Effect of Antitumor Diarylsulfonylureas on in Vivo and in Vitro Mitochondrial Structure and Functions

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ABSTRACT

Diarylsulfonylureas are novel oncolytic agents shown to have therapeutic activity against both rodent solid tumors and xenografts of human tumors in mice. Previous studies have shown that diarylsulfonylureas localize in mitochondria and cause morphological changes in these organelles. We have investigated the mechanism of action of diarylsulfonylureas, namely, \(N\)-(5-indanylsulfonyl)-\(N'\)-(4-chlorophenyl)urea (ISCU) and the \(N\)-4-methyl analogue (MPCU), by studying their effect on mitochondrial morphology and uptake of rhodamine 123 in GC3/cI cells in culture and the oxidative phosphorylation in isolated mitochondria from mouse liver, using pyruvate-malate and succinate as substrates. Morphometric analysis of mitochondria in GC3/cI cells exposed to ISCU showed that ISCU (165 \(\mu\)M) doubled the mitochondrial size after 24-h exposure in culture. Also, ISCU (100 \(\mu\)M), like 40 \(\mu\)M carbonylcyanide \(p\)-trifluoromethoxyphenylhydrazone; FCCP, significantly reduced the rhodamine 123 uptake by GC3/cI cells studied by flow cytometry. In isolated mitochondria both ISCU and MPCU uncoupled oxidative phosphorylation at 50 \(\mu\)M, with pyruvate-malate as substrate, as was indicated by a significant increase in the State 4 oxygen consumption. This resulted in the loss of ADP phosphorylation and, therefore, the ADP/oxygen ratio was reduced to zero and the respiratory control ratio to one. The succinate oxidation was also significantly impaired by ISCU, causing some decrease in ADP phosphorylation. On the other hand, MPCU did not exhibit any significant effect on the oxidation of succinate. At concentrations of lower than 50 \(\mu\)M, both of these compounds exhibited a deleterious effect, causing damage to mitochondrial functions in the presence of pyruvate-malate as substrates. These data confirm, through morphometric analysis, our previous qualitative observations of abnormal mitochondrial morphology observed in GC3/cI cells grown in the presence of high concentrations of ISCU and MPCU and further suggest that diarylsulfonylureas, by uncoupling mitochondrial oxidative phosphorylation, may lower cellular ATP. It is probable that this mechanism contributes, at least partially, to cytotoxicity in GC3/cI cells exposed to high concentrations of ISCU for relatively brief periods (2 to 4 h) and possibly contributes to cytotoxicity at drug concentrations that can be achieved in rodents.

INTRODUCTION

In recent development on new antitumor drugs, DSU\(^3\) have attracted increasing interest (1–8). Classically, these compounds are known as hypoglycemic agents and are used to treat non-insulin-dependent (type II) diabetes mellitus (9). However, studies from Grindey and coworkers (1–4) and those from our laboratory (5–8) have shown that at least two of the compounds in the class of DSU, namely, MPCU (LY 181984) and ISCU (LY 186641, Sulofenur) (Fig. 1), have good therapeutic efficacy against murine solid tumors and human tumor xenografts.

Currently, these compounds are undergoing multicenter Phase I and Phase II clinical trials, but have shown only minimal activity against a spectrum of adult tumors. The major toxic effects exhibited by ISCU were anemia and methemoglobinemia (2, 10, 11), which occurred at drug concentrations in humans that were lower than concentrations of ISCU that cause significant tumor regressions in rodents. In part, this may relate to metabolism in humans, where \(p\)-chloroaniline may contribute to the toxic effects (12). However, DSU seem to have unique antitumor activity because, unlike many other chemotherapeutic agents, they do not inhibit macromolecular synthesis of protein, RNA, or DNA (2–4) except at very high concentration (6). The spectrum of toxicity in preclinical and human trials suggests that rapidly proliferating tissues are not damaged by DSU. Also, our preliminary work has suggested that mitochondria may be the target for these drugs. In cell culture, mitochondria rapidly accumulate MPCU and show altered morphology (7). This accumulation is temperature dependent, and mitochondrial toxins like FCCP, 2,4-dinitrophenol, nigericin, or Na\(_+\) interfere with the uptake (7, 8). Thus, the accumulation of MPCU seems to depend on the intactness of mitochondrial proton motive force, which is largely made up of membrane potential (\(\delta\phi\)) and the pH gradient (\(\delta p\)) across mitochondrial membrane.

