Biochemical Pharmacology, Vol. 45, No. 10, pp. 2135–2142, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

PROLIFERATION-DEPENDENT AND -INDEPENDENT CYTOTOXICITY BY ANTITUMOR DIARYLSULFONYLUREAS

INDICATION OF MULTIPLE MECHANISMS OF DRUG ACTION

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(*Received* 26 *October* 1992; *accepted* 5 *January* 1993)

Abstract—The mechanism(s) by which antitumor diarylsulfonylureas (DSU) cause cytotoxicity has been examined in GC_3/c1 human colon adenocarcinoma cells and a subline selected for resistance to *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea (ISCU). Resistance was stable in the absence of selection pressure. This mutant (designated LYC5) was 5.5-fold resistant to ISCU compared to parental GC_3/c1 cells in serum containing medium when cells were exposed for 7 days. In contrast, LYC5 cells were not resistant to a 4-hr exposure to ISCU. These data indicated two possible mechanisms of action, dependent on concentration and time of exposure to ISCU. Proliferation-dependent and -independent mechanisms of cytotoxicity were identified in wild-type and resistant clones. In serum-free medium containing growth factors, the IC_{50} for parental cells was 0.51 μM and for LYC5 7.0 μM (13.6-fold resistance), whereas without growth factors both lines were 8- to 9-fold resistant relative to conditions of cellular proliferation. Accumulation of ISCU was similar in quiescent and proliferating cells, and was reduced only slightly in resistant LYC5 cells. Analysis of DNA by agarose gel electrophoresis showed that in GC_3/c1 cells nucleosomal ladders were formed only when proliferating cells were exposed to ISCU. No nucleosomal ladders were detected in quiescent cells during exposure to toxic concentrations of drug (IC_{90}), or after removal of ISCU and addition of serum to stimulate growth. These data indicate several mechanisms by which diarylsulfonylurea antitumor agents may cause cell death. In serum-free medium at very high concentration (IC_{50} ~370 μM) for short periods of exposure (4 hr), cytotoxicity was proliferation independent, and GC_3/c1 and LYC5 cells were equally sensitive. This mechanism may relate to the uncoupling activity of ISCU. However, at pharmacological relevant concentrations, the primary mechanism was proliferation dependent and led to formation of nucleosomal DNA ladders (IC_{50} ~0.5 μM). A possible additional mechanism occurred at higher concentration (IC_{50} ~7 μM) in quiescent cells, and was not associated with DNA degradation.

The diarylsulfonylureas represent a novel class of antitumor agents. The preclinical pharmacology of these agents suggested wide-spectrum activity against rodent solid tumors, with little activity against leukemia models [1, 2]. Sulofenur [*N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea; ISCU†] demonstrated significant activity against early stage human tumor xenografts [3] and causes regressions in more advanced colon adenocarcinomas that historically have been refractory to all other agents examined [4]. Very significant activity was seen in a high proportion of xenografts derived from childhood rhabdomyosarcomas [5]. However, clinically sulofenur has been disappointing, with a low response rate in most adult malignancies [6, 7], although this agent has not been evaluated against the childhood sarcomas predicted to be responsive from the preclinical models. In part this low efficacy appears due to dose-limiting toxicity, anemia and methemoglobinemia, being reached at plasma levels well below those associated with antitumor responses in the mouse. This species difference may relate to more rapid metabolism of sulofenur in humans resulting in significant levels of two metabolites which may contribute to toxicity [8, 9]. While the clinical utility of sulofenur may be limited, the broad-spectrum activity particularly against chemo-refractory malignancies in preclinical testing would suggest that this class of agent may identify novel targets that may be exploited therapeutically.

The mechanism(s) by which antitumor diarylsulfonylureas (DSU) are cytotoxic remains obscure, but previous studies have shown that DSU inhibit nucleic acid and protein synthesis only at a very high concentration [3, 10]. DSU are concentrated approximately 4-fold in cells, and influx and efflux appear to be mediated by passive diffusion [11]. However, ionophores that collapse the mitochondrial pH gradient, and agents that uncouple oxidative phosphorylation inhibit drug accumulation, suggesting that DSU may be sequestered in mitochondria

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† Abbreviations: ISCU, *N*-(5-indanylsulfonyl)-*N'*-(4-chlorophenyl)urea (sulofenur); DSU, antitumor diarylsulfonylureas; FBS, fetal bovine serum; EGF, epidermal growth factor; GF, growth factors; and PBS, phosphate-buffered saline.

