Pergamon

Biochemical Pharmacology, Vol. 49, No. 5, pp. 661–668, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00501-X

CHARACTERIZATION OF THE INTRACELLULAR DISTRIBUTION AND BINDING IN HUMAN ADENOCARCINOMA CELLS OF *N*-(4-AZIDOPHENYLSULFONYL)-*N'*-(4-CHLOROPHENYL)UREA (LY219703), A PHOTOAFFINITY ANALOGUE OF THE ANTITUMOR DIARYLSULFONYLUREA SULOFENUR

PETER J. HOUGHTON,*† JANEK SOSINSKI,* JAY H. THAKAR,* GEORGE B. BODER‡ and GERALD B. GRINDEY‡§

*Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38101-0318; and ‡Eli Lilly & Company, Indianapolis, IN 46285, U.S.A.

(*Received* 14 *February* 1994; *accepted* 3 *October* 1994)

Abstract—A photoactivatable diarylsulfonylurea, *N*-(4-azidophenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY219703), has been examined as a potential probe to elucidate the intracellular distribution and binding of antitumor diarylsulfonylureas. Our results demonstrated that against the human colon adenocarcinoma cell line GC_3/c_1, LY219703 is a more potent cytotoxic agent than *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea (Sulofenur; ISCU), whereas a subline selected for resistance to ISCU was cross-resistant to LY219703, suggesting a similar mechanism of action or resistance. Cellular pharmacology studies showed that [^{3}H]LY219703 concentrated in cells, and that its concentrative accumulation could be inhibited by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), thus indicating that it was similar to other antitumor diarylsulfonylurea (DSU) drugs examined. Accumulation of [^{3}H]LY219703 in cells was progressively decreased by co-incubation with increasing concentrations of ISCU, and in cells incubated to steady state with 1 μM [^{3}H]LY219703, ISCU (500 μM) rapidly displaced the photoaffinity analogue. Photoactivation of [^{3}H]LY219703 by UV light (5–30 min) prevented efflux of radiolabeled drug during a 20-min wash in drug-free medium. Subsequent distribution studies showed that 89% of the radiolabel was associated with particulate components, and that ~20% of the radiolabel in the 320,000 *g* pellet could be extracted with acetone. Subcellular distribution showed approximately 6% associated with nuclei, 52% with mitochondria and 26% in the microsomal fraction. The effect of UV photoactivation on the distribution of [^{3}H]LY219703 in soluble and particulate fractions was also examined in GC_3/c_1 cell preparations sonicated prior to being incubated with [^{3}H]-LY219703. A high proportion (83%) of radiolabel associated with the 100,000 *g* pellet, and distribution between soluble and particulate fractions was not altered by UV irradiation. Specific activities of protein in the 100,000 *g* supernatant and pellet were 0.186 and 0.537 nmol/mg, respectively. Putative binding species were analyzed by SDS–PAGE. Using SDS–PAGE, ten major binding proteins were identified in 320,000 *g* pellets from GC_3/c_1 cells: M_r 110, 88, 76, 70, 64, 58, 48, 36, 26, and 24 kDa, and at least four of these (88, 70, 64, and 36 kDa) were also detected in mitochondria isolated from cells after photoactivation, or in mitochondrial preparations that were incubated with [^{3}H]LY219703 and photoactivated after isolation from cells. Results suggested that under conditions of SDS–PAGE some dissociation of radiolabel from proteins also occurred. Binding of [^{3}H]LY219703 to a model substrate, bovine serum albumin, and the effect of denaturing conditions used for sample preparation prior to SDS–PAGE, showed that relatively mild denaturing conditions (23°, 2 hr) caused significant dissociation of radiolabel from BSA. These data suggest that LY219703, and by analogy other antitumor DSU agents, are distributed mainly in membranes, particularly those of organelles such as mitochondria.

Key words: antitumor diarylsulfonylureas; photoactivatable analogue; distribution; mitochondria; membranes; Sulofenur

DSU‖ antitumor agents represent a new class of oncolytics with a potentially novel mechanism of action. At pharmacologically achievable concentrations of free drug (~1 μM), these agents

† Corresponding author: Dr. Peter J. Houghton, Department of Molecular Pharmacology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38101-0318. Tel. (901) 522-0440; FAX (901) 521-1668.

§ Deceased.

‖ Abbreviations: DSU, diarylsulfonylurea; LY219703, *N*-(4-azidophenylsulfonyl)-*N'*-(4-chlorophenyl)urea; ISCU, *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea (Sulofenur); MPCU, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea; TCA, trichloroacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PT, physiological Tris: 20 mM Tris containing 120 mM NaCl, 3 mM K_2HPO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4); RIPA, 0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2% aprotinin, 1 mM PMSF (phenylmethylsulfonyl fluoride) and FBS, fetal bovine serum.

neither inhibit macromolecular synthesis [1, 2] nor arrest cells in any specific phase of the cell cycle. At low, achievable concentrations, cytotoxicity is proliferation dependent, but as the concentration of DSU is increased, cytotoxic activity becomes equal in proliferating and non-proliferating cells [3]. At high concentrations, cell killing may be a consequence of uncoupling oxidative phosphorylation [4, 5]. The cellular pharmacology of two DSU analogues, ISCU (Sulofenur) and MPCU has been reported [2, 6, 7]. Both compounds enter cells through a passive diffusion mechanism, and concentrate 4- to 6-fold in cells at steady state. Accumulation within cells is inhibited by agents that uncouple mitochondria and by ionophores that collapse the pH gradient across the mitochondrial inner membrane. These data have been interpreted to implicate mitochondria as the site of drug sequestration and accumulation [7], and the uncoupling activity of antitumor DSUs would support this. However, a limitation of these studies has been that DSUs rapidly efflux from cells ($T_{1/2} \sim 18$ sec at 37°), and no tight-binding component has been detected in cultured cells [6]. Consequently, it has not been possible to define a cellular target, or to determine the intracellular distribution of DSU. In the present study, we have characterized a new photoaffinity analogue and have examined its intracellular distribution and binding.