In serum, ISCU binds specifically to albumin and has a biological half-life of 5 days in the monkey (10). Our study with GC3/cI cells in culture has shown that ISCU binds to serum albumin (6) and, in a drug-free medium, MPCU is rapidly lost from cells with a \(t\(_1/2\)\) of 132 s at 23°C (8). As there is no tight-binding or nonexchangeable fraction, it suggests that most of the drug exists compartmentalized into mitochondria. In cell culture study, up to 97% of DSU may be recovered in unaltered form (6–8). Earlier studies have shown that sulfonylureas, such as sulfometuron and chlor sulfuron, have herbicidal and bactericidal properties because they inhibit synthesis of branched chain amino acids through inhibition of acetolactate synthase (13, 14). However, this pathway is absent in mammalian cells.

To obtain further insight into the mechanism of action of MPCU and ISCU, we have extended our studies on mitochondria, using GC3/cI cells in culture and organelles from mouse liver and evaluated the effect of these novel antitumor agents on mitochondrial morphology and oxidative phosphorylation.

MATERIALS AND METHODS

Animals. Female CBA/CaJ mice were obtained from Jackson Lab, Bar Harbor, ME. They were maintained in the St. Jude Children’s Research Hospital vivarium according to NIH guidelines, with 12-h cycles of light and dark. Food and water were provided ad libitum.

Chemicals. All the chemical reagents used were of analytical grade. Mannitol was purchased from Fisher Scientific Co., Atlanta, GA. Pyruvate, l-malate, succinate, sucrose albumin, and other reagents were purchased from Sigma Chemical Co., St. Louis, MO.
Cell Line. GC3/c1 cells were derived from a moderately differentiated human colon adenocarcinoma established initially as a xenograft (15), and the GC3/c1 clone was derived by dilution cloning (16). Cells were maintained as monolayers in antibiotic-free RPMI-1640 containing 10% FCS as described before (6, 7). Cultures were fixed immediately upon removal from the incubator by adding glutaraldehyde to a final concentration of 2% (v/v) in the medium. After 1.5 h the cells were pelleted and rinsed in sodium cacodylate buffer (0.1 M, pH 7.2) and postfixed for 1.5 h in buffered 1% osmium tetroxide. Cells were then dehydrated in an ethanol/acetone series and embedded in Poly Bed 812. Thin sections were stained in uranyl and lead salts, and electron microscopy and morphometric analyses were performed at the Memphis State University Center for Electron Microscopy.

Micrographs used in morphometry were all prepared at the same original magnification (calibrated at ×22,400). For each treatment and time point, ten micrographs were used, each from a randomly selected cell and at the standard magnification. Morphometric analysis of the cross-sectional areas of mitochondria in contact prints of the micrographs was made using the Bioquant Image Analysis System IV, equipped with a Summasketch Plus digitizing pad and a statistical analysis package. Data are expressed in μm². Only mitochondria whose entire profiles could be observed in the micrographs were measured. Samples were coded, and analysis was performed without any knowledge of either the treatment or exposure time. To determine the proportion of cellular cross-sectional area occupied by mitochondria, the total cell area and mitochondrial area were determined for 8 untreated cells. The proportion of area occupied by mitochondria was 5.64 ± 2.1% (range, 1.79 to 7.41%).

Isolation of Mitochondria. Mitochondria were prepared by using the procedure of Thakar (17) as modified for liver (18). Briefly, livers from 3 to 4 mice were pooled and homogenized at 0–4°C in a medium containing 0.1 M sucrose, 0.01 M EDTA, 0.046 M KCl, 0.1 M Tris, and 0.5% bovine serum albumin at pH 7.4, using a Teflon pestle, Size C, homogenizer (Thomas Scientific) at 1250 rpm. The homogenates were centrifuged at 478 × g for 10 min to remove debris and nuclei. The supernatants were centrifuged at 12,000 × g for 10 min to harvest mitochondrial pellets. These were then resuspended in the homogenizing medium and centrifuged once more. The resulting pellets were resuspended in small volumes (0.4 to 0.6 ml) of medium which contained 0.23 M mannitol, 0.07 M sucrose, 0.02 mM EDTA, 5.0 mM KH₂PO₄, and 0.02 M Tris buffer at pH 7.4. This also was the medium in which oxidative phosphorylation was studied.

