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EFFECTS OF DIARYLSULFONYLUREA ANTITUMOR AGENTS ON THE FUNCTION OF MITOCHONDRIA ISOLATED FROM RAT LIVER AND GC3/c1 CELLS

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Abstract—Diarylsulfonylureas, such as *N*-(4-chlorophenyl)aminocarbonyl-2,3-dihydro-1-indene-5-sulfonamide (LY186641, Sulofenur) and *N*-(4-chlorophenyl)aminocarbonyl-4-methylbenzene sulfonamide (LY181984), have been shown to be effective antitumor agents in a variety of *in vivo* and *in vitro* animal models. Their mechanism of action is unknown but does not appear to be the result of nonselective destruction of actively dividing cell populations. Mitochondria have been shown to accumulate Sulofenur and therefore may be targets of drug action. The purpose of these investigations was to examine the effects of a variety of diarylsulfonylureas in mitochondria and attempt to determine the relevance of these changes to antitumor activity. Many of the diarylsulfonylureas which were effective antitumor agents in animal models were also uncouplers of mitochondrial oxidative phosphorylation. They increased state 4 respiration and dissipated the mitochondrial membrane potential in a concentration-related fashion. The mechanism of uncoupling appeared to be related to a dissociable hydrogen ion as these molecules had pK_a values that ranged from 6.0 to 6.2 and were highly lipophilic. Thus, the uncoupling action appears to be the result of hydrogen ion translocation. The mechanism of antitumor activity does not appear to be the result of uncoupling as no correlation was evident between inhibition of cell growth and uncoupling action of a variety of active and inactive diarylsulfonylureas. *In vitro*, Sulofenur is cytotoxic at high concentrations and inhibits cell growth at lower concentrations in the absence of any overt cell kill. The inhibition of cell growth also did not appear to be related to the uncoupling action of these drugs. In contrast, uncoupling may have played a partial role in the early, high exposure cell kill that can occur with these compounds.

Diarylsulfonylureas have been shown recently to have therapeutic efficacy in a variety of *in vitro* and *in vivo* tumor models [1, 2]. They are particularly active in models of human colon adenocarcinoma, which are resistant to other forms of chemotherapy [3, 4]. The mechanism of action of these agents is unknown but does not appear to involve inhibition of DNA, RNA or protein synthesis [2]. Toxicology studies have demonstrated that, unlike cytotoxic chemotherapeutic agents, normal proliferating cells are not targets of these compounds, and typical chemotherapy toxicities (such as GI disturbances and bone marrow depression) are not observed in animals or humans [1]. Anemia and methemoglobinemia appear to be the dose-limiting toxicity in humans and animals [1, 5]. Houghton *et al.* [6] demonstrated that one compound of this series, *N*-(4-chlorophenyl)aminocarbonyl-4-methylbenzene sulfonamide (LY181984)† is taken up rapidly into GC3/c1 cells in a concentration-related fashion. This uptake is also sensitive to azide, suggesting an energy-dependent process. Efflux from these cells is also very rapid. Cellular uptake of LY181984 is dependent on an intact mitochondrial pH gradient across the mitochondrial membrane as uncouplers of oxidative phosphorylation such as carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone and 2,4-dinitrophenol inhibit the accumulation, but not the initial uptake, of the drug [6]. Oligomycin, which inhibits mitochondrial ATP synthetase with little acute action on the mitochondrial pH gradient, has no effect on LY181984 accumulation into GC3/c1 cells. Electron microscopic evaluation of tumor cells exposed to LY181984 and LY186641 (Sulofenur, *N*-(4-chlorophenyl)aminocarbonyl-2,3-dihydro-1-indene-5-sulfonamide) demonstrated evidence of enlarged mitochondria without any obvious changes in cellular organelles or membranes [6]. These data suggest that the mitochondria may be a target organelle in the mechanism of antitumor activity of these compounds.