MATERIALS AND METHODS

[^{3}H]LY219703 (sp. act. 984 mCi/mmol) and LY219703 were prepared as previously described [6]. RPMI-1640 was obtained from Whittaker (Walkersville, MD), and other cell culture reagents were from GIBCO (Grand Island, NY).

Cell culture. GC_3/c_1, a cell line derived from a human colon adenocarcinoma, and its subline, LYC5, selected for resistance to ISCU, and conditions of growth have been described previously [3]. Briefly, GC_3/c_1 cells were grown in RPMI-1640 supplemented with 2 mM glutamine, 10% FBS without antibiotics. For the experiments described, LYC5 cells were subcultured in the absence of ISCU for 1 or 2 days. LYC5 cells maintain resistance for at least 2 years in the absence of drug selection pressure. Colony-forming assays were as described previously [3].

Accumulation of [^{3}H]LY219703 was as described previously for studies with [^{3}H]MPCU [6]. Cells were seeded at 1.5×10^6/35 mm culture dish, and allowed to attach overnight. Monolayers were washed (2×2 mL) with PT buffer to remove serum. Buffer was aspirated and replaced with drug-containing medium. In studies where drug accumulation was examined in the presence of FCCP, cells were preincubated for 10 min with 10 μM FCCP in PT buffer (without glucose). Medium was aspirated and replaced with medium containing [^{3}H]LY219703 (1 μCi/mL) and FCCP (10 μM).

Photoaffinity labeling. Cells ($1.7–2.0 \times 10^7$) were seeded per 100 mm culture dish and allowed to attach overnight. Monolayers were washed extensively (2×15 mL PT buffer) over 15 min at room temperature. Buffer was aspirated and replaced with 2 mL of [^{3}H]LY219703 containing medium (3–6 μCi/mL), and cells were incubated for periods specified in the experiments described. Monolayers were subsequently irradiated at a distance of 5 cm using a germicidal UV lamp for various periods up to 30 min, or were left unirradiated. Drug-containing buffer was aspirated, and monolayers were washed with 3×15 mL PT buffer over 20 min. Monolayers were trypsinized, pipetted to give a uniform suspension, and centrifuged at 500 *g* for 10 min, 4°. The pellet was resuspended in PT containing soybean trypsin inhibitor (2%) and recentrifuged. The pellet was resuspended in Ca^{2+}/Mg^{3+}-free PBS and recentrifuged before being suspended in 0.25 M sucrose/50 mM HEPES, pH 7.4, or PT-P buffer (PT containing 1 mM PMSF). Cells from 2 to 6 dishes were pooled for specific experiments.

Cell fractionation. To determine distribution of radiolabel in soluble or particulate fractions, the cells suspended in ice-cold PT buffer were homogenized on ice (polytron setting No. 10; 2×15 sec), and immediately centrifuged (320,000 *g*, Beckman Ti100A, 30 min, 4°). The supernatant was removed, and radiolabel was determined on an aliquot. The pellet was washed (0.5 mL PT buffer) and dissolved in RIPA lysis buffer on ice, and then radioactivity was determined. A further aliquot of supernatant (250 μL) was mixed with 250 μL of 10% TCA, and 100 μL of FBS was added as carrier. Samples were centrifuged as before (320,000 *g*, 30 min, 4°), and radiolabel in the supernatant was measured. To determine whether radiolabel in the initial 320,000 *g* pellet was associated with lipid, the pellet was washed several times with PT-P buffer, until no radiolabel was detected in washings (<100 dpm). The pellet was then homogenized in acetone (−20°), left on ice for 30 min, and then recentrifuged as described above. After removing the supernatant, the pellet was dissolved in lysis buffer, and radiolabel in each fraction was determined.

Photoaffinity labeling of disrupted cell preparations. GC_3/c_1 cells were harvested and resuspended at 10^8/mL in PT buffer, and sonicated on ice (3×10 sec). Suspensions were incubated at room temperature for 40 min with 7.8 μM [^{3}H]LY219703 in the absence or presence of 500 μM ISCU. Samples were then exposed to UV irradiation (20 min) or were not irradiated. Total radiolabel, and [^{3}H]LY219703 associated with soluble and particulate fractions, were determined after centifuging samples (100,000 *g*, 4°, 60 min). The proportional distribution between soluble and particulate matter in irradiated or non-irradiated samples, and the specific activity of protein in each fraction, were determined.

