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STUDIES ON THE CELLULAR PHARMACOLOGY OF *N*-(4-METHYLPHENYLSULFONYL)-*N'*-(4-CHLOROPHENYL)-UREA

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Abstract—The cellular pharmacology of *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)-urea (MPCU) has been examined in a cloned line of GC_3 human colon adenocarcinoma cells. There was a rapid concentrative accumulation of drug, which could be separated into energy-independent and -dependent phases. Accumulation over 15 sec was linear and temperature dependent, but not energy dependent (azide insensitive). The rate of uptake was a linear function of concentration over a wide range (0.0026 to 5 mM). No saturation kinetics were demonstrated. Steady state was achieved within 10 min, and drug levels associated with GC_3/c_1 cells exceeded the extracellular concentration by 4- to 6-fold. This second phase "concentrative accumulation" of drug was azide sensitive. When cells were incubated to steady state in the presence of azide, removal of azide (with addition of glucose) resulted in a further uptake of sulfonylurea to a higher steady state. When [^{3}H]MPCU was removed from the medium after achieving steady state, loss of drug from cells was rapid ($T_i \approx 130$ sec), and no tight-binding component was apparent. After achieving steady state, cell-associated drug was lost into drug-containing medium reaching a lower steady state if 10 mM azide (± glucose) was added. These data indicate that MPCU may enter cells by a non-saturable energy-independent process (passive diffusion) and bind weakly to some intracellular component or become sequestered to some compartment in an energy-dependent manner.

Sulfonylurea drugs have a wide spectrum of pharmacologic activity, the most recognized being hypoglycemic activity. However, sulfonylureas were initially synthesized as antibacterial agents [1]. Recently carbutamide *N*-(*p*-NH_2-phenylsulfonyl)-*N'*-(*n*-butyl)urea, which differs from tolbutamide in substitution of p-NH_2 for p-CH_3, has been demonstrated as being effective in treatment of *Pneumocystis carinii* [2]. The sulphonylurea sulfometuron methyl is a potent herbicide that inhibits the growth of many bacteria, yeasts and plants [3, 4]. Substantial data support toxicity being a consequence of accumulation of α-ketobutyrate caused by inhibition of the synthesis of branched-chain amino acids. In bacteria, the site of inhibition is the enzyme acetolactate synthase [5] required for synthesis of L-isoleucine and L-leucine.

Recently, the antitumor activity of a series of diarylsulfonylureas has been identified, these compounds having very significant activity against rodent solid tumors [6] and human tumor xenografts [7, 8]. At present the mechanism of cytotoxicity and the basis for therapeutic selectivity are unknown. However, as branched-chain amino acids are not synthesized by mammalian cells, the site of action is presumably different from that of the sulfonylurea herbicides. In work reported here, we have examined the accumulation of *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)-urea (MPCU§; Fig. 1) in a human cell line, GC_3/c_1, developed from a colon adenocarcinoma xenograft sensitive to the sulfonylurea class of antitumor agents.

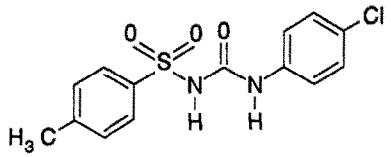

Fig. 1. Chemical structure of *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)-urea.

MATERIALS AND METHODS

A cloned line of human colon adenocarcinoma, GC_3/c_1 [9], was routinely grown in antibiotic-free RPMI 1640 supplemented with 2 mM glutamine and containing 10% fetal calf serum (Hiclone, Gibco, Grand Island, NY). [^{3}H]MPCU (941.2 mCi/mmol) was synthesized as described (European Patent 166615) and purified by reverse phase HPLC. Tris base (ultrapure) was obtained from Boehringer (Indianapolis, IN); all other chemicals were obtained from the Sigma Chemical Co. (St Louis, MO) or through Fisher Scientific (Springfield, NJ).