Measurements of Oxidative Phosphorylation. Oxygen consumption in mitochondria was measured in the above medium using a YSI Model 5300 biological oxygen monitoring system equipped with a Clark-type oxygen electrode and water-jacketed incubation chamber having a 1.5-ml volume maintained at 30°C. The RCR and ADP/O were calculated according to the method of Chance and Williams (19). The protein concentration of the mitochondrial pellet was measured using the reagents and the procedure supplied by Bio-Rad Laboratories (20).

Rhodamine 123 Uptake Study. Flow cytometry of cells after exposure to rhodamine 123 has been used previously to determine mitochondrial function in the presence of inhibitors such as rotenone and monensin (21). For rhodamine 123 uptake studies, GC3/c1 cells were routinely grown in Falcon No. 3001 tissue culture dishes as before. In overnight or 48-h log-phase growing cultures, FCCP (40 μM) or ISCU (100 μM) was added, and the monolayers of cells were incubated at 37°C for 30 min. Next, an isotonic concentrated stock of 100 μg/ml of rhodamine 123 (Fisher Scientific, Pittsburgh, PA) in phosphate-buffered saline was added to each cell culture dish to yield a final rhodamine 123 concentration of 5 μg/ml. Cells were returned to 37°C to incubate for 30 min, after which the rhodamine-containing medium was aspirated and replaced with RPMI 1640 supplemented with 10% fetal bovine serum and FCCP or ISCU. After 10-min incubation at 37°C, cells were released by trypsinization, resuspended in complete medium, filtered through 44 μm nylon mesh, and placed on ice. Fluorescence at wavelengths of 525 nm ± 10 nm from stained cells was detected by a Coulter EPICS 753 flow cytometer (Coulter Corp., Hialeah, FL) after excitation with 600 mW of laser light at 488 nm. Mean fluorescence levels of individual samples were compared to stained samples receiving no drug pretreatment to evaluate the relative fluorescence intensity.

RESULTS

Morphometric Studies. The morphometric analysis of GC3/c1 cells in culture revealed that, in the presence of ISCU (165 μM), mitochondria increased in size (Fig. 2). At 24 h ISCU-treated mitochondria had twice the area of control organelles (P < 0.005). On the contrary, after 48 and 72 h of treatment the area of ISCU-treated mitochondria decreased significantly as indicated by area measurements (P < 0.005 and P < 0.05). Other subcellular organelles did not show any appreciable change during this study.

Mitochondrial Oxidative Phosphorylation. Since the isolation of functional mitochondria from GC3/c1 cells grown in culture is a formidable task, we have evaluated the effect of ISCU and MPCU on isolated mitochondria from mouse liver. The oxygen consumption rates and the two other parameters of oxidative phosphorylation in mouse liver mitochondria are presented in Fig. 3 and Tables 1 and 2. As depicted in Fig. 3, using pyruvate-malate as substrate, and adding small quantities of ADP control rates of oxygen consumption, RCR and ADP/O were obtained for each preparation. A similar procedure was also used to obtain control values for succinate oxidation by mitochondria (Tables 1 and 2). When a small quantity of ADP (0.1 to 0.3 μmol) is added to mitochondria which are functionally intact and incubated with substrates and oxygen, the consumption of oxygen is stimulated (State 3 oxygen rate). Upon the phosphorylation of added ADP, the oxygen consumption in mitochondria decreases to its original background level, which is known as State 4 oxygen consumption. The ratio of State 3 oxygen rate to State 4 oxygen rate is called RCR.
concentrations below 50 mM, ISCU was fairly active in impairing mitochondrial functions with pyruvate-malate as substrate. The State 4 $O_2$ rate was increased by 50% at 10 mM ISCU. In the presence of succinate as substrate, this impairment was less significant. There was very little change in the State 4 $O_2$ rate, and the RCR and ADP/O values decreased by a small fraction only. Studies at concentrations of MPCU lower than 50 mM indicated that this compound exhibited some deleterious effect on mitochondrial oxidative phosphorylation at concentrations as low as 20 mM (Fig. 5).

