sera pooled from normal individuals than from pooled cancer sera. In other respects the two gel profiles were identical.

Since the NADH oxidase activity of the drug-responsive form was completely inhibited by 0.1  $\mu$ M sulfonylurea, it was not possible to assay sulfonylurea-inhibited NADH oxidase activity with the eluted fractions. Additionally, the components unique to cancer serum were still minor components of the



mixture of proteins bound to the affinity support. That the sulfonylurea-inhibited NADH oxidase activity did bind to the affinity column was confirmed by a failure of the activity to appear in the column flow-through. Activity could be partly restored by adding excess human serum albumin to bind the excess sulfonylurea followed by overnight dialysis.

The protein band with a  $M_r$  of 33.5 kDa and specific to the pooled sera of cancer patients, however, was reactive with polyclonal antisera prepared to a comparable 33.5 kDa protein shed from HeLa cells into culture media conditioned by growth of HeLa cells (arrowhead, Fig. 8). Whether or not the 29.5 kDa band from the sulfonylurea affinity column was related to the NADH oxidase could not be determined. On the immunoblots the cancer-specific band at 29.5 (29–30.5) kDa may have been present as the upper band of a poorly resolved doublet (small arrows, Fig. 8). The  $\approx$  29.5 kDa band was largely obscured by reactivity with material present in both normal and cancer sera but also reactive with the preimmune sera (single arrows, Fig. 8).

The polyclonal antisera from HeLa that cross-reacted with the 33.5 kDa material retained by the sulfonylurea affinity column and eluted with 1  $\mu$ M LY181984 specifically inhibited as well as immunoprecipitated the drug-responsive NADH oxidase activity of HeLa plasma membranes and the corresponding drug-responsive NADH oxidase activity shed by HeLa cells into media conditioned by the growth of HeLa cells [5]. Preimmune sera or sera to antigens other than the 34 (33.5) kDa protein (e.g. antiserum albumin) did not inhibit the activity. The polyclonal antisera also inhibited the drug-responsive NADH oxidase activity of pooled sera from cancer patients and was without effect on sera pooled from healthy volunteers or from patients with disorders other than cancer. On Western blots, immunoreactivity of the 33.5 kDa component from sera of cancer patients was not given by preimmune sera nor by secondary antisera alone.

#### 4. Discussion

The chloroaniline structure of the A ring of the antitumor sulfonylureas was critical to activity in the inhibition of xenograft growth [7]. The structure of

the B ring was less critical. The 4-aminophenyl B ring gave rise to an active antitumor sulfonylurea and afforded an opportunity to immobilize the sulfonylurea to an impermeant support.

Since  $\alpha$ -cyclodextrin has six primary hydroxyl and twelve secondary hydroxyl groups, it was an attractive candidate for drug conjugation. Succinic anhydride provided a four carbon linker between the  $\alpha$ -cyclodextrin and the LY237868. The degree of substitution of  $\alpha$ -cyclodextrin by succinyl groups was 14.1 and this high degree of substitution led to an effective conjugation.

When attached to  $\alpha$ -cyclodextrin, the sulfonylurea LY237868 not only retained activity but the conjugates appeared to be more active than the parent sulfonylurea. The conjugates effectively blocked the NADH oxidase of right-side-out [4] plasma membrane vesicles. Since the  $\alpha$ -cyclodextrin–LY237868 conjugate would be unlikely to penetrate the isolated and sealed plasma membrane vesicle, these results support previous findings [4] that the antitumor sulfonylurea-inhibited site of NADH oxidation was at the external cell surface.

The possibility of an external NADH oxidase in HeLa cells was first raised by studies with sealed plasma membrane vesicles of known absolute orientation [4]. Membrane integrity of the isolated vesicles after treatment with sulfonylureas or with the sulfonylurea– $\alpha$ -cyclodextrin conjugate was verified using electron microscopy. After repeated freezing and thawing, some of the vesicles were everted so that populations of both right-side-out and inside-out were present. The right-side-out vesicles exhibited an electrophoretic mobility greater than that of the inside-out vesicles. Sidedness was confirmed by measurements of ATPase latency and binding of immunogold-labeled concanavalin A. When the two vesicle populations were resolved by preparative free-flow electrophoresis, the active antitumor sulfonylurea LY181984 inhibited only the NADH oxidase activity of the right-side-out vesicles. These findings suggested two NADH sites or activity forms for the plasma membrane NADH oxidase. One activity, inhibited by LY181984, appeared to be accessible to external NADH (an impermeant substrate) only with sealed right side-out vesicles. The other, not inhibited by LY181984, was accessible to NADH with sealed inside-out vesicles. After membrane disruption by

Triton X-100, both NADH oxidase forms were accessible to NADH. The findings demonstrate that the NADH oxidation site inhibited as a result of binding the active antitumor sulfonylurea LY181984 was at the external cell surface. Plasma membrane vesicles from HeLa cells were able to oxidize NADH supplied to either membrane surface, but only with right-side-out vesicles was NADH oxidation sensitive to inhibition by the antitumor sulfonylurea.