Therefore, the purposes and specific aims of these investigations were: (1) to examine the effects of diarylsulfonylureas in rat liver mitochondria. Rat liver mitochondria were chosen initially for the ease of isolation in adequate quantities and the extensive database in this organelle; (2) to examine the effects of these compounds in GC3/c1 cells and mitochondria isolated from GC3/c1 tumors grown in nude mice;

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† Abbreviations: LY181984, *N*-(4-chlorophenyl)-aminocarbonyl-4-methylbenzene sulfonamide; LY186641, Sulofenur, *N*-(4-chlorophenyl)aminocarbonyl-2,3-dihydro-1-indene-5-sulfonamide; LY221236, *N*-(3-chlorophenyl)-aminocarbonyl-4-methylbenzene sulfonamide; LY91337, 1-(*p*-tolylsulfonyl)-3-(2,6-xylyl)urea; DMSO, dimethyl sulfoxide; EGTA, ethylene-bis(oxyethylene)tetraacetic acid; CCCP, carbonyl cyanide-3-chlorophenylhydrazone; and RCR, respiratory control ratio.

and (3) to determine if the effects of Sulofenur observed in isolated mitochondria were relevant to the mechanism of antitumor activity.

MATERIALS AND METHODS

Materials. Sulofenur, *N*-(3-chlorophenyl)aminocarbonyl-4-methylbenzene sulfonamide (LY221236), LY181984, and 1-(*p*-tolylsulfonyl)-3-(2,6-xylyl)urea (LY91337) were synthesized at Eli Lilly & Co. ADP, rotenone, safranin-O, antimycin, dimethyl sulfoxide (DMSO), succinate and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased from the Sigma Chemical Co. Cell culture medium was purchased from GIBCO. Fetal bovine serum was purchased from HYCLONE. All other reagents were of the highest grade commercially available.

Isolation of mitochondria. Liver mitochondria were prepared from fasted male Fisher 344 rats (100–150 g) by differential centrifugation essentially as described previously [7, 8]. Briefly, rats were euthanized by CO_2 exposure and the livers removed, chopped finely, rinsed and homogenized in ice-cold 0.25 M sucrose, 4 mM HEPES, 4 mM K_2HPO_4 and 1 mM ethylene-bis(oxyethylene)tetraacetic acid (EGTA) (pH = 7.3) (SHKE buffer). The homogenate was centrifuged at 600 *g* for 10 min, and the resulting supernatant was decanted into fresh tubes and centrifuged at 15,000 *g* for 5 min. The pellet was resuspended in approximately 20 mL of SHKE buffer and recentrifuged at 15,000 *g* for an additional 5 min. The final mitochondrial pellet was resuspended in approximately 5 mL of 0.25 M sucrose, 8 mM K_2HPO_4, 2 mM KH_2PO_4, 5 mM $MgCl_2$ and 1 mM EGTA (pH = 7.2) (respiration buffer) and filtered through gauze. Mitochondrial protein was determined by the method of Bradford [9]. Mitochondria from GC3/c1 tumors implanted into nude mice for approximately 21 days were prepared essentially as described above for rat liver mitochondria. Tumor tissue was removed, minced and homogenized in a polytron. The tissue was then centrifuged as described above.

Determination of mitochondrial respiratory functions. Mitochondria (1.0 mg protein/mL) were incubated in respiration buffer, and oxygen consumption was monitored with a Clark-type electrode at 25° using a YSI O_2 monitor (Yellow Springs Instruments Co., Yellow Springs, OH). State 4 respiration was initiated by the addition of 5.0 mM glutamate/malate. State 3 respiration was initiated by the addition of 450 nmol ADP. The respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 respiration. The ADP/O ratio was calculated as the nanoatoms of O_2 consumed for every nanomole of ADP added. The potency of each molecule was determined by calculating the concentration of test article that caused a 50% reduction in the RCR (EC_{50}). Each experiment was conducted in duplicate with liver or tumor mitochondria on at least 3 separate study days (N = 3).

Determination of mitochondrial membrane potential. Semi-quantitative measurements of the potential difference across the inner mitochondrial membrane were determined spectrophotometrically using the indicator dye, safranin-O [10, 11]. Measurements were performed on an SLM-Aminco DW-2000 dual beam spectrophotometer at the wavelength pair 533-511 nm. Mitochondria, (1 mg protein/mL) in respiration buffer containing 2 μM rotenone and 9.6 μM safranin-O, were mixed at 25°. Succinate (5.0 mM) was added to energize the mitochondria and the absorbance changes were digitized and recorded on disk. Test articles were added in 0.5 to 2.0 μL volumes dissolved in DMSO.

In vitro *effects of Sulofenur in GC3/c1 cells.* A cloned line of human colon adenocarcinoma (GC3/c1) cells was grown on antibiotic-free RPMI 1640 supplemented with 2 mM glutamine and 10% fetal bovine serum. Cell protein was measured by the method of Bradford [9].