[12]. Data that suggest some action through mitochondria include mitochondrial enlargement in drug-treated cells [12, 13], and direct uncoupling activity of DSU in isolated mitochondria [13]. The cytotoxicity of ISCU also appears to be independent of cellular proliferation when cells are exposed for up to 24 hr [10, 14], consistent with a mitochondrial-targeted mechanism. Preliminary data, derived using a cell line with acquired resistance to DSU, suggested that there may be two distinct cytotoxic mechanisms for these agents [15]. In this report we have extended these studies and have characterized mechanisms of cell killing by ISCU that occur after short exposure periods to high concentrations of drug, and mechanisms that may pertain to pharmacologically achievable concentrations that are dependent and independent of cellular proliferation.

MATERIALS AND METHODS

A cloned line of human adenocarcinoma GC_3/c1 [16] was routinely grown in antibiotic-free RPMI 1640 medium (Whittaker, Walkersville, MD) supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS, Hyclone, Logan, UT). [^{3}H]ISCU (698.1 mCi/mmol) was synthesized as described (European Patent 166615), purified by reverse-phase high-performance liquid chromatography, and supplied by Dr G. B. Grindey, Eli Lilly & Company, Indianapolis, IN. All chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) or through Fisher Scientific (Springfield, NJ).

Selection of GC_3/LYC5. GC_3/c1 cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS), and surviving cells were exposed to 300 μM ISCU for 24 hr in the presence of 10% FBS. Four surviving colonies were isolated using cloning rings. One of these clones, designated LYC5, was exposed to increasing concentrations of ISCU. After 10 weeks it was capable of growing in medium containing 120 μM ISCU. Compared to parental GC_3/c1 cells, LYC5 was 4- to 6-fold resistant to ISCU (determined by colony formation assay after a 7-day exposure to ISCU). For the experiments reported, LYC5 cells had been maintained in drug-free medium for over 1 year. Resistance was stable over this period.

ISCU accumulation studies. Studies with [^{3}H]-ISCU were essentially as described previously [17], with the following modifications. Near confluent cultures of GC_3/c1 and LYC5 cells were grown for 3 days in medium containing 0.5% FBS, and then plated in RPMI 1640 with or without supplementation with 10 ng/mL epidermal growth factor (EGF), 10 μg/mL insulin and 5 μg/mL transferrin. Accumulation of [^{3}H]ISCU was examined 5 or 7 days after plating of GC_3/c1 and LYC5 cells, respectively. At these periods after plating, cultures grown in the presence of growth factors (+GF) were in logarithmic growth, whereas cells in growth factor-deficient (−GF) medium remained quiescent but retained viability. For there to be similar numbers of cells/dish for the drug accumulation studies, 0.5×10^6 cells were plated/35-mm dish in the presence of growth factors and 1.6×10^6 in the absence of growth factors. For drug accumulation, medium was aspirated, cells were washed with phosphate-buffered saline (PBS), and 1 mL of RPMI 1640 ± growth factors (prewarmed to 37°) containing [^{3}H]ISCU (0.66 to 20 μM) was added. Plates were returned to the incubator (37°, 5% CO_2) for 60 min. To terminate drug accumulation, medium was aspirated, and monolayers were washed rapidly in four successive changes of ice-cold PBS. Monolayers were drained, and to each dish 1 mL of trypsin–EDTA solution was added. After 5 min monolayers were gently pipetted to give a uniform suspension of cells and 0.75 mL was added to 10 mL of ScINT-AXF liquid scintillation fluid (Packard, Meriden, CT), and radioactivity was determined. A 0.2-mL aliquot of this suspension was used to determine the cell number per dish as described previously [16].

Clonogenic assays. GC_3/c1 cells and LYC5 cells were seeded (6×10^3 per 35-mm culture dish), and allowed to attach overnight. ISCU was dissolved in dimethyl sulfoxide. For all studies, including controls, the final concentration of dimethyl sulfoxide was <0.6%, which did not alter the growth rate or clonogenic efficiency of either cell line. Colony formation (>50 cells) was determined in triplicate for each concentration of ISCU, and experiments were repeated at least once. Colonies were enumerated after drying and staining (0.1% crystal violet) using an Artek model 880 counter. For both cell lines colonies were determined 7 days after exposure to ISCU.

Growth in serum-free medium. GC_3/c1 or LYC5 cells were transferred to medium containing 0.5% FBS for 3 days prior to seeding at cloning density. After plating, cells were grown in RPMI 1640 alone or supplemented with 10 ng/mL EGF, 10 μg/mL insulin and 5 μg/mL transferrin. Cells maintained 96 ± 8% colony-forming ability after 7 days in unsupplemented RPMI 1640. To examine the effect of a 4-hr exposure to ISCU, cells were plated ± GF and exposed to drug after 3 days (GC_3/c1) or 5 days (LYC5) when cells were in logarithmic growth. After exposure to ISCU, cells were washed to remove drug, and refed with RPMI 1640 containing 10% FBS. Colonies were enumerated after an additional 7 days. In parallel experiments, cells were processed as described, but cell number was enumerated as the end point to determine the effect of ISCU.