Intracellular distribution. GC_3/c_1 cells were photolabeled and harvested as described and resuspended in 0.25 M sucrose–HEPES, pH 7.4. Cells were disrupted by a Dounce homogenizer. Unbroken cells were removed by centrifugation (500 *g*, 5 min). The supernatant was subsequently centrifuged at 4° to derive pellets containing nuclear (1500 *g*, 10 min), mitochondrial (10,000 *g*, 10 min) or post-mitochondrial (microsomal) fractions (320,000 *g*, 30 min). Pellets were subsequently solubilized in RIPA buffer (containing 2% aprotinin

and 1 mM PMSF) for electrophoresis, and radiolabel was determined.

Photoaffinity labeling of isolated mitochondria. Alternatively, mitochondria were isolated from GC_3/c_1 cells and labeled with [^{3}H]LY219703. Mitochondria were isolated as described above, and resuspended in PT buffer at 1.28 mg/mL protein. Mitochondria were incubated with 7.8 μM [^{3}H]LY219703 for 40 min and UV irradiated for 20 min. Mitochondria were centrifuged, and the pellets were dissolved in RIPA buffer.

One-dimensional SDS–PAGE was carried out using standard procedures [8]. Samples of 320,000 *g* or mitochondrial pellets (200 μL) in RIPA containing 2% aprotinin, 1 mM PMSF were mixed with 80 μL of 10 × SDS sample buffer and allowed to denature for 2 hr at room temperature. Gels were stained and destained to visualize molecular weight markers, and dried for fluorography.

Photoaffinity labeling of bovine serum albumin. Diarylsulfonylurea antitumor agents bind to BSA with high affinity [9]. To examine the photoaffinity labeling of this substrate, and the potential dissociation of radiolabel during denaturing conditions employed for SDS–PAGE, BSA (781 μM) in PT buffer was incubated at room temperature for 40 min with [^{3}H]LY219703 (7.8 μM). Protein-associated radiolabel was determined by G25 chromatography in samples that received either no further treatment or photoactivation by UV light (20 min) with or without denaturing in SDS sample buffer. Samples were denatured at room temperature for 2 hr or at 100° for 5 min. Samples were eluted from Sephadex-G25 columns (bed volume 9.1 mL) in PT buffer, 0.25-mL fractions were collected, and radioactivity and protein concentration were determined.

RESULTS

Cross-resistance to LY219703 in ISCU-resistant cells. We have reported previously the derivation of a clone of GC_3/c_1 cells selected for resistance to ISCU [3] and cross-resistant to several other DSU analogues [10]. This clone, designated LYC5, is resistant to low concentrations of ISCU when exposed for 7 days, but not resistant to short exposures to very high concentrations of ISCU. LYC5 cells thus appear to be altered at the locus responsible for cytotoxic activity of DSU relevant to the therapeutic activity of these agents. Results in Fig. 1 show that LYC5 cells were approximately 9-fold resistant to LY217903, a slightly greater level of resistance compared with ISCU [3]. LY219703 was about 2-fold more potent than ISCU against GC_3/c_1 cells, having a potency similar to MPCU in this cell line [9]. These data suggest that the photoaffinity analogue LY219703 has a mechanism of action and/or resistance similar to that of ISCU.

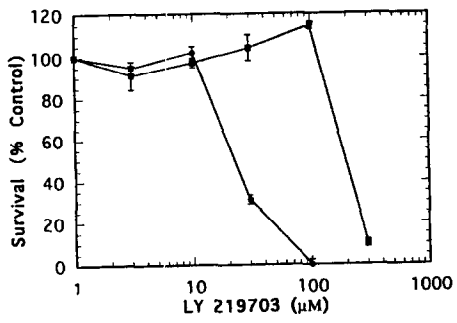

Fig. 1. Sensitivity of GC_3/c_1 and LYC5 cells to LY219703. GC_3/c_1 (●) and LYC5 cells (■) were exposed continuously to increasing concentrations of LY219703 for 7 days, at which time colonies were enumerated. Each value represents the mean ± SD of 9 determinations and is expressed as percent of control colony number. Calculated IC_{50} values were 19.5 and 187 μM for GC_3/c_1 and LYC5 cells, respectively. Control (100%) ± SD colony numbers were: GC_3/c_1 = 411 ± 15; LYC5 = 378 ± 20.

Accumulation of [^{3}H]LY219703. To further characterize this photoaffinity analogue, its accumulation into GC_3/c_1 cells was examined in the presence or absence of the ionophore FCCP (Fig. 2). [^{3}H]-LY219703 accumulated rapidly in cells, reaching steady-state levels in 10–15 min (data not shown). In the presence of FCCP, accumulation was reduced to 25% of that in control cells, essentially identical to results obtained with MPCU [6] and ISCU (unpublished). Thus, the concentrative accumulation of the photoactivatable analogue was energy dependent and similar to that of other DSUs studied. Accumulation of [^{3}H]LY219703 was progressively reduced in the presence of increasing concentrations (200–1000 μM) of ISCU (Fig. 2). Further, ISCU (500 μM) rapidly displaced >75% [^{3}H]LY219703 when cells were first incubated to steady state with 1 μM photoaffinity analogue (data not shown).