In vivo *antitumor effects of Sulofenur.* Male nude mice bearing 21-day-old subcutaneous GC3/c1 tumors were administered Sulofenur (in 2.5% Emulphor EL-620/saline) at 300 mg/kg daily for a course of 10 days. Control animals received vehicle (Emulphor/saline) alone. Each day the tumor weight was approximated by the formula [(width)(width)(length)/2]. Tumor lengths were measured in millimeters. At selected time points, mice were anesthetized with pentobarbital (100 mg/kg). A small incision was made over the tumor site, and the tumor carefully exposed to minimize changes in blood flow was freeze-clamped and stored in liquid nitrogen. Tissue ATP was quantitated using a bioluminescent assay. Samples stored in liquid nitrogen were weighed and pulverized with a mortar and pestle on dry ice. The resulting frozen powder was then precipitated with 10% trichloroacetic acid/EDTA and an aliquot of the acid supernatant (1.5 mL) was added to an equal volume of 0.4 M TRIZMA/EDTA buffer (pH 7.4) and extracted three times with water-saturated ether. ATP in the samples was determined upon the addition of luciferin/luciferase in an LKB-Wallac 1250 Luminometer.

Statistics. Linear regression curves were calculated from log concentration–response curves for individual experiments, and EC_{50} values were determined from the regression formula (Cricket Graph). All data, where appropriate, were analyzed statistically by analysis of variance using SAS [12]. The level of significance was $P \leq 0.05$.

RESULTS

Sulofenur caused a concentration-dependent increase in rat liver mitochondria state 4 respiration with an EC_{50} of approximately 20 μM (Fig. 1). At 50 μM, Sulofenur caused a steep increase in state 4 respiration immediately after drug administration which was followed by an inhibition of mitochondrial respiration (not shown). The increased state 4 respiration caused by 25 μM Sulofenur was inhibited totally with antimycin which is a potent inhibitor of QH_2-cytochrome *c* reductase of the mitochondrial electron transport chain (Fig. 1). LY181984 which has a 4-methyl phenyl group as opposed to the indanyl of Sulofenur also was capable of increasing state 4 respiration, although less potently than Sulofenur (Table 1). Addition of oligomycin to the

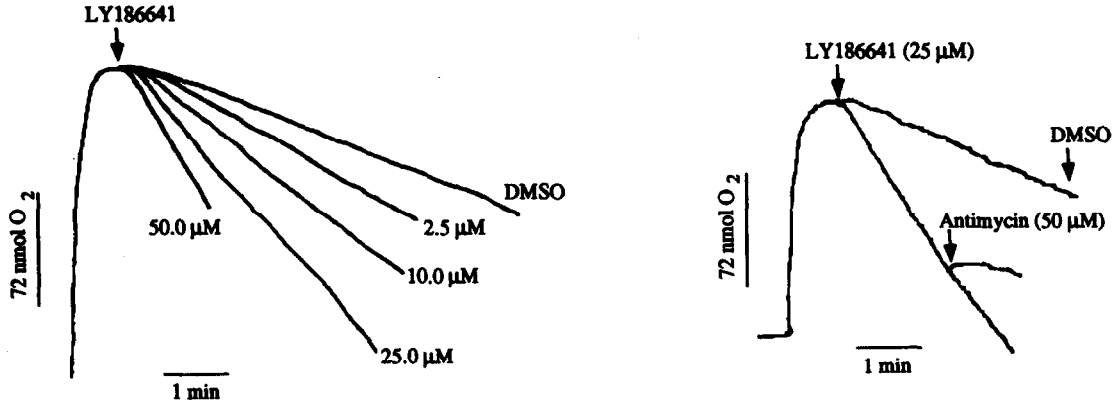

Fig. 1. Effects of Sulofenur on pyruvate/malate-supported rat liver mitochondrial state 4 respiration (left panel) and the effect of antimycin on Sulofenur-induced stimulation of state 4 respiration (right panel). Rat liver mitochondria (1 mg protein/mL) were prepared as described in Materials and Methods. Sulofenur was added dissolved in DMSO. Antimycin was dissolved in ethanol.