Accumulation studies. Near confluent cultures

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§ Abbreviations: MPCU, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)-urea; PT buffer, physiologic Tris, pH 7.4; and PA buffer, PT buffer without glucose containing 10 mM sodium azide.

were dispersed into single cell suspensions by gentle trypsinization (0.05% trypsin, 0.53 mM EDTA, Gibco) and seeded at 2×10^6 cells per 35 mM culture dish (No. 3001, Falcon, Oxnard, CA). Cells were allowed to attach overnight before being washed twice in PT buffer (physiologic 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). Monolayers were incubated at room temperature for 15 min in PT buffer (or other buffer) prior to aspiration and adding buffer containing radiolabeled sulfonylurea. For experiments using sodium azide, monolayers were washed as above with a further wash in PA buffer (PT buffer without glucose, containing 10 mM sodium azide) and incubated for 15 min in PA buffer prior to examining drug uptake. To determine accumulation of [^{3}H]MPCU in the absence of Na^+, 120 mM *N*-methylglucamine HCl was substituted for NaCl in PT buffer. To terminate drug accumulation, medium was rapidly aspirated, and monolayers were washed in four successive changes of ice-cold phosphate-buffered saline (PBS). Monolayers were drained, and to each dish 1 mL of trypsin–EDTA solution was added. After 5 min, monolayers were triturated to give a uniform suspension of cells and 0.75 mL was added to 10 mL of ACS liquid scintillation mixture (Amersham, Arlington Heights, IL); then, radioactivity was determined. Cell number per dish was determined on 200 μL of suspension, using the method of Butler [10] as described for this cell line previously [9].

Efflux studies. Monolayers were processed as above, and incubated in 1 mL of drug-containing medium for 60 min. Buffer was aspirated and replaced with PT buffer without radiolabeled drug or in PT or PA buffer containing [^{3}H]MPCU at the same concentration used during the 60-min incubation. At the appropriate time buffer was aspirated and monolayers were washed and processed as before.

Determination of cell volume. Cell volume was determined on suspensions of GC_3/c_1 cells after mild trypsinization of monolayer cultures, using ^3H_2O and [^{14}C]sucrose or [^{14}C]inulin. The volume was calculated to be 3.05 ± 0.45 (SD) μL/10^6 cells.

Metabolism of MPCU. Monolayers of GC_3/c_1 cells were washed twice in PT buffer and incubated (60 min, room temperature) with 5.3 μM [^{3}H]MPCU. Cells were washed in ice-cold PBS as described, and radiolabel was extracted twice into 50 mM sodium phosphate (pH 7.0)–acetonitrile (65:35). Cells and supernatant fractions were centrifuged and the supernatants used for analysis by HPLC. Extraction of radiolabel was ≥ 95% in duplicate determinations. Samples mixed with 2.5 μg MPCU were analyzed by reverse phase HPLC (Partisil 5 ODS-3 RAC, Whatman, Whippany, NJ), and eluted with 50 mM sodium phosphate (pH 7.0)–acetonitrile (59:41) at a flow rate of 1 mL/min. Absorbance was detected at 254 nm, and 0.5-min fractions were collected. MPCU eluted at 6.5 min, and [^{3}H]MPCU was >99% radiochemically pure. Recovery of radiolabel from biologic samples was 94.1 to 108.5% of that loaded on the column.

RESULTS

Data presented in Fig. 2 summarize extensive experiments in which the accumulation of [^{3}H]MPCU was determined over 60 sec in the presence of metabolic inhibitors, the absence of glucose or Na^+, or at low temperature. Accumulation was not dependent upon Na^+, or glucose but was temperature dependent. Ouabain, an inhibitor of Na^+, K^+-ATPase had no significant effect upon accumulation, but accumulation of [^{3}H]MPCU was decreased in PA buffer (no glucose + 10 mM NaN_3). Sodium azide (10 mM) in glucose containing PT buffer had only a slight effect upon drug accumulation.

Effect of NaN_3 on accumulation. The effect of NaN_3 upon accumulation of [^{3}H]MPCU over 10 min is shown in Fig. 3. In PA buffer [^{3}H]MPCU accumulated to a low steady state within 2 min. At the concentration used (2.65 μM), cell-associated drug appeared to be equal to that in the medium. In contrast, cellular accumulation of [^{3}H]MPCU in PT buffer was far greater (≈4-fold).

To determine whether the effect of NaN_3 was reversible, monolayers were incubated for up to 10 min in PA buffer containing 2.65 μM [^{3}H]MPCU. After 4 min, buffer was aspirated from half the dishes and replaced by PT buffer containing 2.65 μM [^{3}H]MPCU, and drug accumulation was examined (Fig. 4). As shown, in PA buffer radiolabeled drug reached a low steady state (equal to that in buffer) after approximately 2 min, which was maintained for a further 8 min. When PT buffer containing [^{3}H]MPCU was substituted, there was a rapid accumulation of drug to a higher steady state. Glucose added to 20 mM (after 4 min of incubation) did not stimulate further uptake of drug in the presence of 10 mM NaN_3 (data not shown).