Rhodamine 123 Uptake Study. Rhodamine 123 is a unique fluorescent probe highly specific for mitochondrial localization and function (21-23). The cellular content of rhodamine 123, which may reflect mitochondrial membrane potential ($\Delta \psi$), can be conveniently measured by flow cytometry. As shown in Table 3 and Fig. 6, the treatment of GC3/c1 cells with FCCP (40 mM)

Data showing the effect of ISCU (50 mM) on various parameters of oxidative phosphorylation are shown in Fig. 3A and Table 1. With the NAD-linked substrate pyruvate-malate, ISCU consistently uncoupled mitochondria in all the preparations. This resulted in the stimulation of State 4 oxygen consumption and absence of State 3 oxygen utilization. Therefore, theoretically the RCR was decreased to one and also the loss of phosphorylation of ADP resulting in decreasing ADP/O to zero. All these changes were statistically highly significant ($P < 0.001$). The stimulation of State 4 oxygen consumption was 3- to 4-fold. In the presence of an FAD-linked substrate succinate, again ISCU at 50 mM showed some significant impairment of various parameters of oxidative phosphorylation (Table 1). The State 4 oxygen consumption was increased by 1- to 2-fold. However, this did not totally uncouple the phosphorylation of ADP from substrate oxidation; thus, the values of RCR and ADP/O decreased, but to a smaller extent.

In a separate experiment (Fig. 4), it was observed that, at concentrations below 50 mM, ISCU was fairly active in impairing mitochondrial functions with pyruvate-malate as substrate. The State 4 $O_2$ rate was increased by 50% at 10 mM ISCU. Thus, the study indicated that ISCU is an effective uncoupler of oxidative phosphorylation, although less potent than FCCP.

Studies with MPCU (50 mM) demonstrated that oxidative phosphorylation was significantly impaired in the presence of this compound when pyruvate-malate was used as substrate (Fig. 3B; Table 2). Again, the State 4 $O_2$ rate was stimulated, and RCR and ADP/O ratios were significantly decreased. However, in the presence of succinate as substrate, this impairment was less significant. Therefore, the values of RCR and ADP/O were significantly decreased. How-

**Table 1** Effect of ISCU on various parameters of mitochondrial oxidative phosphorylation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State 3 $O_2$ rate*</th>
<th>State 4 $O_2$ rate*</th>
<th>RCR</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate (5 mM) + L-malate (1 mM)</td>
<td>76.1 ± 18.1</td>
<td>20.4 ± 6.35</td>
<td>4.1 ± 1.4</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>ISCU (50 mM)</td>
<td>0</td>
<td>64.9 ± 14.8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Succinate (6 mM)</td>
<td>211.0 ± 30.0</td>
<td>67.5 ± 16.9</td>
<td>3.2 ± 0.7</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>ISCU (50 mM)</td>
<td>165.5 ± 31.8</td>
<td>120.5 ± 7.3</td>
<td>1.4 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

* $O_2$ rates are expressed as nanomols of oxygen/min/mg of mitochondrial protein at 30°C.
* Mean ± SD of 8 preparations for pyruvate + malate and 5 preparations for succinate as substrates.
* $P < 0.001$.
* $P < 0.005$.
* $P < 0.01$.

Table 2 Effect of MPCU on various parameters of mitochondrial oxidative phosphorylation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State 3 $O_2$ rate*</th>
<th>State 4 $O_2$ rate*</th>
<th>RCR</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate (5 mM) + L-malate (1 mM)</td>
<td>93.4 ± 20.3</td>
<td>30.2 ± 8.7</td>
<td>3.2 ± 0.8</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>MPCU (50 mM)</td>
<td>45.4 ± 50.7</td>
<td>63.2 ± 18.4</td>
<td>1.5 ± 0.5</td>
<td>1.0 ± 0.9</td>
</tr>
<tr>
<td>Succinate (6 mM)</td>
<td>222.5 ± 48.8</td>
<td>94.6 ± 8.1</td>
<td>2.4 ± 0.6</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>MPCU (50 mM)</td>
<td>192.2 ± 2.3</td>
<td>99.5 ± 5.6</td>
<td>1.9 ± 0.4</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Details are the same as those given under Table 1.
* $P < 0.01$. With pyruvate + malate five preparations were tested, and with succinate as substrate three preparations were tested.