incubations should inhibit the mitochondrial ATP synthetase and did inhibit ADP-dependent state 3 respiration (Fig. 2). LY181984 (100 μM) still increased mitochondrial state 3 respiration despite the presence of oligomycin (Fig. 2). Calculation of the respiratory control ratio following ADP addition demonstrated that Sulofenur had an EC_{50} of 19.1 ± 3.2 μM (Fig. 3). Sulofenur-induced increases in state 4 respiration were accompanied by a collapse of the mitochondrial membrane potential with an EC_{50} of 43.2 ± 10.9 μM (Fig. 4). In this figure, CCCP, an uncoupler of oxidative phosphorylation, is shown as a positive control. Sulofenur was as equipotent and equi-effective in collapsing the membrane potential in mitochondria isolated from GC3/c1 tumors as those isolated from rat liver (Figs. 4 and 5). These data in mitochondria isolated from either rat liver or GC3/c1 tumor cells suggest that Sulofenur is an uncoupler of mitochondrial oxidative phosphorylation.

The relationships between these mitochondrial

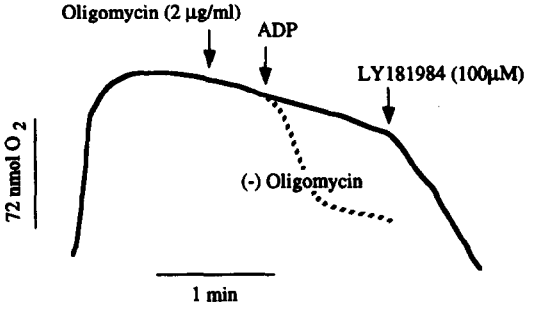

Fig. 2. Effect of LY181984 on state 3, ADP-stimulated respiration in rat liver mitochondria in the presence and absence of oligomycin. Rat liver mitochondria (1 mg protein/mL) were prepared as described in Materials and Methods and were respiring on pyruvate/malate (5 mM). Sulofenur was added dissolved in DMSO. Oligomycin was dissolved in ethanol.

Table 1. Comparison of the effects of different sulfonylurea structures on inhibition of mitochondrial function and inhibition of tumor growth *in vivo* and *in vitro* in relationship to the pK_a

Compounds	Respiratory control ratio EC_{50} (μM)	Membrane potential EC_{50} (μM)	% Inhibition *In vivo**	EC_{50}† (μg/mL) *In vitro*	pK_a‡
LY186641	19.1 ± 3.3	43.2 ± 10.9	100	11.2	6.2
LY221236	49.1 ± 12.1	150.6 ± 19.4	13	> 20	6.0
LY181984	51.3 ± 12.6	182.0 ± 19.6	100	8.9	6.1
LY91337	> 200	NT	23	> 20	7.3

All values are means ± SEM (where indicated) of at least four experiments. NT = not tested. EC_{50} represents the drug concentration that caused a 50% decrease in either membrane potential or RCR.

* Each mouse received 300 mg/kg, p.o. × 8. C3H mice bearing 6C3HED lymphosarcoma were used.

† Cultured CCRF-CEM cells, 72-hr exposure.

‡ Determined in dimethylformamide/water.

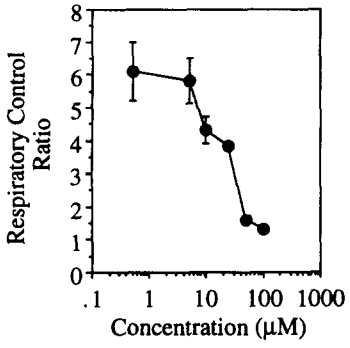

Fig. 3. Concentration–response relationships of the effects of Sulofenur on rat liver mitochondrial respiratory control ratio. Rat liver mitochondria (1 mg protein/mL) were prepared as described in Materials and Methods and were respiring on pyruvate/malate (5 mM). Sulofenur was added dissolved in DMSO. The EC_{50} was 19.1 ± 3.2 μM and was calculated by regression analysis from the linear portion of the curve. Data points represent means ± SEM of four experiments.

effects of Sulofenur and antitumor activity were investigated with GC3/c1 cells both in culture and implanted into nude mice. Sulofenur, at 300 mg/kg daily for 10 days, caused a significant decrease in tumor size to approximately 50% of control weight (Fig. 6). Mice tolerated this course of drug treatment well with no observable clinical signs of toxicity. If mitochondrial dysfunction was contributing to the antitumor activity of Sulofenur, tumor ATP content should be decreased. However, the only detectable changes that were observed occurred within the first 6 hr after a single dose in the liver (as a non-target tissue) causing a 60% decrease in tissue ATP treatment. Tumor ATP content was not significantly different from control at any time point examined (Fig. 7).