Kinetics of [^{3}H]MPCU accumulation. To examine whether the initial uptake or subsequent accumulation of drug was a saturable process, drug uptake was examined over a range from 2.65 μM to 5 mM. To achieve solubility MPCU was dissolved in dimethyl sulfoxide (DMSO) such that all cultures were exposed to drug in PT buffer containing a final concentration of 1% DMSO. In preliminary experiments it was found that 1% DMSO did not alter drug accumulation, and that accumulation was linear for up to 15 sec (see inset, Fig. 6). As shown in Fig. 5a, initial rate of uptake calculated over 15 sec was linear over the range of concentrations examined. For higher concentrations uptake was essentially complete within 30 sec (Fig. 5a, inset). In contrast, steady-state levels determined at 10 min demonstrated marked deviation from linearity (by extrapolation of steady-state levels achieved at 2.65 and 5.3 μM MPCU).

Differential effect of NaN_3. It was therefore of interest to determine whether the initial uptake or steady-state level was sensitive to the effect of azide. As shown in Fig. 6, accumulation of [^{3}H]MPCU over 15 sec was not reduced in PA buffer over a 100-fold range of drug concentration (4.65 to 500 μM). In contrast, steady-state levels were decreased in the presence of NaN_3 (Fig. 7). Data are presented as the accumulation of drug above the level in the extracellular compartment (i.e. cell: buffer = 1). In PA buffer there was only slight accumulation of drug relative to the concentration in buffer (≈ 1.15-fold),