Effect of UV photoactivation on cellular retention of [^{3}H]LY219703. Initial studies showed that accumulation of [^{3}H]LY219703 was similar to that of other DSUs. When cells were incubated with [^{3}H]-LY219703 (1 μCi/mL), drug rapidly effluxed from cells when it was removed from the medium, with ≤10% radiolabel remaining cell-associated after 20 min. When cells were exposed to higher drug concentrations (6–10 μCi/mL, 7.8–13 μM), approximately 20% of the radiolabel was cell-associated after a 20-min efflux. To examine the effect of UV irradiation on retention of [^{3}H]-LY219703, cells were exposed to drug (7.8 μM) for periods of 20–70 min; then cells were irradiated for 5–30 min in the presence of drug or received no UV radiation. At the end of this period, medium was aspirated, and non-irradiated monolayers were washed rapidly in four changes of ice-cold PBS, as described in Materials and Methods. Monolayers were trypsinized, and radioactivity associated with the cells was determined (time of efflux = 0, Fig. 3). For UV-irradiated monolayers, medium was aspirated and 15 mL of drug-free PT medium was added for an additional 20 min, after which time cell-associated radiolabel was determined as above. Data are presented in Fig. 3. When GC_3/c_1 cells were incubated with [^{3}H]LY219703 for 20 or 70 min, but not UV irradiated, only 17 or 19% of the radiolabel associated with cells at the end of drug incubation remained after a 20-min efflux. Thus, prolonged incubation (i.e. 70 min) did not increase

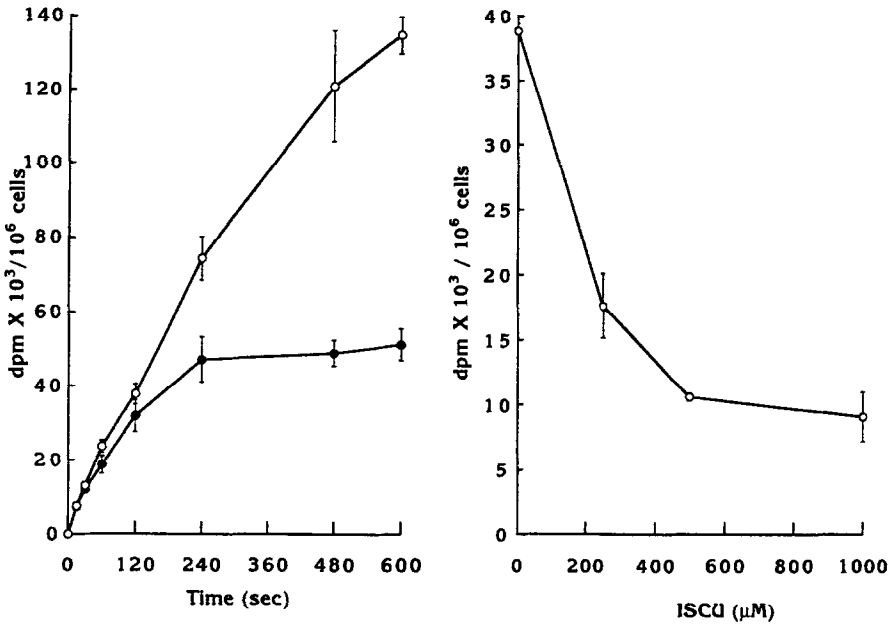

Fig. 2. (Left panel) Accumulation of [^{3}H]LY219703 in GC_3/c_1 cells. Monolayer cells were incubated with PT buffer without (○) or with 10 μM FCCP (●) for 10 min prior to replacement of the same medium containing [^{3}H]LY219703 at a final concentration of 0.98 μM. At various times over 10 min, buffer was aspirated and monolayers were rapidly washed and processed as described in Materials and Methods. Each point is the mean ± SD for 3 determinations, expressed per 10^6 cells. (Right panel) Effect of co-incubation with ISCU on the steady-state level of [^{3}H]LY219703. GC_3/c_1 cells were exposed simultaneously to 0.25 μM [^{3}H]LY219703 in the presence of increasing concentrations of ISCU. Cell-associated radiolabel was determined after 15 min. Values are means ± SD, N = 3.