In vitro, Sulofenur was effective in inhibiting the growth of GC3/c1 cells, measured as the protein/culture plate, in a time and concentration-dependent fashion (Fig. 8). These growth curves revealed markedly different concentration–response curves depending on the sampling time. At 144 hr of exposure, the EC_{50} to Sulofenur was calculated to be ~8 μg/mL whereas after 48 hr of exposure the EC_{50} was ~80 μg/mL (Fig. 9). Furthermore, early inhibition of cell growth by high Sulofenur concentration was associated with cell death, whereas cell death was not observed after 144 hr at low exposures. To examine if the early (48 hr) inhibition of GC3/c1 cell growth and cell death after exposure to high Sulofenur concentrations were due to mitochondrial dysfunction, the temporal relationships between cell ATP content and cell growth were compared. Sulofenur, at 25 μg/mL, had only minimal effects on cell growth after 48 hr of exposure and only minimal effects on cellular ATP (Fig. 10). However, at 100 μg/mL, Sulofenur caused a rapid decrease in cellular ATP that preceded the onset of inhibition of cell growth and cell death. ATP was decreased by 35% 3 hr following exposure to 100 μg/mL Sulofenur (Fig. 10).

Examination of the structure–activity relationships for these mitochondrial effects revealed that the EC_{50} for uncoupling and dissipation of the membrane potential correlated well with the pK_a for the molecule. In contrast, the antitumor efficacy of these different molecules in CCRF-CEM cells *in vitro* or C3H mice bearing 6C3HED lymphosarcoma *in vivo* did not correlate with either the pK_a or the EC_{50} values for inhibition of mitochondrial function. Of particular interest was a series of molecules which were not weak acids (pK_a values ~ 12) (data not shown). These compounds, which did not uncouple mitochondrial respiration, had activity in the *in vivo* but not the *in vitro* models.

DISCUSSION

The mechanism of antitumor activity of Sulofenur

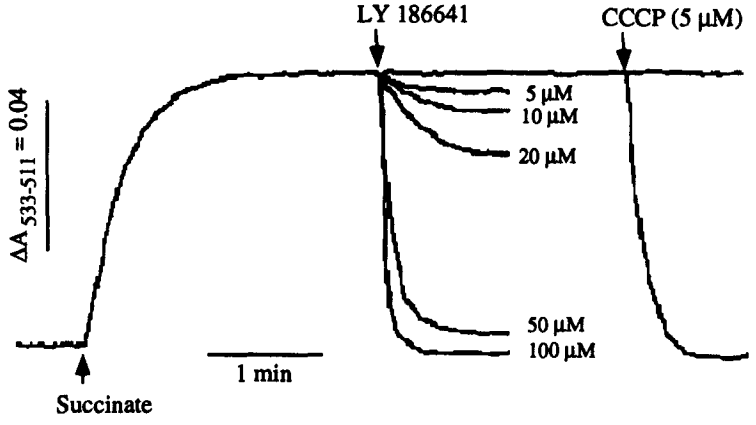

Fig. 4. Effects of Sulofenur on the mitochondrial membrane potential. Rat liver mitochondria were energized with succinate (5 mM) in the presence of rotenone (2 μM). Membrane potential was monitored at the wavelength pair 533-511 in the presence of 9.6 μM safranin-O. Sulofenur was added dissolved in DMSO.

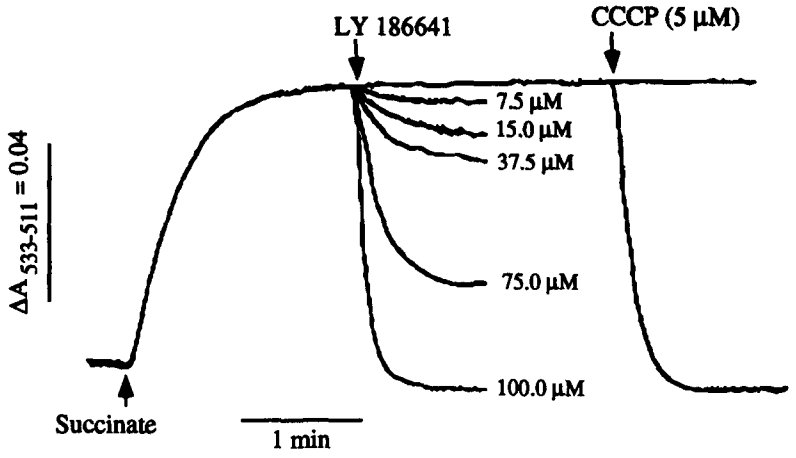

Fig. 5. Effects of Sulofenur on tumor mitochondrial membrane potential. Mitochondria were prepared from GC3/c1 tumors implanted into nude mice and energized with succinate (5 mM) in the presence of rotenone (2 μM). Membrane potential was monitored at the wavelength pair 533-511 in the presence of 9.6 μM safranin-O. Sulofenur was added dissolved in DMSO.