DNA analysis by agarose gel electrophoresis. GC_3/c1 cells were exposed to ISCU in serum-free medium without GF or medium containing 10% FBS, harvested in Hanks–trypsin and washed four times in PBS. Cell pellets were stored at −70° until DNA extraction. Samples were thawed in 2 mL TE, pH 8.0 (10 mM Tris–HCl, 1 mM EDTA), containing 200 μg proteinase K. After suspending the pellet by gentle pipetting, 10% sodium dodecyl sulfate (200 μL) was added and the sample incubated at 56° overnight. Samples were extracted twice at 23° with an equal volume of phenol:chloroform (1:1) and once with chloroform. Samples were made 0.1 M with 5 M NaCl and mixed by inversion; DNA was precipitated by the addition of 3 vol. of ice-cold ethanol. After centrifuging, the pellet was dried under a stream of N_2, resuspended in 2 ml TE, pH 8.0, containing 10 mg/mL RNase, and incubated at 37° overnight. Samples were re-extracted, and DNA was pre-

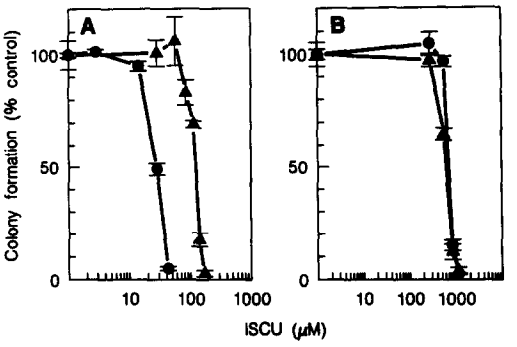

Fig. 1. Sensitivity of GC_3/c1 (●) and LYC5 (▲) cells to different periods of exposure to ISCU in medium containing 10% FBS. (A) Continuous exposure of cells for 7 days; and (B) exposure for 4 hr. Results show a representative experiment. Values are means ± SD, N = 3. Control (100%) colony numbers were: GC_3/c1 = 462 ± 5; LYC5 = 398 ± 25.

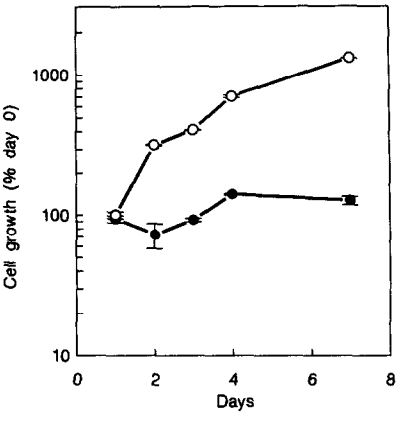

Fig. 2. Growth of GC_3/c1 cells with or without growth factor supplementation. Cells were grown for 3 days in RPMI 1640 containing 0.5% FBS, and then were plated in serum-free medium supplemented with insulin, transferrin and EGF (○) or without growth factors (●). Cell number was enumerated over 7 days (mean ± SD, N = 3; 100% = 10^5 cells).

cipitated and dried as above. DNA was resuspended in TE, pH 8.0, and concentration was determined by absorbance at 260 and 280 nm. Samples (10 μg) were analyzed by electrophoresis in 1.2% agarose in TBE buffer (22.5 mM Tris–borate, 1 mM EDTA).

RESULTS

Cross-resistance dependence on ISCU concentration. To investigate further the mechanism of action of DSU, a stable line resistant to ISCU was selected as described in Materials and Methods. The phenotype of this clone, designated LYC5, will be presented in detail elsewhere.* LYC5 was approximately 5.5-fold resistant to ISCU (Fig. 1A), and was stable in the absence of drug selection pressure for >1 year.

Previous data using LYC5 cells had suggested that at high concentration most DSU had similar potency against the resistant clone, whereas the potency for the same series of compounds differed by >30-fold against wild-type GC_3/c1 cells. These data were interpreted to indicate a potential second site of action of DSU at high concentration, to which LYC5 cells would not be resistant. To test this, GC_3/c1 and LYC5 cells were exposed to ISCU for 4 hr, and colony formation was determined 7 days after removal of drug. As shown in Fig. 1B, concentrations of ISCU reducing colony formation by 50% (IC_{50}) were similar, being 641 and 715 μM for LYC5 and GC_3/c1 cells, respectively. Thus, under conditions of short exposure time LYC5 cells were not resistant to ISCU. These data suggested that ISCU may have two distinct mechanisms of action. LYC5 cells were resistant only to the effect of continuous exposure to low concentration, but not to 4-hr exposure to high concentrations of ISCU.