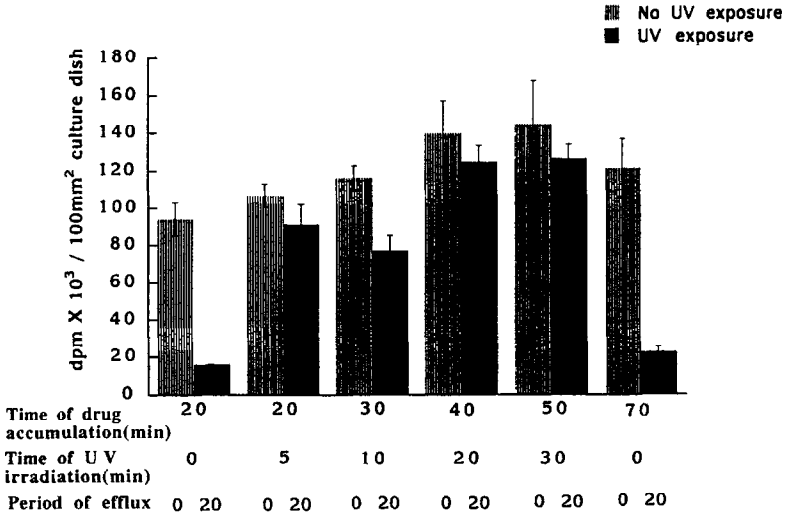

Fig. 3. Accumulation and retention of [^{3}H]LY219703 in GC_3/c_1 cells subjected to UV irradiation for various periods of time (0–30 min). GC_3/c_1 monolayers were exposed for 20–70 min to 7.8 μM [^{3}H]-LY219703. At the end of the accumulation period, groups of three monolayers each were treated as follows: (i) medium was aspirated and the radiolabel associated with cells was determined (designated 0 efflux), or (ii) 15 mL PT buffer was added, and cell-associated radiolabel was determined after 20 min efflux, or (iii) at the end of the accumulation period, monolayers were UV irradiated for periods of 5–30 min in the presence of radiolabeled drugs. Medium was aspirated, and these monolayers were incubated for an additional 20 min in 15 mL of drug-free PT buffer, at which time cell-associated radiolabel was determined. Values are the means ± SD (N = 3) for a representative experiment.

significantly the non-exchangeable fraction of LY219703. Drug accumulation reached steady state by 40–50 min. Exposure to UV light for 5–30 min increased the proportion of non-exchangeable radiolabel associated with the cells. After a 20-min efflux, the proportion of radiolabel still associated with cells receiving 5, 10, 20 or 30 min of UV irradiation was 85, 66, 89 and 87% of that associated with unirradiated cells at the end of the drug exposure period (i.e. no efflux).

Cellular distribution and binding of [^{3}H]LY219703. To determine whether UV activated [^{3}H]LY219703 was associated with soluble or particulate matter, homogenates were prepared and centrifuged at 320,000 *g* for 30 min (4°). The pellet was solubilized, and radiolabel associated with homogenate, supernatant and pellet was determined. Approximately 89% of the radiolabel was associated with the 320,000 *g* pellet (Table 1). Washing the pellet with acetone (−20°) showed that 31% of the radiolabel could be extracted into acetone, and acidification of the 320,000 *g* supernatant precipitated approximately half of the radioactivity (Table 1).

To examine the intracellular distribution of radiolabel after photoactivation, cells were fractionated and radiolabel in each fraction was determined (Table 2). Approximately 52% of total radioactivity was associated with the mitochondrial fraction and 26% with the microsomal components.

Distribution of [^{3}H]LY219703 in cell-free preparations. Binding of radiolabeled LY219703 was examined in sonicated GC_3/c_1 cell preparations. Cells were suspended at 10^8/mL in PT buffer and sonicated on ice. Preparations were incubated with [^{3}H]LY219703 for 40 min at 23°, followed by a further 20-min incubation with or without UV irradiation. Radiolabel in whole preparations, supernatant and pellet was determined after centrifugation (100,000 *g*, 60 min, 4°). The proportion of [^{3}H]LY219703 associated with the 100,000 *g* pellet was 80.05 ± 2.7% (SD N = 8). The specific activity for the 100,000 *g* supernatant and pellet fraction was 402 ± 96 × 10^3 and 1162 ± 27 × 10^3 dpm/mg protein, respectively. Distribution into particulate fraction was not altered by photoactivation or by competition with 500-fold unlabeled ISCU (data not shown).

Table 1. Distribution of [^{3}H]LY219703 in GC_3/c_1 cells after photoactivation

Sample	Total dpm	%*
Homogenate	1,559,440	100
320,000 *g* Supernatant	163,570	10.5
320,000 *g* Pellet	1,395,870	89.5
Acid precipitation		
320,000 *g* Supernatant		
Supernatant after TCA	77,670	47.5
Pellet after TCA	85,900	52.5
Acetone extraction		
Acetone	697,410	31.2
Pellet	1,539,170	68.8

* Average of duplicate determinations from a representative experiment.

Cells were incubated for 60 min in the presence of [^{3}H]-LY219703 (3.9 μM), and irradiated for 20 min prior to aspiration of medium and washing cells. Monolayers were trypsinized and resuspended in 2 mL PT buffer (4°); then homogenates were prepared and centrifuged at 320,000 *g* for 30 min (4°). The pellet was solubilized in RIPA buffer, and radiolabel associated with homogenate, supernatant and pellet was determined.

Table 2. Subcellular distribution of [^{3}H]LY219703 in GC_3/c_1 cells after photoactivation

Fraction	dpm	% Total radioactivity
Nuclear	107,880	6.4
Mitochondrial	895,490	53.4
Microsomal	449,070	26.8
320,000 *g* Supernatant	225,320	13.4

GC_3/c_1 cells were exposed to [^{3}H]LY219703 and UV irradiated, washed and harvested. Cells were disrupted (0.25 M sucrose–50 mM HEPES, pH 7.4), and samples were centrifuged at 500 *g* to remove unbroken cells. Nuclear pellets and mitochondrial fractions were collected after 10 min of centrifugation at 1500 *g* and 10,000 *g*, respectively. Microsomal pellets were prepared after centrifugating at 320,000 *g* for 30 min. Results are the means of duplicate determinations and are representative of other experiments.