It is unlikely, however, that an ectoenzyme which requires as much as 0.15 mM NADH as an extracellular substrate could function physiologically as an NADH oxidase. Therefore, the physiological electron acceptor for the sulfonylurea-inhibited NADH oxidase has been assumed to be some constituent endogenous to the plasma membrane rather than NADH, since an extracellular source of NADH in the body would be highly unlikely. Protein thiols and disulfides were indicated as the physiological substrates for the activity rather than NADH. In the presence of NADH there was an increase in protein disulfides and a corresponding decrease in membrane thiols both of which were inhibited by 1  $\mu$ M LY181984 [10]. In the absence of NADH, the protein appears to function as a protein disulfide-thiol interchange activity [11]. This latter activity also was inhibited by the active antitumor sulfonylureas [11].

In support of the above hypothesis as well as in support of an external form of the NADH oxidase were results with the impermeant thiol reagent 5,5'-dithiobis(2-nitrophenylbenzoate) (DTNB). DTNB inhibited specifically the NADH oxidase activity of HeLa cells and was without effect on the corresponding activity of plasma membrane vesicles prepared from rat liver [12].

The inhibition of growth of HeLa cells by the  $\alpha$ -cyclodextrin-LY237868 conjugate also would support the conclusion that the principal site of antitumor activity of the sulfonylureas was at the external cell surface. In contrast to plasma membrane vesicles, we did not exclude endocytic uptake of conjugate or release of free drug with HeLa cells. However, since the LY237868- $\alpha$ -cyclodextrin conjugate, on a sulfonylurea basis, was even more effective than free LY237868 or even the more active LY181984, such explanations to account for the conjugate activity seem less likely. If the drug site for the sulfonylureas was indeed at the external surface of the cells, the

conjugate might be expected to be more active on a molar sulfonylurea basis. The sulfonylurea was lipid soluble and would be able to enter and cross the membrane. By restricting the drug to the cell surface, it appeared that overall effectiveness was increased perhaps by as much as one order of magnitude.

The concept of a cell surface site of action for anticancer drugs is not new. Inhibition of cell growth by adriamycin and other anthracyclines covalently linked to polymers to prevent entrance into cells was demonstrated earlier by Tritton and Yee [13] and Tökes et al. [14]. Growth inhibition was observed when the adriamycin was attached to agarose beads [13], polyvinyl alcohol [15], polyglutaraldehyde microspheres [14,16], *N*-(2-hydroxypropyl)methacrylamide [17] or diferric transferrin [18]. It was concluded from these studies that the anthracyclines disrupt cellular growth processes without actually entering cells (see also Ref. [19]). As with the sulfonylurea- $\alpha$ -cyclodextrin conjugate reported here, the anthracycline conjugates often were more effective than was the free drug on a per mole of drug basis. With adriamycin attached to agarose, immobilized adriamycin was 100- to 1000-times more active than was free adriamycin [13]. With adriamycin bound to the polyglutaraldehyde microspheres [14,16,19], increased effectiveness on an adriamycin basis also was found. Transferrin-adriamycin conjugates also have proven to be disproportionately more effective than free adriamycin [20]. The latter were tested clinically and found to be therapeutic in the treatment of leukemia [21,22].

Since the LY237868- $\alpha$ -cyclodextrin complex did appear to interact with the NADH oxidase, the LY237868 was attached to an agarose affinity support. As a source of starting material, serum from cancer patients and from normal individuals were compared. Sera from cancer patients, but not that from normal individuals, contained a NADH oxidase activity either stimulated or inhibited by the antitumor sulfonylurea LY181984, a sulfonylurea closely related chemically to LY237868. The inhibitions ranged from 10% to 50% and were given by sera over a wide range of tumor types including both solid and cellular cancers [6].

When applied to the affinity column with elution by 1  $\mu$ M LY181984, two protein bands of apparent molecular masses of 33.5 and 29.5 kDa not present

with pooled sera of normal individuals were observed with sera of cancer patients. The 33.5 kDa component corresponded to a comparable protein shed into culture media conditioned by growth of HeLa cells [5]. Polyclonal antisera raised to the 33.5 kDa component from the conditioned media, reacted with a 33.5 kDa component from cancer sera. No 33.5 kDa immunoreactive protein was observed with sera from normal individuals. The nature of protein in the vicinity of 29.5 (29–30.5) kDa associated with the pooled sera from cancer patients and eluted with 1  $\mu$ M sulfonylurea remains problematic. Although apparently present on the immunoblots as the upper band of a poorly resolved doublet, it was largely obscured by material also reactive with preimmune sera and present in sera from both cancer patients and healthy volunteers. Despite the latter difficulty and the fact that the affinity support bound a mixture of several proteins that were eluted with sulfonylurea, two proteins were identified as being unique to sera of cancer patients. One of these, with a  $M_r$  of 33.5, corresponded to the shed form of the sulfonylurea-binding protein previously identified from culture media conditioned by growth of HeLa cells. This component, and possibly the 29–30.5 kDa component as well, may provide one or more targets suitable for exploitation as cancer markers with potential utility in the development of new diagnostic and therapeutic strategies.

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