Dependence on proliferation for cytotoxicity. Previously we reported that cytotoxicity of ISCU is independent of cellular proliferation, when cells are exposed for up to 24 hr in serum-free medium [10]. One problem encountered with these previous experiments was that in proliferating cultures 10% FBS was used, and ISCU binds avidly to serum albumin. Consequently, in serum-free conditions bovine serum albumin was included to give equivalent binding of ISCU; however, it was not possible to determine accurately free drug concentration under either condition of growth. To obviate this problem serum-free conditions for growth of GC_3/c1 and LYC5 cells were established (see Materials and Methods) and were used to determine the relationship between proliferation and cytotoxicity in each line. The growth of GC_3/c1 cells with or without growth factors (± GF) is presented in Fig. 2. In the absence of GF, cell number increased by 27.5 ± 8.8%, whereas in the presence of GF the cell number increased to 1020 ± 90% over 7 days. The effects of proliferation conditions on the cytotoxicity of continuous (7-day) or short (4-hr) exposure to ISCU in GC_3/c1 and LYC5 cells are presented in Fig. 3. As before, for both cell lines cytotoxicity was independent of cellular proliferation for 4-hr exposure to high concentrations of ISCU (Fig. 3, panels B and D). For GC_3/c1 cells the IC_{50} values were 370 and 390 μM under proliferating and quiescent conditions, respectively. Similar results were obtained with LYC5 cells. However, for continuous exposure there were clearly proliferation-dependent and -independent mechanisms for both GC_3/c1 and LYC5 cells, (Fig. 3, panels A and C). Under conditions of cellular proliferation, the mean IC_{50} values for ISCU were 0.51 (N = 3) and 7.0 μM, respectively, for GC_3/c1 and LYC5 cells. Thus, under these conditions, LYC5 cells were 13.6-fold resistant to ISCU relative to GC_3/c1 cells. When exposed for 7 days to ISCU under non-proliferating conditions both cell lines demonstrated resistance.

* Sosinski J, Thakar JH, Germain GS, Kuttesch JF and Houghton PJ, manuscript in preparation.

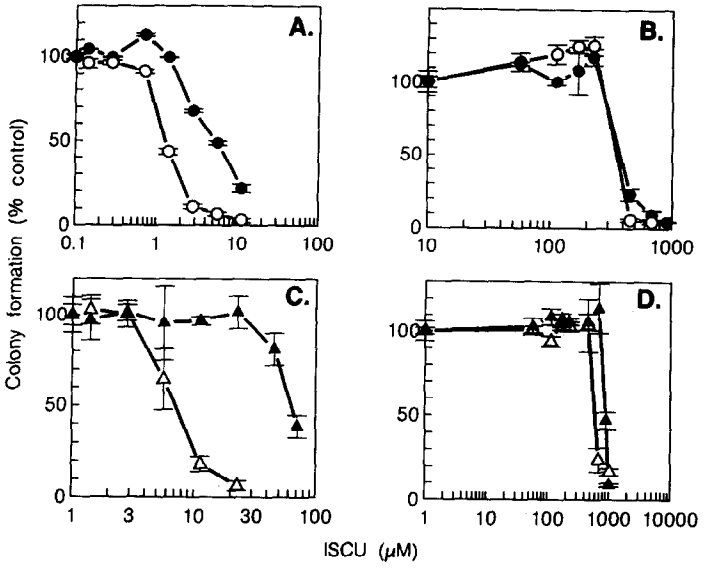

Fig. 3. Effects of proliferation and exposure time for ISCU cytotoxicity. GC_3/c1 (panels A and B), and LYC5 (panels C and D) cells were grown for 3 days in 0.5% FBS-containing medium, and seeded at cloning density. Cells were exposed for 7 days (panels A and C) or 4 hr (panels B and D) in the presence (open symbols) or absence (closed symbols) of growth factors. Values are means ± SD, N = 3. For GC_3/c1 cells 100% = 521 ± 7 (+GF) and 397 ± 7 (−GF); for LYC5 cells 100% = 414 ± 25 (+GF) and 313 ± 33 (−GF).

Table 1. Effect of growth conditions on cytotoxic potency of ISCU*

Cell line	IC_{50} (μM) +GF	IC_{50} (μM) −GF	Difference (-fold)
GC_3/c1	0.51	4.4	8.6
LYC5	7.0	62.7	8.9
Difference (-fold)	13.6	14.2	

* Cells were adapted to serum-free conditions, as described in Materials and Methods, and exposed to ISCU for 7 days at which time colonies were enumerated. The IC_{50} concentrations were compared for different conditions of growth within a cell line, and for similar conditions of growth between cell lines.