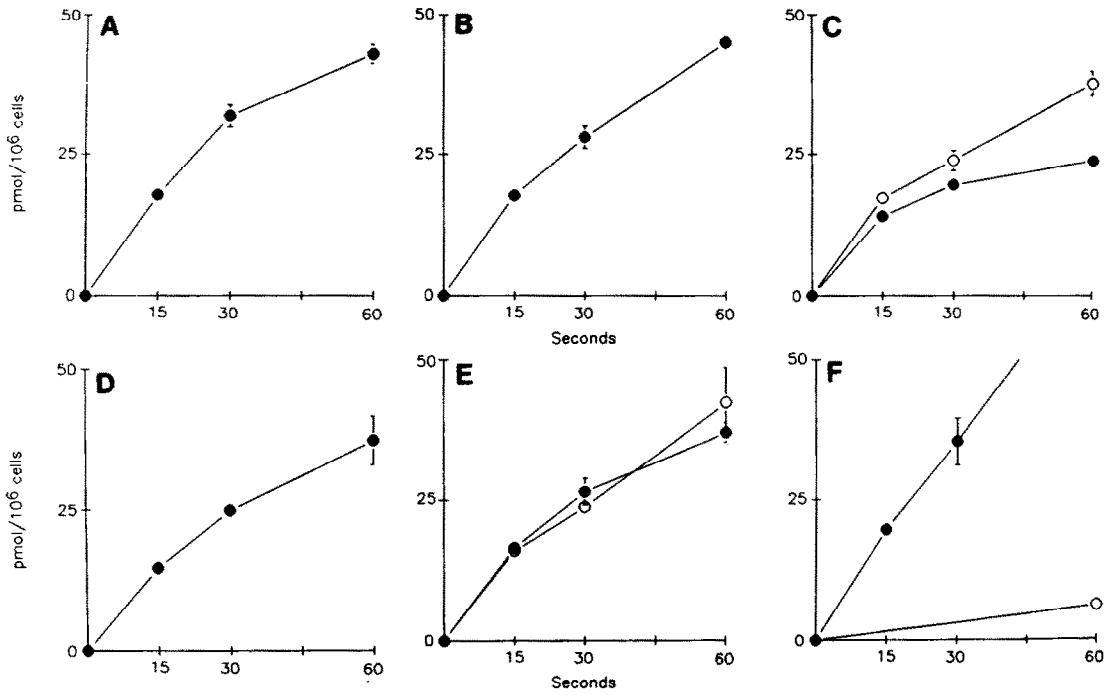

Fig. 2. Accumulation of [^{3}H]MPCU (2.65 μM) in GC_3/c_1 monolayer cultures. Cultures were washed twice in PT buffer, and incubated in the appropriate buffer for 10 min, prior to addition of radiolabeled drug in the same buffer. (A) physiologic Tris (PT; control); (B) PT buffer without glucose; (C) 10 mM NaN_3 in PA (no glucose, ●) or PT buffer (○); (D) 1 mM ouabain in PT buffer; (E) PT buffer (○) or sodium-free buffer (●); and (F) uptake at 23° (●) or 2° (○) in PT buffer. Each point is the mean of 3 determinations. Bars (± 1 SD) are shown where these exceed the symbol size. Accumulation was determined at room temperature unless stated, and results are expressed as pmol/10^6 cells.

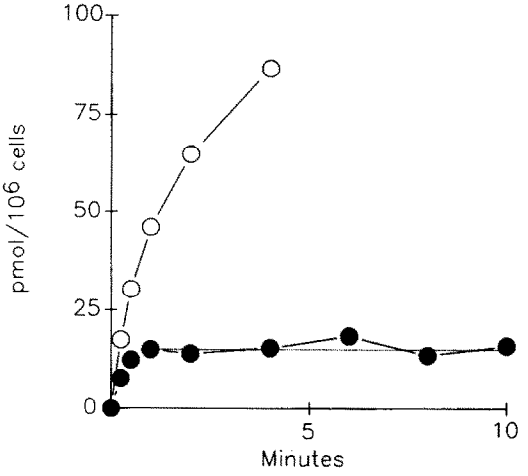

Fig. 3. Effect of NaN_3 on the accumulation and steady-state level of [^{3}H]MPCU in GC_3/c_1 cells. Monolayers were incubated in PT or PA buffer for 10 min at which time buffer was replaced with PT (○) or PA (●) containing 2.65 μM [^{3}H]MPCU. Each point is the mean of 3 determinations (SD < symbol size). Horizontal dotted line indicates equilibrium level between buffer and cells.

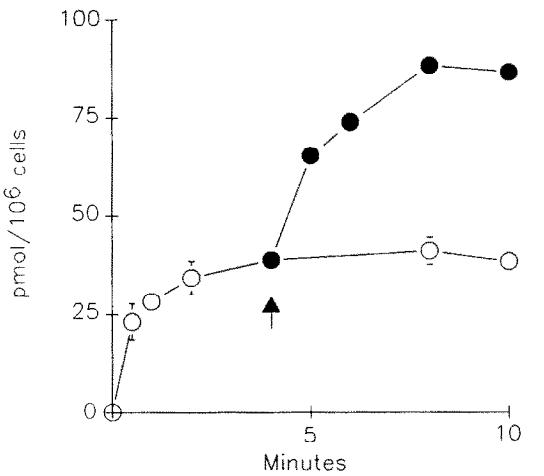

Fig. 4. Reversal of the effect of NaN_3 in GC_3/c_1 cells. Cells were incubated in PA buffer for 10 min. Buffer was replaced with PA containing 2.65 μM [^{3}H]MPCU (○) and accumulation examined over 10 min. After 4 min of uptake (arrow), buffer was aspirated and replaced with PT buffer containing 2.65 μM [^{3}H]MPCU (●), and accumulation of radiolabel was determined. Results are means ± SD for 3 determinations (bars indicate ± 1 SD where this exceeded symbol size).