SDS–PAGE analysis of [^{3}H]LY219703 binding. Association of radiolabel with proteins was examined in whole cell lysates, in pellets derived after centrifugation at 320,000 *g*, or in mitochondrial preparations. Co-incubation of GC_3/c_1 cells with 500-fold excess of unlabeled LY219703 completely eliminated binding of photoactivated [^{3}H]LY219703 (data not shown). A representative autoradiogram demonstrating distribution of radiolabeled proteins from 320,000 *g* pellets of GC_3/c_1 cells is shown in Fig. 4A. The apparent M_r values of major bands were 110, 88, 76, 70, 64, 58, 48, 36, 26, and 24 kDa. Shown also is the photoaffinity labeling of proteins in the mitochondrial preparation together with the Coomassie Blue staining pattern (Fig. 4B). Four major bands detected in the 320,000 *g* pellet (88, 70, 64 and 36 kDa) appeared to be represented in the mitochondrial preparation derived from labeled cells. Of note was that most of the abundant proteins in the mitochondrial fraction were radiolabeled. After purification of mitochondria, photoactivation of [^{3}H]LY219703 also gave a similar pattern of radiolabeled proteins (Fig. 4C). Heavily radiolabeled bands had mobilities consistent with peptides of 70, 64 and 43 kDa, with minor bands at 110, 88, 84, 80, 77, and 21 kDa. Of note, however, was a substantial amount of radiolabel migrating with the solvent front on SDS–PAGE.

Labeling of bovine serum albumin with [^{3}H]-LY219703. To examine possible dissociation of radiolabel under conditions used to denature samples for SDS–PAGE, a model substrate was used. Diarylsulfonylurea antitumor agents have been shown to bind with high affinity to serum albumin [9]. Consequently, BSA was used as a model substrate. Binding of [^{3}H]LY219703 to BSA was almost complete (96%). As shown in Table 3, UV irradiation caused some displacement of radiolabel

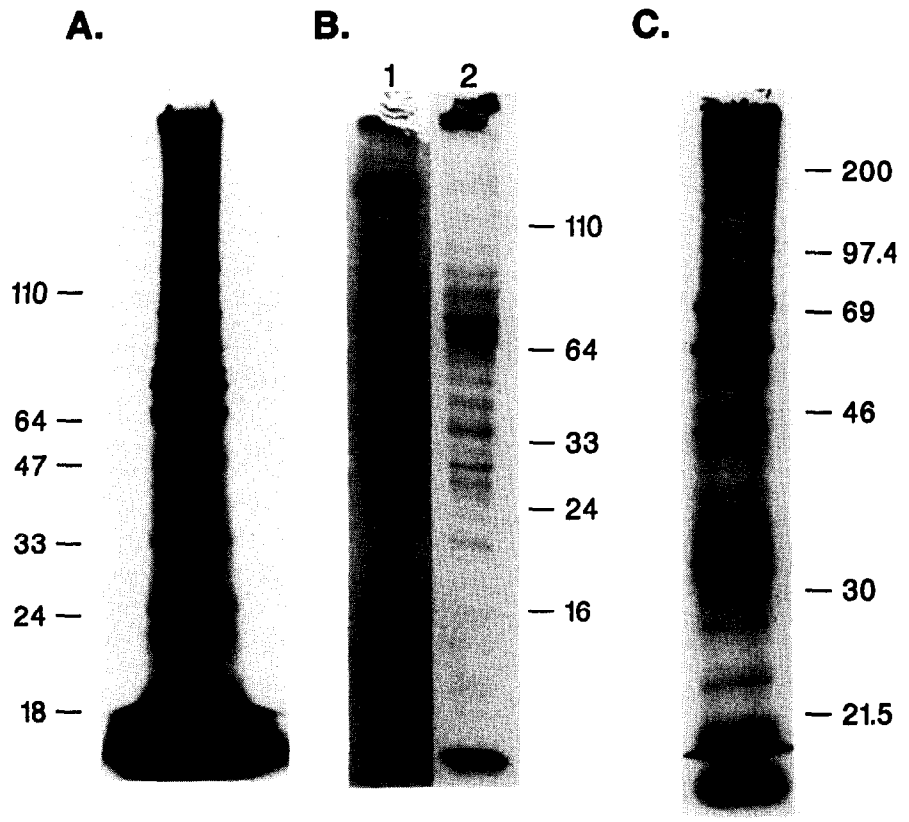

Fig. 4. Radiolabeling of particulate fractions and mitochondria of GC_3/c_1 cells by [^{3}H]LY219703. Monolayer GC_3/c_1 cells were incubated with 7.5 μM [^{3}H]LY219703 for 60 min, and UV irradiated for 20 min. Monolayers were extensively washed, trypsinized, homogenized, and centrifuged. Pellets were suspended in RIPA buffer (containing 2% aprotinin and 1 mM PMSF), and kept on ice until particulate material had dissolved. After addition of 10 × SDS sample buffer (40%, v/v), samples were mixed and allowed to denature for 2 hr at room temperature. Samples were analyzed by SDS–PAGE, and autoradiograms of gels were prepared. (A) Radiolabeling pattern for proteins in the 320,000 g pellet; (B) lane 1: Coomassie Blue stained gel of a fraction enriched for mitochondria; lane 2: autoradiogram from the gel shown in lane 2. (C) Radiolabeling pattern for proteins in samples of mitochondria isolated from cells prior to incubation and photoactivation of [^{3}H]LY219703. Molecular weight markers indicate the mobilities for each set of results. Results are representative of several individual experiments.

from BSA eluted from Sephadex-G25. To determine the effect of denaturing BSA under conditions used in SDS–PAGE, samples were mixed with an equal volume of 2 × SDS sample buffer and denatured at room temperature (23°, 2 hr) or at 100°, 5 min. Denaturing for 2 hr caused almost complete dissociation of radiolabel from BSA in samples that were not UV irradiated. There was also a substantial decrease in radiolabel associated with BSA after UV photoactivation, and this was increased when samples were denatured at 100° (Table 3).