The IC_{50} concentrations were 4.4 and 62.7 μM for GC_3/c1 and LYC5, respectively. As shown in Table 1, the relative differences in potency for growth (+GF) and quiescent conditions (−GF) were similar for both cell lines (8.6- and 8.9-fold), and the differential between sensitivity of GC_3/c1 and LYC5 was also quite similar under the different growth conditions (13.6- and 14.2-fold).

Effect of growth conditions on accumulation of ISCU. The data presented above are consistent with resistance in LYC5 being a consequence of reduced accumulation of ISCU, as the relative resistance to ISCU was independent of the condition of growth (i.e. ± GF there was a 13- to 14-fold resistance in LYC5 cells relative to GC_3/c1 cells). Thus, for LYC5 cells the curves for proliferation-dependent and -independent cytotoxicity were equally displaced to higher concentrations of ISCU. To examine ISCU accumulation, cells were grown ± GF for 5 days (GC_3/c1) or 7 days (LYC5) to maximize the growth rate differential between proliferating and quiescent cells. The plating density was adjusted such that approximately equal numbers of proliferating and non-proliferating cells per dish were used in the drug accumulation experiments. Preliminary studies demonstrated that steady-state levels of [^{3}H]ISCU were achieved in both cell lines within 30 min (Fig. 4, insets). Steady-state accumulation of [^{3}H]ISCU over the concentration range used in the cytotoxicity assays was next examined in both cell lines ± GF. For GC_3/c1 cells, grown for 5 days ± GF, uptake of ISCU was similar, irrespective of the proliferative status of the cultures (Fig. 4A). Data for LYC5 were similar with no significant difference in the steady-state level of ISCU achieved in proliferating or quiescent cells (Fig. 4B). Over the range of concentrations used (0.66 to 20.66 μM), accumulation of ISCU in LYC5 cells was 78.5 ± 11% (+GF) and 66.0 ± 6.1% (−GF) compared to that in GC_3/c1 cells under identical conditions.

Effect of growth conditions on DNA nucleosomal ladder formation. Proliferating cells exposed to ISCU appear to undergo a typical apoptosis, characterized by nuclear chromatin condensation, and plasma membrane blebbing.* It was therefore of interest to determine whether formation of DNA nucleosomal

* Boder G, personal communication, cited with permission.

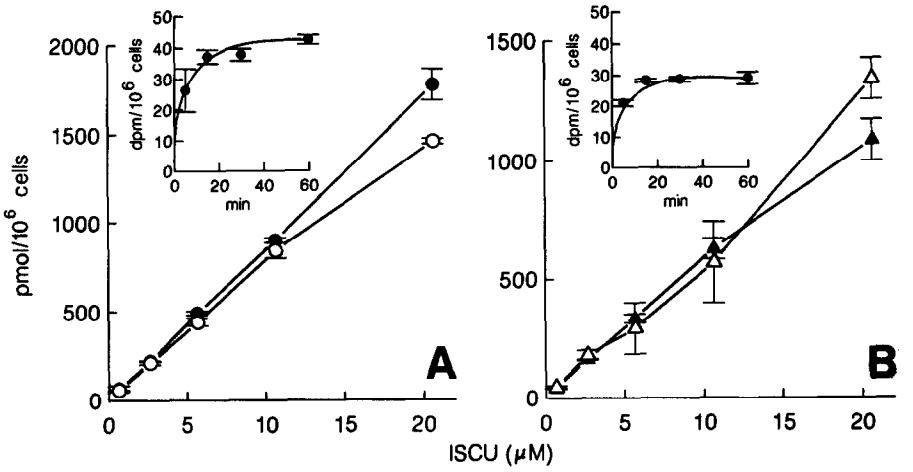

Fig. 4. Steady-state accumulation of ISCU in proliferating and quiescent cells. Cells were harvested from 0.5% FBS medium, and seeded as described in Materials and Methods. Steady-state levels of [^{3}H]ISCU were determined after a 45-min incubation with drug under conditions of cellular proliferation (●, ▲) or quiescence (○, △). (A) GC_3/c1; (B) LYC5. Values are means ± SD, N = 3. Results show a representative set of experiments; insets demonstrate the time course for ISCU accumulation.

ladders (a characteristic of apoptotic death) was dependent on the proliferative status of cells at the time of exposure to ISCU.