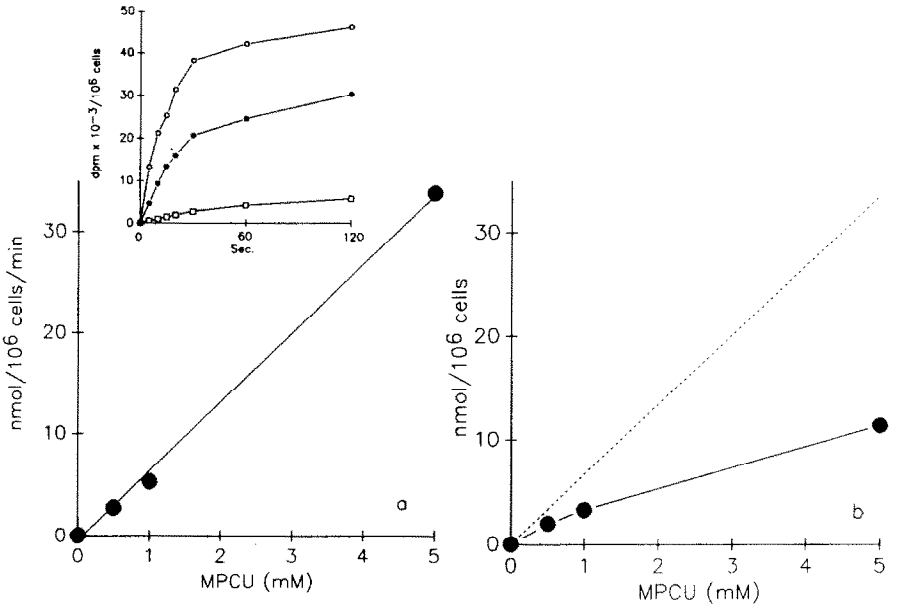

Fig. 5. Effect of concentration of MPCU upon the initial accumulation and steady-state levels of [^{3}H]MPCU. Cells were incubated in PT buffer for 10 min, after which buffer was replaced with PT containing 2.65 μM to 5 mM MPCU. Accumulation was examined at (a) 15 sec; and (b) 10 min. The inset (panel a) shows the time course for accumulation of [^{3}H]MPCU at concentrations of 1 mM (○), 0.5 mM (●) and 0.0625 mM (□). The dotted line (panel b) indicates the steady-state level anticipated from that determined at low drug concentrations (2.65 and 5.3 μM). All buffers contained 1% DMSO. Results are means of 3 determinations (SD < symbol size).

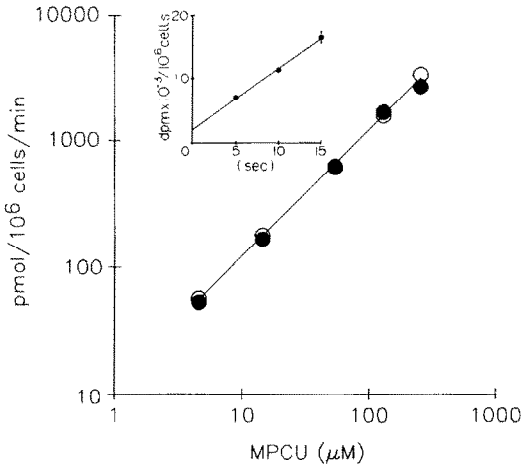

Fig. 6. Lack of effect of NaN_3 on the initial accumulation of [^{3}H]MPCU in monolayer cultures of GC_3/c_1 cells. Monolayers were washed in PT buffer and incubated for 10 min in PT or PA buffer. Buffer was replaced with PT (○) or PA (●) containing [^{3}H]MPCU (4.6 to 500 μM), and accumulation was determined after 15 sec. Inset: linear uptake of [^{3}H]MPCU (4.6 μM) over 15 sec. Values are means ± 1 SD (shown where these exceed symbol size, N = 3).

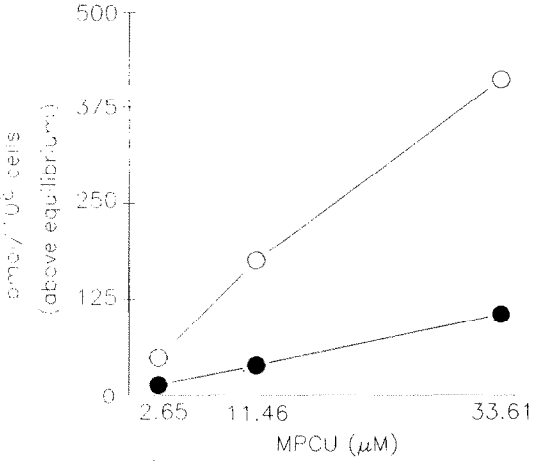

Fig. 7. Effect of NaN_3 upon concentrative accumulation of [^{3}H]MPCU. Monolayers were processed as described (Fig. 5), and uptake was determined at 10 min in (○) PT buffer, or (●) PA buffer. Results are expressed as pmol/10^6 cells above that at equilibrium level (buffer:cells = 1). A volume of 3.05 μL/10^6 cells was used for calculation. Results are means for 3 determinations (SD < symbol size).

whereas in PT buffer this was dependent upon the drug concentration over the range examined (4.6 to 6.4-fold).