DISCUSSION

Previous studies have suggested at least two potential mechanisms of action of antitumor DSU. At low pharmacologically achievable concentrations in humans (~0.5 to 1 μM free drug), cytotoxic effects of ISCU are proliferation dependent [3]. Under these conditions, LYC5 cells are 5.5-fold resistant to ISCU. At concentrations required to kill cells during brief exposure (4–24 hr), cytotoxicity is independent of proliferation, and LYC5 cells are not resistant under these conditions [3]. This second mechanism appears to correlate with DSU-induced uncoupling of mitochondria [4]. Other data have implicated accumulation of DSUs in mitochondria; however, the rapid rate of redistribution of both ISCU and MPCU within cells and a non-detectable tight binding component have not allowed determination of the site(s) of drug accumulation or putative cellular receptor(s).

To facilitate these studies, the DSU analogue LY219703 was synthesized as a photoaffinity probe. Initial studies characterized its cytotoxic potency in GC_3/c_1 and the DSU-resistant derivative LYC5. LY219703 was slightly more potent than ISCU, but LYC5 cells had a slightly greater level (about 9-fold) of cross-resistance to both LY219703 and ISCU under the culture conditions used. We next examined the uptake of [^{3}H]LY219703 relative to that of [^{3}H]MPCU [6] and ISCU (unpublished results).

Table 3. Association of [^{3}H]LY219703 with bovine serum albumin (BSA) after Sephadex-G25 gel filtration chromatography

Conditions for sample treatment	% Total radioactivity associated with BSA*
No UV irradiation	
No SDS denaturation	96
SDS sample buffer (2 hr, 23°)	2.6
UV irradiation (20 min)	
No SDS denaturation	73
SDS sample buffer (2 hr, 23°)	35
SDS sample buffer (5 min, 100°)	18

* Results show a representative experiment. Recovery of radiolabel from the column was >93%. BSA (781 μM) was incubated with 7.8 μM [^{3}H]LY219703 for 40 min, and then processed as described in Materials and Methods. Samples were chromatographed on Sephadex-G25 columns to separate protein-bound and free drug, and radiolabel associated with the protein peak was determined.

LY219703 had essentially similar kinetics of accumulation as other DSUs and achieved a cellular concentration about 4-fold above the extracellular level. Concentrative accumulation of [^{3}H]LY219703 was inhibited by FCCP. Uptake of [^{3}H]LY219703 was reduced by co-incubating cells with increasing concentrations of unlabeled ISCU, and ISCU rapidly displaced the radiolabeled LY219703. In addition, LY219703 uncoupled mitochondria at 50 μM [10]. Hence, LY219703 appeared to be similar to MPCU and ISCU and showed potential to be a useful reagent to study distribution and putative intracellular receptors.

In GC_3/c_1 cells, incubated to steady state with [^{3}H]LY219703 (1 μCi/mL), drug efflux was rapid after removing extracellular drug. We investigated the effect of various periods of UV exposure on cellular retention of [^{3}H]LY219703. Results showed that irradiation essentially prevented loss of drug during a 20-min period of efflux, with >85% of accumulated drug remaining. Subsequently, the intracellular distribution of [^{3}H]LY219703 was examined in GC_3/c_1 cells exposed for 60 min to drug, with an additional 20-min UV exposure. Initially, cells were disrupted, and radiolabel in soluble and particulate fractions was determined; approximately 90% of radiolabel associated with particulate material. Extraction of the pellet in cold acetone showed that approximately 31% could be extracted into the organic phase, whereas <5% could be extracted into ice-cold PT buffer. These data suggest that LY219703 (and by inference other antitumor DSUs) associates predominantly with particulate components, possibly membranes. This pattern of distribution would be anticipated because of the hydrophobic characteristics of antitumor DSU [11]. Fractionation of cells into nuclear, mitochondrial, microsomal and cytosolic components revealed about 50% of drug associated with the mitochondrial fraction and a significant fraction (25%) associated with the post-mitochondrial pellet (microsomal). Relatively little drug was associated with nuclear and soluble fractions; however, 52% of radiolabel in the soluble fraction was precipitated by 10% TCA, suggesting covalent binding to soluble proteins.

We further examined the distribution before and after UV photoactivation of [^{3}H]LY219703, where the analogue was incubated with sonicated GC_3/c_1 preparations. A high proportion of radiolabel (83%) was associated with the 100,000 *g* pellet, and this was not altered by UV irradiation. These data indicate that, under the conditions used, photoactivation of [^{3}H]LY219703 did not alter the distribution of the analogue. The specific activity of protein in the pellet was 2.9-fold greater than that of the supernatant, suggesting that distribution was not necessarily a consequence of the mass distribution of proteins in the two fractions.