![Fig. 3. Oxygen consumption curves for isolated mouse liver mitochondria. A, studied for the effect of ISCU (50 mM); B, studied for the effect of MPCU (50 mM). In the incubation system various additions were made as depicted by number. 1, medium; 2, substrate, i.e., pyruvate + malate or succinate; 3, mitochondria (1 to 2 mg of protein); 4, ADP (100 to 300 nmol); 5, ISCU (50 mM); and 6, MPCU (50 mM). ST3 and ST4, States 3 and 4, respectively.](image)

**Fig. 4. Effect of ISCU (0 to 50 mM) on parameters of oxidative phosphorylation of mitochondria from mouse liver studied in vitro. A, State 4 oxygen consumption rate; B, respiratory control ratio (RCR); C, ADP/O ratio. Pyruvate-malate was used as substrate.**
It is noteworthy that the impairment of mitochondrial function and toxic cell death are closely related events (25, 26). As observed previously (7) and confirmed here by our present study (Fig. 2), enlargement of mitochondria may represent a crucial pathogenic event and, if not controlled, can lead to cell death. In experimental essential fatty acid deficiency (27), radiation pancreatitis (28), or thyrotoxic myopathy (29), enlargement of mitochondria seems to be a prominent feature of cell pathology associated with biochemical functional impairment.

We reported previously that GC3/c1 cells exposed to high concentrations of ISCU and MPCU exhibited enlarged mitochondria (7). However, although this effect appeared dependent upon the concentration of DSU, no morphometric analysis or time-course study was undertaken. Therefore, in the current study the effect of a single concentration of ISCU was examined over 72 h. In the present study there appears to be a highly significant enlargement of mitochondrial volume after ~24 h of exposure, followed by condensation of these organelles. Exposure of cells at this concentration for 24 h resulted in 75% reduction in subsequent colony formation. Although this phenomenon cannot be directly related to cytotoxicity, by 24 h most (75%) of the cells have committed to death. Hence, the increase in mitochondrial volume appears to be an early event in this process. It is of note also that, in a subline of GC3/c1 selected for 3- to 4-fold resistance to ISCU, the enlargement of mitochondria is far smaller and occurs at ~36 to 48 h after continuous exposure to ISCU (data not presented). Enlargement of these organelles is consistent with impairment of function; however, the decrease in area at later time points and its significance are not understood at this time. The subsequent decrease in mitochondrial size could reflect enrichment of the population for cells not lethally damaged (i.e., doomed cells had detached from the culture plate), or it may be a later event in the process of cell death. Although the number of cells/plate was not quantitated over the time-course of drug exposure, cell density did not decrease over the first 24 h; however, there was a subsequent loss of cells. Thus, at longer time points the data may be influenced by selective loss of doomed cells.

The data presented in Fig. 3 and Tables 1 and 2 have shown that both ISCU and MPCU are efficient uncouplers of oxidative

and ISCU (100 μM) significantly reduced the fluorescence intensity of rhodamine 123 as compared to that of rhodamine 123 control (P < 0.002 and P < 0.05, respectively).

**DISCUSSION**

Previous studies from our group have shown that DSU accumulate in mitochondria and at high concentration cause enlargement of these organelles. Mitochondria, therefore, are a possible target for the action of DSU. However, the exact mechanism of action of these compounds remains uncertain. In the past, for the reason that the isolated mitochondria from tumor cells have generally not shown any considerable difference in their properties as compared to the normal cells, these organelles have remained relatively unexplored as the target for antitumor therapy (22-24). Chen and coworkers have pointed out that mitochondria have high membrane potential and are capable of accumulating large quantities of certain positively charged lipophylic toxic antimetabolites (22-24). Our present study seems to be timely in implicating mitochondria as a viable target for the chemotherapeutic amelioration of solid tumors.