Retention of MPCU. Data suggested that there was a rapid accumulation of MPCU (azide insensitive) followed by an azide-sensitive concentrative component. The second component could represent binding, metabolism, or compartmentalization. To examine potential binding, monolayers were incubated for 60 min in PT containing 2.65 μM

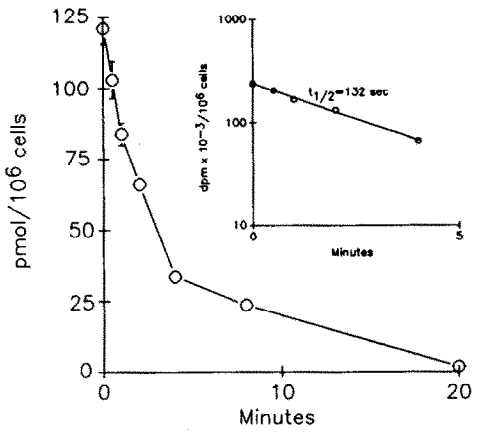

Fig. 8. Efflux of [^{3}H]MPCU into drug-free medium. Monolayer cultures were incubated for 60 min with 2.65 μM [^{3}H]MPCU in PT buffer. Buffer was aspirated and replaced with 3.5 mL PT, and radiolabel associated with cells was determined over 20 min. Inset: semilogarithmic transformation of initial efflux data. Each point is the means ± 1 SD, N = 3.

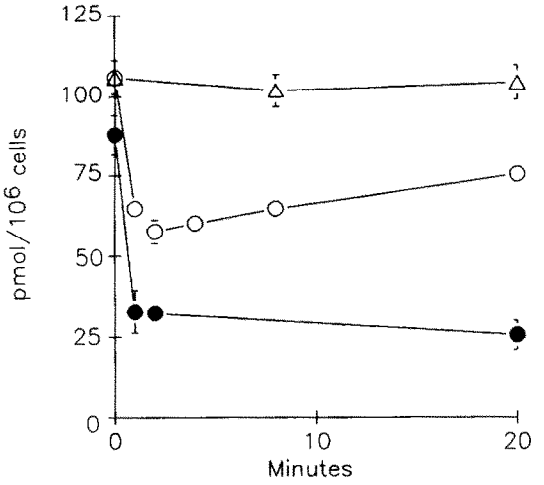

Fig. 9. Effect of NaN_3 and glucose upon the steady-state level of [^{3}H]MPCU in GC_3/c_1 cells. Monolayers were incubated for 60 min in PT containing 2.65 μM [^{3}H]MPCU. At this time buffer was replaced with (△) PT; (○) PT containing 10 mM NaN_3; or (●) PA buffer, each containing 2.65 μM [^{3}H]MPCU. Results are means for 3 determinations ± 1 SD (where this exceeds symbol size). Levels of radiolabel associated with cells were determined over 20 min.

[^{3}H]MPCU. Buffer was aspirated, and replaced with 3.5 mL PT without drug, and retention of radiolabel was determined over 20 min (Fig. 8). Loss of drug was rapid, with an initial $T_{\frac{1}{2}} \approx 132$ sec. By 20 min there was virtually no drug associated with GC_3/c_1 cells.

Effect of NaN_3 *on retention.* To examine whether NaN_3 caused loss of accumulated drug, cells were incubated for 60 min in PT containing 2.65 μM [^{3}H]MPCU. At this time buffer was aspirated and replaced with 1 mL PT buffer, PT buffer containing 10 mM NaN_3, or PA buffer each containing 2.65 μM [^{3}H]MPCU. Data are presented in Fig. 9. Replacing drug-containing PT buffer with the same buffer caused no change in cell-associated drug. The diarylsulfonylurea effluxed to a low steady state (approximately equal to the extracellular level) when PA buffer containing drug was substituted, and to an intermediate level when PT buffer containing radiolabeled drug and 10 mM NaN_3 was substituted.

Metabolism of [^{3}H]*MPCU.* To determine whether concentrative uptake was due to intracellular metabolism, cells were incubated for 60 min with 5.3 μM [^{3}H]MPCU. Extracts analyzed by HPLC demonstrated a single peak of radiolabel, which co-eluted with authentic MPCU (data not shown).