To examine this further, initial experiments with GC_3/c1 cells determined the rate at which cells lost colony-forming ability when deprived of serum in the presence or absence of 2.85 μM ISCU. GC_3/c1 cells were grown in 0.5% FBS for 3 days, plated at cloning density in serum-free medium without GF for various periods (24–168 hr), at which time cells were washed and refed with medium containing 10% FBS. Colonies were enumerated after an additional 7 days. Cells maintained >96% colony-forming ability after serum deprivation for up to 7 days, whereas in the presence of 2.85 μM ISCU there was a progressive loss of cloning efficiency (Fig. 5). Exposure to ISCU for 48, 96 or 168 hr resulted in 15, 60 and 96% reduction in colony formation, respectively. Thus, 2.85 μM ISCU in serum-free medium produced a reduction in colony formation equivalent to that of 57 μM ISCU in medium containing 10% FBS. As shown in Fig. 6A, DNA nucleosomal ladders were detected as early as 72 hr during exposure to 57 μM ISCU in the presence of 10% FBS. However, in non-proliferating GC_3/c1 cells no DNA nucleosomal ladders were observed (Fig. 6). In a separate, but similar experiment, cell number was enumerated at various times in cultures growing under the same conditions. In the presence of ISCU, there was no significant loss of cells during the initial 96 hr of drug exposure (97% of the cell number 24 hr after exposure to drug). It was possible, therefore, that DNA nucleosomal ladders may have been produced only after subsequent cellular proliferation. To examine this cells were exposed to ISCU (2.85 μM) for 48 or 96 hr in serum-free medium, and then were washed and refed with drug-free medium containing 10% FBS. Cells were harvested 24, 48 or 72 hr after addition of serum,

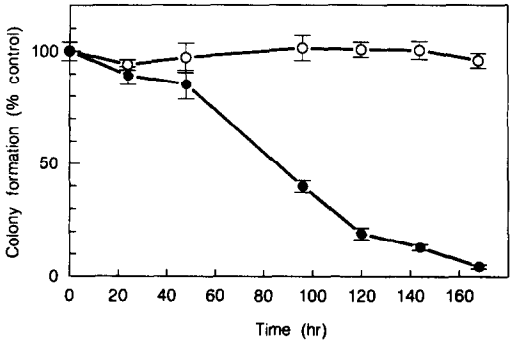

Fig. 5. Time-dependent loss of colony formation in non-proliferating GC_3/c1 cells. Cells were grown for 3 days in 0.5% FBS medium and seeded at cloning density in RPMI 1640 without supplementation. Cells were maintained for up to 7 days in the presence (●) or absence (○) of ISCU (2.85 μM), were washed and then refed with growth medium (10% FBS). Colonies were enumerated after a further 7 days. Values are means ± SD, N = 3. Control value (100%) = 331 ± 13 colonies.

DNA was extracted and analyzed by agarose gel electrophoresis. No DNA degradation was determined under these conditions (Fig. 6B). Similarly, no DNA degradation was observed in cells treated for up to 72 hr with 57 μM ISCU (20-fold the IC_{25} concentration for this period of exposure) under non-proliferating conditions (data not shown).

DISCUSSION

Previous studies have indicated that DSU may have similar potencies against both proliferating and non-proliferating cells [10, 14]. However, in these