Table 3  **Mean rhodamine 123 fluorescence intensity of FCCP- and ISCU-treated GC3/c1 cells**

Log-phase cultures of GC3/c1 cells having 1 to 2 × 10⁶ cells in RPMI 1640 medium were exposed to FCCP (40 μM) or ISCU (100 μM) in duplicates and incubated at 37°C in a CO₂ incubator. Rh-123 was added to these cells and control cells at a final concentration of 5 μg/ml and further incubated for 30 min at which time the medium was replaced and cells were washed with phosphate-buffered saline, trypsinized, suspended in RPMI 1640, kept on ice, and studied using a flow cytometer.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>183.76 ± 6.81*</td>
</tr>
<tr>
<td>FCCP (40 μM)</td>
<td>117.20 ± 13.94*</td>
</tr>
<tr>
<td>ISCU (100 μM)</td>
<td>160.32 ± 12.60*</td>
</tr>
</tbody>
</table>

* Mean ± SD of three separate experiments. Details are given in the text.

* P < 0.002.

* P < 0.05; both probabilities versus control.

Fig. 5. Effect of MPCU (0 to 50 μM) on the oxidative phosphorylation of mouse liver mitochondria studied in vitro using pyruvate-malate as substrates. A, State 4 oxygen consumption rate; B, RCR; C, ADP/O ratio.

![Graph showing the effect of MPCU on oxidative phosphorylation](image)

**Fig. 6.** Flow cytometric measurement of the fluorescence due to rhodamine 123 uptake by GC3/c1 cells. A, unstained control; B, rhodamine 123 treatment alone; C, FCCP (40 μM); D, ISCU (100 μM) treatment. A total of 5,000 to 10,000 cells were used for obtaining each spectrum. A decrease in the fluorescence intensity in ISCU-treated and FCCP-treated cells is depicted by a shift to the left on the abscissa. A decrease in rhodamine 123 fluorescence was highly significant for FCCP-treated cells, P < 0.002 (B versus C), and significant at P < 0.05 for ISCU-treated cells (B versus D).
phosphorylation, although in this study ISCU was found to be superior to MPCU. Also, it is noteworthy that ISCU exhibited its uncoupling action against both NAD-linked as well as FAD-linked substrates, whereas MPCU seems to be more effective in uncoupling the oxidation of pyruvate-malate. It seems that the oxidation of pyruvate is probably more crucial for cell survival because, when this is inhibited by a compound such as cyperquat, a potent neurotoxic agent, the neurons of substantia nigra degenerate (Ref. 30 and references therein). Furthermore, in isolated mitochondria both these compounds are active at concentrations lower than 50 µM, and ISCU has a significant effect at 20 µM (Figs. 4 and 5). We have shown previously that ISCU and MPCU accumulate in cells to a level 4- to 6-fold that in the extracellular medium, and that this concentrative accumulation is probably accounted for by sequestration into mitochondria. The uptake of DSU was not studied in isolated mitochondria; hence, the intramitochondrial concentration is, at this time, unknown. However, in medium containing 10% fetal bovine serum, the 50% inhibitory concentration for ISCU by continuous exposure (7 days) in GC3/ci cells is ≈40 µM. Assuming that 95% ISCU is bound to serum albumin (determined by ultrafiltration), then the free concentration would be ≈2 µM, and the steady-state level in cells would be ≈8 µM. If most, or all, of the DSU partitions into mitochondria, which occupy ≈5% of the volume of GC3/ci adenocarcinoma cells, it is apparent that concentrations of DSU in the mitochondrial matrix may exceed 160 µM, high enough to cause partial or complete uncoupling of oxidative phosphorylation.

It has been shown that uncouplers of mitochondrial oxidative phosphorylation decrease rhodamine 123 uptake (21-24). To examine whether ISCU caused uncoupling under conditions used for cytotoxicity assays, we have examined the uptake of rhodamine 123 in cells exposed to ISCU (100 µM) and FCCP (40 µM). As ISCU is rapidly eliminated from cells when extracellular drug is removed, the effect of ISCU on accumulation of rhodamine 123 was examined in the presence of ISCU, rather than after sequential ISCU treatment followed by uptake of the dye.