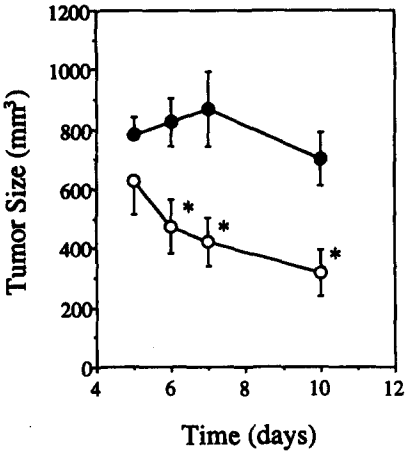

Fig. 6. Effect of Sulofenur (300 mg/kg/day) on tumor size. Sulofenur was dissolved in 2.5% Emulphor EL-620/saline and administered as an i.p. injection. Tumor size was estimated as described in Materials and Methods. Key: (●) control data, and (○) data from treated animals. An asterisk indicates significantly different ($P \leq 0.05$) from the untreated control. Data points represent means ± SEM of four experiments.

and related materials is not understood. Houghton *et al.* [13] have indicated that the mitochondria may be one site of accumulation of LY181984 and Sulofenur and therefore may also be targets in the biological action of these drugs. In the present report, Sulofenur caused a rapid increase in rat liver mitochondrial state 4 respiration rate, a total loss of respiratory control and a dissipation of the mitochondrial membrane potential, effects which are consistent with uncoupling as a mechanism of action for these diarylsulfonylureas. Increased respiration was due to increased electron transport activity as antimycin completely shut down Sulofenur-stimulated mitochondrial respiration rate. In addition, oligomycin inhibition of ADP-stimulation mitochondrial respiration was also reversed with LY181984 and Sulofenur, indicating that stimulation of oligomycin-sensitive ATPase was also not involved. These effects were not unique to mitochondria isolated from rat liver as uncoupling was also observed in mitochondria isolated from GC3/c1 tumors implanted into nude mice. Sulofenur caused dissipation of the mitochondrial membrane potential (Fig. 4) and increased state 4 respiration (data not shown). The mechanism(s) of uncoupling of mitochondria by the diarylsulfonylureas appears to be the result of their weak acid properties. Classically, uncouplers of this type have a pK_a of between 4.5 and 6.5 [14]. In previous studies, the chemical nature of the acidic group (amide, hydrazone, phenolic hydroxyl, etc.) did not appear to be important in the uncoupling action of these types of molecules [14]. In addition to the acidic group, the rest of the molecule was usually a bulky lipophilic moiety with no steric similarities between molecules. This pattern also fits the diaryl-sulfonylureas Sulofenur, LY221236 and LY181984 which were examined in the present report and they are all highly lipophilic containing an ionizable hydrogen with pK_a values ranging from 6.0 to 6.2. Molecules such as LY91337 have pK_a values greater than 6.5 and were not found to be uncouplers of oxidative phosphorylation. Therefore, these diarylsulfonylureas which were effective uncouplers appeared to be acting as lipophilic, reversibly dissociable molecules that penetrate the mitochondrial membrane and shuttle protons across the inner mitochondrial membrane thereby dissipating the hydrogen ion gradient and interrupting oxidative phosphorylation [15]. Sulofenur is as potent an uncoupler as dinitrophenol (both molecules have an EC_{50} of approximately 20 μM) [15]. At 50 μM, Sulofenur first stimulated, then inhibited mito-