DISCUSSION

The diarylsulfonylureas represent a new class of antitumor agents which may have a novel mechanism of action. Several analogues have demonstrated significant antitumor activity and high therapeutic index in rodent tumor and human tumor models [6–8]. Of interest is that certain human colon adenocarcinomas, which as xenografts are intrinsically resistant to all established oncolytic agents, are responsive to therapy with diarylsulfonylureas ([7] and unpublished data). One of these xeongrafts, $HxGC_3$, has been established *in vitro*, and several clonal lines have been isolated. The concentration of MPCU required to reduce clonogenic potential of GC_3/c_1 cells (72-hr exposure) was 14.8 μM. Thus, GC_3/c_1 appeared to be a suitable cell line in which to study the cellular pharmacology of these agents.

Our initial studies have attempted to examine cellular accumulation of [^{3}H]MPCU in monolayer cultures. Association of drug with cells was rapid and temperature dependent; initial rates of uptake (15 sec) at 2.6 μM MPCU were 1.4 and 38.0 pmol/10^6 cells at 2° and 23°. At 37° the rate of uptake was 84 pmol/10^6 cells/min (data not shown). Uptake was not altered by ouabain and was not dependent upon Na^+. Accumulation over 10–15 sec was linear at all concentrations of drug examined (2.65 μM to 5 mM), and showed no evidence of saturation kinetics. As MPCU is highly lipophilic, these data suggest that this phase is not carrier mediated. Further, depletion of ATP by 10 mM NaN_3, which uncouples oxidative phosphorylation [11], did not alter this phase of accumulation. That ouabain did not alter accumulation suggests that membrane potential, dependent on Na^+, K^+-ATPase of the plasma membrane, is not influencing this phase of drug association. The antitumor diarylsulfonylureas have structural similarity to oral hypoglycemic agents, and indeed MPCU does have hypoglycemic activity *in vivo*. That binding to GC_3/c_1 cells is relatively weak suggests that MPCU has a mechanism different from those of sulfonylurea hypoglycemic agents which bind tightly to membrane receptors ($K_D \approx 10^{-9}$ M), on pancreatic β-cells [12, 13]. Results presented here suggest entry into GC_3/c_1 cells by a passive process.

The second phase of accumulation was concentrative, in which steady-state levels reached ≈ 4- to 6-fold that in the extracellular compartment. This

phase was sensitive to NaN_3. The effect of NaN_3 was rapid, and rapidly reversible. Cells loaded to steady state in the absence of glucose, and 10 mM NaN_3, rapidly accumulated MPCU to a higher steady state when glucose was replaced and NaN_3 removed. Conversely, in cells loaded to steady state in the presence of glucose, the addition of NaN_3 resulted in a new lower steady state. In the absence of glucose, NaN_3 caused rapid loss of MPCU to the equilibrium level (cell : buffer = 1). These results support a model in which concentrative accumulation is energy dependent. However, when drug-loaded cells were incubated in non-drug-containing PT buffer (Fig. 8), there was rapid loss of drug ($T_{\frac{1}{2}} \approx 132$ sec) with no apparent non-exchangeable fraction. Thus, although MPCU is concentrated in cells, "binding" to a cellular component appears to be weak. No metabolism of MPCU was apparent in GC_3/c_1 cells exposed to drug for 1 hr.

Several models can be suggested to account for the data presented. Of interest is the effect of NaN_3 and apparent antagonism by glucose. In GC_3/c_1 cells NaN_3 reduced accumulation, and glucose reduced the efflux of sulfonylurea in drug-loaded cells exposed to NaN_3 (Fig. 9). These results are the exact opposite of those observed in multidrug resistant (MDR) cells in which NaN_3 stimulates the uptake, and glucose causes the efflux, of anthracyclines or Vinca alkaloids [14, 15]. In MDR cells, considerable data support efflux via a unidirectional "pump" (gp170) (reviewed in Ref. 16), the product of the *mdr*1 gene [17]. Thus, our data could suggest an inward pump for sulfonylureas in GC_3/c_1 cells. However, the lack of saturation kinetics and rapid loss of drug when monolayers were incubated in drug-free medium argue against this. Two alternatives are that (i) MPCU binds weakly to some intracellular (or membrane?) component in an energy-dependent manner, and that ATP is a requirement for this binding; or (ii) MPCU is sequestered into a cellular compartment, and that sequestration is determined by virtue of either a membrane potential or pH gradient dependent upon cellular energy.

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