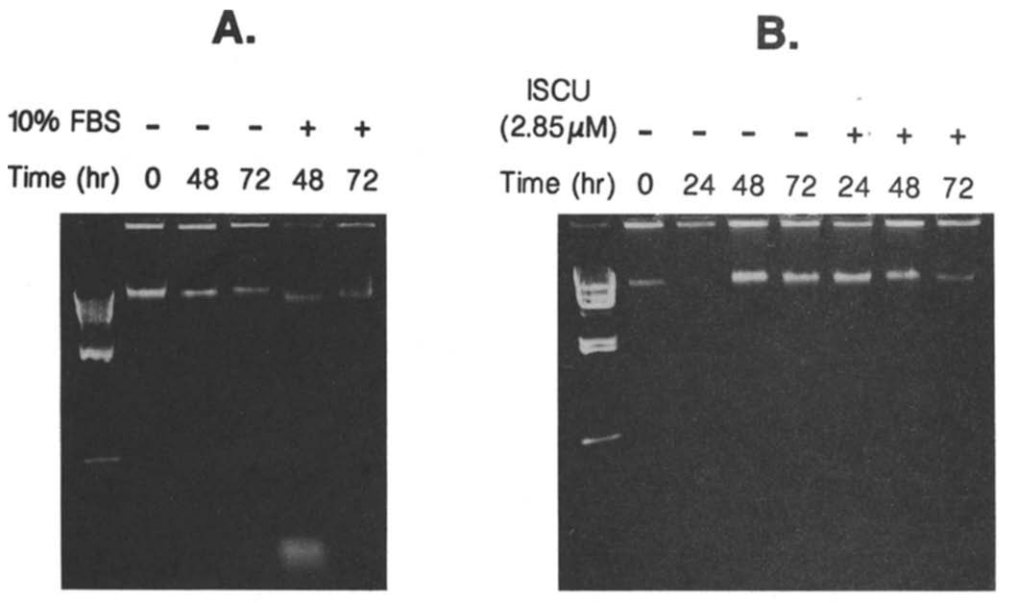

Fig. 6. Formation of DNA nucleosomal ladders in GC_3/c1 cells treated with ISCU. (A) Cells were grown in 0.5% FBS for 3 days, were washed and then refed with serum-free or growth medium (10% FBS) containing 57 μM ISCU. DNA was extracted 48 or 72 hr after the addition of drug, and was analyzed by electrophoresis in 1% agarose gels. Lane: (1) DNA standards; (2) 0 hr (3 days in 0.5% FBS); (3) 48 hr 0% serum; (4) 72 hr 0% serum; (5) 48 hr 10% serum; and (6) 72 hr 10% serum. Samples in lanes 3–6 were from cultures exposed to 57 μM ISCU. (B) Cells were incubated for 96 hr ±2.85 μM ISCU in serum-free medium without GF, were washed and then refed medium containing 10% FBS. Cells were harvested 24, 48 or 72 hr after addition of serum-containing medium.

experiments exposure to high concentrations of ISCU was relatively short (24 or 4 hr, respectively). Other data [15] suggested two independent mechanisms of action: a primary action at low concentration and a secondary mechanism of toxicity at high concentration. Precedent exists for multiple mechanisms of action, determined by drug concentration. For example, the antimetabolite 5-fluorouracil, subsequent to metabolic conversion to 5-fluoro-2′-deoxyuridylate, probably inhibits growth of cells by inhibition of thymidylate synthase, whereas in cells protected from thymineless death by co-incubation with dThd, a secondary cytotoxic mechanism exists, related to incorporation of FUTP into RNA [18, 19]. To examine potential mechanisms, clones of GC_3/c1 adenocarcinoma cells were selected for ISCU resistance. The clone reported, LYC5, was 5.5-fold resistant in serum-containing medium, and maintained this level of resistance in the absence of selection pressure for >1 year. Of interest was that while LYC5 cells were resistant to continuous exposure to low concentration of ISCU, there was no resistance to short exposure times and high concentration of drug, either in the presence or absence of serum or growth factors. These data supported our previous suggestion that distinct mechanisms of action may relate to the concentration of DSU to which cells were exposed. However, these data are in contrast to those of Trump *et al.* [20] obtained using trypan blue exclusion as a marker of viability. Using GC_3/c1 and LYC5 cells (supplied from our laboratory), Trump *et al.* [20] found that significantly less toxic effects of ISCU were observed in LYC5 cells exposed for up to 8 hr to high concentrations of drug in serum-free medium (125–1000 μM). Whether these differences relate to methodologies used (± serum, or clonogenicity vs trypan blue exclusion) is unknown. However, the inclusion of a mitochondrial target as a component of initial damage in their study is in agreement with our earlier data [12, 13].

To examine further potential sites of action we used serum-free culture methods to define proliferation-dependent and -independent mechanisms of cytotoxicity. These conditions allowed more accurate determination of free ISCU concentration, as this class of compound binds avidly to serum albumin and complicated interpretation of previous studies; in those studies, bovine serum albumin was added to equalize free-drug in serum-containing and serum-free media. Optimal growth of GC_3/c1 and LYC5 was found in serum-free medium supplemented with insulin, transferrin and EGF. Without growth factors cells remained essentially quiescent, but retained full viability for at least 7 days (determined by subsequent colony-forming efficiency). Cytotoxicity of ISCU was then examined in both sensitive and resistant cells exposed continuously (7 days) or for short periods (4 hr). For continuous exposure cytotoxicity was clearly dependent on cellular proliferation in both cell lines (Table 1). Under growth conditions (+GF), LYC5 cells were 13.6-fold resistant to ISCU relative to GC_3/c1 cells, and in both lines the IC_{50} was increased

to a similar extent in growth factor deficient medium (8.9- and 8.6-fold). In contrast there was no proliferation dependence when cells were exposed to ISCU for 4 hr. It is unlikely that the mechanism of cytotoxicity for short exposure to very high concentrations of ISCU is the same as that occurring in quiescent cells exposed for 7 days to this drug as LYC5 cells were resistant under the latter conditions whereas both LYC5 and GC_3/c1 had similar sensitivity exposed under the former conditions. However, continuous exposure at low concentration, conditions more relevant to therapeutic use of ISCU, demonstrated two potential mechanisms of cytotoxicity.