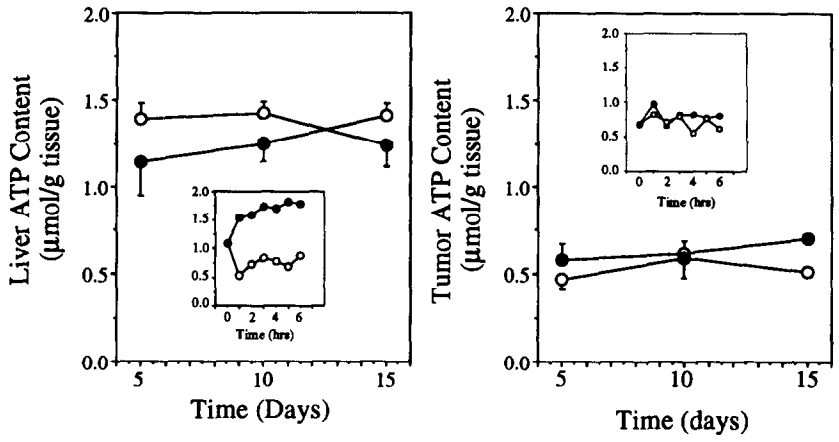

Fig. 7. Effect of Sulofenur (300 mg/kg/day) on mouse liver and GC3/c1 tumor ATP content. Sulofenur was dissolved in 2.5% Emulphor EL-620/saline and administered as an i.p. injection. Key: (●) control data, and (○) data from treated animals. Tissue ATP was extracted and assayed as described in Materials and Methods. The effects of a single dose within hours of dosing are shown in the inset. Data points represent means ± SEM of four experiments.

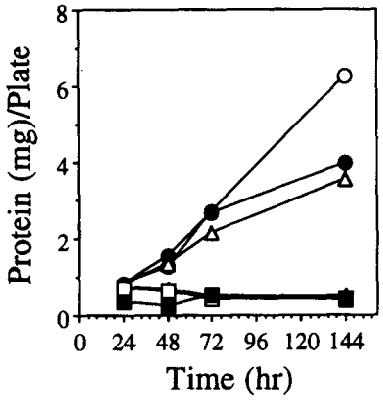

Fig. 8. Effect of various concentrations of Sulofenur on the growth of GC3/c1 cells in culture. Sulofenur was added in DMSO. Cell growth was assessed as the protein/plate. Drug concentrations were as follows; (○) 0.00, (●) 3.12, (△) 12.5, (▲) 25.0, (□) 50.0, and (■) 100.0 μg/mL. Data points represent means ± SEM of four experiments (error bars are within the symbols).

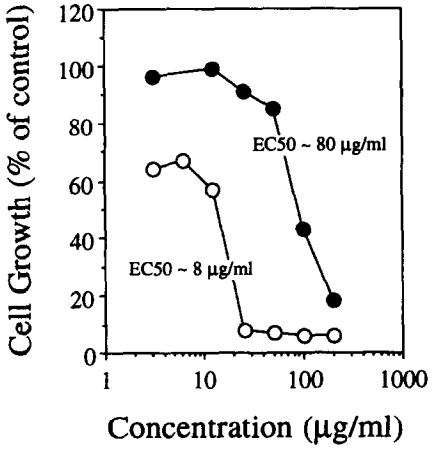

Fig. 9. Log concentration–response curves of the effects of Sulofenur on the growth of GC3/c1 cells in culture. Sulofenur was added in DMSO. Open circles represent cells incubated with Sulofenur for 144 hr (control = 6.23 mg protein/plate), whereas closed circles represent 48-hr incubations (control = 1.25 mg protein/plate). Cell growth was assessed as the protein concentration/plate. Data points represent means ± SEM of four experiments (error bars are within the symbols).

chondrial state 4 respiration (Fig. 1). This is a common, well-described phenomenon with classical uncouplers [15, 16].

Studies by Houghton *et al.* [13] have indicated that the mitochondria may selectively accumulate Sulofenur and other diarylsulfonylureas and that this uptake was dependent on an intact mitochondrial membrane potential. If these compounds reach high intramitochondrial concentrations selectively in the tumor tissue, then the uncoupling action may play an important role in the antitumor activity of these compounds. This scenario, however, does not appear to occur. First, the effectiveness of a series of the molecules *in vivo* and *in vitro* did not correlate with either the pK_a of the molecule or the EC_{50} for uncoupling of mitochondrial respiration. Second, Sulofenur at 300 mg/kg/day was effective *in vivo* in reducing the size of the GC3/c1 tumor over a 10-day course of treatment by approximately 60% (Fig. 6). However, there were no changes in tumor ATP levels at any treatment day. Liver ATP was decreased slightly within hours after dosing but returned to control by day 5 demonstrating the *in vivo* uncoupling action of the drug. Uncoupling of mitochondrial