The rhodamine 123 uptake study (Table 3; Fig. 6) was of short time duration, during which cells were exposed to drugs for a total of 60 min. Both FCCP and ISCU significantly reduced the uptake of this vital dye, although FCCP was considerably more potent. Moreover, these experiments were performed in the presence of 10% fetal calf serum in the incubation medium. ISCU tightly binds to albumin in fetal calf serum; hence, the concentration of unbound ISCU in medium was ≈5 µM.

The hypoglycemic action of sulfonylureas is probably related to the inhibition of the K+ channels. However, recently it was pointed out by White et al. (31) that sulfonylureas like glyburide and tolbutamide stimulate mitochondrial oxygen utilization and decreased the ATP content. Also Mojena et al. (32) have shown that sulfonylureas, like glipizide, increase glycogen phosphorylase activity and lead to higher lactate formation (32). This may be due to the uncoupling of oxidative phosphorylation, resulting in a lower ATP/ADP ratio. Our study supports these observations and the view that sulfonylureas may function as an uncoupler of cellular oxidative phosphorylation and that pharmacologically this may present a novel mechanism by which antitumor DSU exert their cytotoxic effects, at least in vitro.

Generally, the uncouplers of oxidative phosphorylation exhibit protonophoric action by having a weak acidic dissociable group, with an electron withdrawing property and high lipophylic character (33). DSU, such as ISCU and MPCU, exhibit all these characteristics (4), making them good candidates for uncoupling of oxidative phosphorylation. Therefore, they may function as a catalyst in transporting H+ across the inner membrane of mitochondria and may not show a tight binding to mitochondrial protein (8). Also, our earlier observation of abnormal mitochondria in GC3/ci cells in the presence of MPCU and ISCU could have been related to the accumulation and uncoupling action of DSU. This supports previous observations of Chen et al. (22-24) that mitochondria may function as "source or sink" for antimetabolites.

We have proposed that, in cell culture, there appear to be at least two distinct mechanisms by which DSU exert cytotoxicity (34). Exposure of cells in serum-free medium for 4 h to ISCU results in 50% cytotoxicity (reduction in colony formation) at ≈40 µg/ml (≈114 µM). At this concentration incorporation of radiolabeled thymidine and uridine into nucleic acid is inhibited. This probably relates to the uncoupling activity reported in this study. Whether this mechanism is important for cytotoxicity in a therapeutic setting is less clear. If ISCU is concentrated 4-fold within cells, and most of the intracellular drug is sequestered within mitochondria, which occupy 5% of the cellular volume, then for an external concentration of 0.5 µM (0.18 µM/ml), the intramitochondrial concentration would approach 40 µM. Consequently, under these conditions intramitochondrial concentrations of ISCU could approach the level that, in vitro, would cause partial uncoupling of mitochondria. It is of note, therefore, that the concentration of ISCU that reduces clonogenic survival of GC3/ci cells exposed for 24 h to drug, in medium containing 0.5% serum, is 1.9 µg/ml (5.4 µM) (7). In low-serum conditions, the free-drug concentration will be sufficient to achieve intramitochondrial concentrations associated with mitochondrial effects described in the current work.

Whether the effect of uncoupling is a primary or secondary mechanism of cellular cytotoxicity remains unclear. It seems likely that in mice, at doses that cause only minimal regressions of highly sensitive rhabdomyosarcoma xenografts, the concentration of unbound ISCU may approach the range required for mitochondrial impairment. For example, daily dosing of mice at 100 mg/kg results in plasma concentrations of 250 to 300 µg/mL. Assuming 99.97% protein-bound drug (10), this would result in 0.1 µg/ml of free ISCU, close to the estimated concentration that in vitro results in drug accumulation sufficient to partially uncouple mitochondria. In the study of Taylor et al. (10), the maximum tolerated dose of 1200 mg/kg/day of ISCU gave peak plasma levels of total drug of 284.1 µg/ml and a free-drug concentration of 0.08 µg/ml, relatively close to that required to exert the mitochondrial effects described in this current study. Clearly, the toxicity of ISCU, which may be related to metabolism and production of p-chloroaniline, prevents the achieving of adequate free drug necessary to exert the antitumor effects against many types of cancer. Further studies with GC3/ci and an ISCU-resistant subline recently developed by us (34) will be of value in defining the significance of the uncoupling activity of DSU both in vitro and in vivo.

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* Unpublished data.
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