We next examined the potential for [^{3}H]LY219703 to identify cellular targets for DSU binding. Proteins in the 320,000 *g* or mitochondrial pellet from cells incubated with analogue prior to photoactivation, or mitochondria that were photoaffinity labeled after isolation, were resolved by SDS–PAGE. Unlabeled LY219703 or ISCU completely inhibited labeling in intact cells, probably as a consequence of reduced accumulation of [^{3}H]LY219703. Approximately ten major bands were detected, and the identity of these proteins remains to be determined. The major bands detected in the 320,000 *g* pellet had mobilities consistent with peptides of 110, 88, 76, 70, 64, 58, 48, 36, 26, and 24 kDa. At least four bands were represented in samples enriched for mitochondria (M_r 88, 70, 64, and 36 kDa). Of note, however, was that radiolabeled bands on gels coincided with the major protein bands from mitochondrial preparations. Hence, this labeling pattern may reflect the relatively high concentration of drug accumulated in these organelles, rather than specific high-affinity binding sites. The pattern of photoaffinity labeling of proteins in mitochondria radiolabeled after isolation from GC_3/c_1 cells was similar to that of mitochondrial fractions photoaffinity-labeled in intact cells. Of note was that in all experiments a high proportion of radiolabel migrated with or in front of the dye front. To examine this further, binding of [^{3}H]LY219703 to BSA and the effect of denaturing samples in SDS sample buffer were determined. Results indicate that under relatively mild conditions of denaturing protein samples prior

to SDS–PAGE analysis, a significant proportion of radiolabel dissociated from BSA. Whether this represents dissociation of weakly bound drug or dissociation of radiolabel from the analogue remains to be determined. However, these data suggest that migration of radiolabel with the solvent front on SDS–PAGE may be a consequence of the conditions used for processing samples.

Our data suggest that the photoaffinity analogue LY219703 may be of value in elucidating the distribution and possibly the mechanism(s) of action of antitumor DSU. Results indicate that this photoactivatable analogue has similar cellular pharmacology to other antitumor DSUs. Our studies show that intracellular distribution of LY219703 is predominantly into particulate components, and drug may distribute into membranes. The drug predominantly distributed into the mitochondrial fractions of cells, and several proteins associated with mitochondria were radiolabeled by this photoactivatable analogue, both in intact cells and in isolated mitochondrial preparations. These data are consistent with previous results that suggested accumulation in these organelles as a consequence of the pH gradient across the mitochondrial inner membrane.

Acknowledgements—This work was supported, in part, by PHS award CA51949 and CA21765 (CORE) from the National Cancer Institute and by the American Lebanese Syrian Associated Charities (ALSAC).

REFERENCES

1. Grindey GB, Identification of diarylsulfonylureas as novel anticancer drugs. *Proc Am Assoc Cancer Res* **29**: 525, 1988.
2. Houghton PJ, Bailey FC, Germain GS, Grindey GB, Witt BC and Houghton JA, *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea, a novel agent equally cytotoxic to nonproliferating human colon adenocarcinoma cells. *Cancer Res.* **50**: 318–322, 1990.
3. Sosinski J, Thakar JH, Germain GS, Harwood FC and Houghton PJ, Proliferation-dependent and -independent cytotoxicity by antitumor diarylsulfonylureas. Indication of multiple mechanisms of drug action. *Biochem Pharmacol* **45**: 2135–2142, 1993.
4. Thakar JH, Chapin C, Berg RH, Ashmun RA and Houghton PJ, Effect of antitumor diarylsulfonylureas on *in vivo* and *in vitro* mitochondrial structure and functions. *Cancer Res* **51**: 6286–6291, 1991.
5. Rush GF, Rinzel S, Boder GB, Heim RA, Toth JE and Ponsler GD, Effects of diarylsulfonylurea antitumor agents on the function of mitochondria isolated from rat liver and GC3/c_1 cells. *Biochem Pharmacol* **44**: 2387–2394, 1992.
6. Houghton PJ, Bailey FC, Germain GS, Grindey GB, Howbert JJ and Houghton JA, Studies on the cellular pharmacology of *N*-(4-methylphenylsulfonyl)-*N*-(4-chlorophenyl)-urea. *Biochem Pharmacol* **39**: 1187–1192, 1990.
7. Houghton PJ, Bailey FC, Houghton JA, Murti KG, Howbert JJ and Grindey GB, Evidence for mitochondrial localization of *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea in human colon adenocarcinoma cells. *Cancer Res* **50**: 664–668, 1990.
8. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
9. Schultz RM, Andis SL, Toth JE, Boder GB, Rinzel SM and Grindey GB, Effect of albumin on antitumor activity of diarylsulfonylureas. *Anticancer Res* **13**: 1939–1944, 1993.
10. Sosinski J, Thakar JH, Germain GS, Dias P, Harwood FC, Kuttesch JF and Houghton PJ, Cross-resistance to antitumor diarylsulfonylureas and collateral sensitivity to mitochondrial toxins in a human cell line selected for resistance to the antitumor agent *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea. *Mol Pharmacol* **45**: 962–970, 1994.
11. Howbert JJ, Grossman CS, Crowell TA, Rieder BJ, Harper RW, Kramer KE, Tao EV, Aikins J, Poore GA, Rinzel SM, Grindey GB, Shaw WN and Todd GC, Novel agents effective against solid tumors: The diarylsulfonylureas. Synthesis, activities, and analysis of quantitative structure–activity relationships. *J Med Chem* **33**: 2393–2407, 1990.