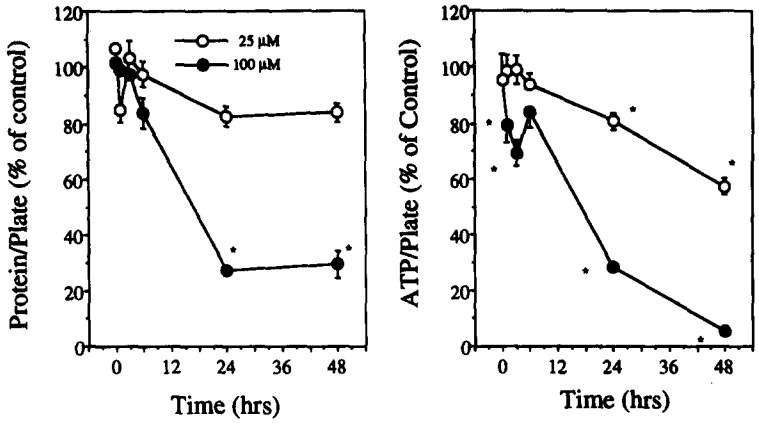

Fig. 10. Effects of Sulofenur on cell growth and ATP content in GC3/c1 cells. Sulofenur was added in DMSO. Cell growth was assessed as the milligrams of protein/plate. Open and closed circles represent exposure to 25 and 100 μM, respectively. Data points are expressed as a percentage of the appropriate time control and represent means ± SEM of four experiments. Asterisks indicate values significantly different ($P \leq 0.05$) from control. Initial protein and ATP values were 0.77 ± 0.08 mg protein/plate and 1.30 ± 0.11 nmol ATP/mg protein, respectively.

oxidative phosphorylation by diarylsulfonylureas could reduce cellular ATP by two mechanisms. If the tumor cells were aerobic and dependent on mitochondrial respiration for energy, then ATP should have been reduced dramatically by this course of drug therapy as it was effective in shrinking the tumor. If the tumor cells were anerobic in nature, a decrease in ATP would still be expected as uncoupling should reverse the membrane potential and cause the mitochondrial ATP synthetase to function as a ATPase, consuming cellular ATP. Dinitrophenol can deplete cells of fructose-derived glycolytic ATP in isolated rat hepatocytes (mitochondrial ATP synthesis was blocked with oligomycin) demonstrating that reversal of the mitochondrial ATP synthetase occurs with uncouplers and that the "ATPase" activity of the ATP synthetase consumes large amounts of cellular ATP under these conditions (Rush GF, unpublished observations). In any event, decreases in GC3/c1 cell ATP were not observed *in vivo* and thus did not correlate with Sulofenur-induced reduction in tumor size.

The inhibition of GC3/c1 cell growth *in vitro* by Sulofenur could be described by two different concentration–response curves, depending on the observation time. Following 144 hr of exposure, the cells were very sensitive to the growth-inhibiting effects of Sulofenur with an EC_{50} of approximately 8 μg/mL. In contrast, the potency of Sulofenur in inhibiting cell growth after 48 hr of exposure was approximately 80 μg/mL, one full log unit to the right of the 144-hr concentration–response curve (Figs. 9 and 10). Exposure of GL3/c1 cells to high concentrations of Sulofenur *in vitro* was associated with rapid cell death (a loss of the seeding population), whereas exposure to low concentrations of drug was not. The inhibition of cell growth at 144 hr at low drug concentrations (25 μg/mL) was probably not related to any uncoupling action of Sulofenur on these cells as ATP was decreased only modestly in the absence of any overt cell death. However at high concentrations, uncoupling of mitochondrial respiration may have played a partial role in the cell death as cellular ATP was decreased significantly by 35% 3 hr after exposure, well before any signs of significant cell death suggesting a causal relationship between ATP depletion and cell death.

In conclusion, many of the diarylsulfonylureas that are effective antitumor agents in animal models are also uncouplers of mitochondrial oxidative phosphorylation. The mechanism of uncoupling appears to be related to the presence of a dissociable hydrogen ion as these molecules have pK_a values that range from 6.0 to 6.2 which is aided by the high lipophilicity of these molecules. Thus, the uncoupling action appears to be the result of hydrogen ion translocation. The mechanism of antitumor activity does not appear to be the result of uncoupling whereas uncoupling may play a partial role in the early, high exposure cell kill *in vitro* that can occur with these compounds.

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