To determine whether relative resistance in quiescent cells could be a consequence of reduced accumulation of ISCU, this accumulation was examined in proliferating and quiescent cultures of GC_3/c1 and LYC5 cells. Steady-state accumulation was measured over a range of concentrations used in the cytotoxicity assay (concentrations below 0.66 μM were precluded as the specific activity of [^{3}H]ISCU was inadequate to accurately detect cell-associated drug). However, accumulation of ISCU at all extracellular concentrations examined was essentially identical in proliferating and non-proliferating cultures of GC_3/c1 cells. Similar results were obtained in LYC5 cells, although the accumulation of ISCU was slightly lower than in GC_3/c1 cells, averaging 78 ± 11 and 66 ± 6% of the steady-state levels in parental cells for proliferating and non-proliferating cultures, respectively. Previously we have shown that accumulation of DSU was increased by lowering the extracellular pH [17], and that a 4-fold increase in drug accumulation corresponded to a 4-fold decrease in IC_{50} concentration for the ISCU analogue *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea. Similar results have been obtained with ISCU (Sosinski J and Houghton PJ, unpublished results). Consequently, the small decrease in ISCU accumulation found in LYC5 cells appears inadequate to explain the 13-fold resistance, and cannot explain relative resistance in quiescent cells. From this we conclude that LYC5 is not a drug uptake mutant, although we have not examined intracellular distribution extensively.

Trump *et al.* have reported that exposure to high concentrations of ISCU (125–1000 μM) for up to 8 hr results in increased intracellular Ca^{2+} associated with mitochondrial swelling [20], whereas in CHO cells low concentrations of ISCU (≈2 μM in serum-free medium) result in morphological changes associated with disruption of actin [21], and characteristics of apoptosis. It was of interest, therefore, to determine whether there were biochemical markers that may distinguish between proliferation-dependent and -independent mechanisms involved in ISCU cytotoxicity. In the presence of serum, nucleosomal DNA ladders were detected in GC_3/c1 cells within 72 hr of drug exposure. However, in non-proliferating cells nucleosomal ladders were not observed at concentrations of ISCU causing similar cytotoxicity. Further studies showed that after exposure to ISCU for 96 hr, at a concentration consistent with >60% loss in clonogenic survival in non-proliferating cells, washing cells to remove drug, and refeeding with medium containing 10% FBS, did not result in DNA degradation. We conclude from these studies that DNA nucleosomal ladders occur only in proliferating cells exposed to ISCU, and that exposure of quiescent cells, although toxic, does not result in DNA degradation.

Although the studies reported do not directly address the biochemical mechanisms responsible for the cytotoxic activity of ISCU, they indicate at least two independent mechanisms. This hypothesis is based upon the following observations: (1) LYC5 cells were resistant (13-fold) to low concentration continuous exposure to ISCU, but were not resistant to high concentrations of drug when exposed for short time periods. These results suggest that the mechanism of action of ISCU at high concentration (short exposure) may be distinct from the mechanism that pertains to pharmacologically achievable concentrations of unbound ISCU (<1 μM). At concentrations of ISCU that are cytotoxic to GC_3/c1 and LYC5 cells exposed for 4 hr to drug, there is a rapid loss of ATP (Thakar J, unpublished data) consistent with the known uncoupling activity of these compounds. LYC5 cells were equally as sensitive as GC_3/c1 cells to this action of ISCU. (2) In contrast, LYC5 cells were resistant to prolonged exposure to low concentrations of ISCU, suggesting that cytotoxicity under these conditions is mediated through a different mechanism than uncoupling mitochondria. Under these conditions, ATP levels appear not to be decreased (Thakar J, unpublished data). (3) Cytotoxicity of high concentration exposure for 4 hr was proliferation independent, whereas cytotoxicity was proliferation dependent when cells were exposed for 7 days. These data indicate that the cytotoxic mechanisms at high and low concentrations of ISCU are distinct.

When cells were exposed continuously for 7 days, cytotoxicity was proliferation dependent. For both GC_3/c1 and LYC5 cells there was an 8-fold increase in the IC_{50} when non-proliferating cells were exposed to ISCU. Exposure of proliferating cells to ISCU resulted in formation of nucleosomal ladders, whereas at equivalent cell killing no DNA degradation was observed when quiescent cells were exposed to drug. These results could imply two mechanisms of cytotoxicity at low concentrations of ISCU (distinct from uncoupling), or alternatively a common target but having different consequences in proliferating (e.g. DNA ladders) compared to non-proliferating cells. However, available data in rodents suggest that at doses and schedules of ISCU that cause tumor regressions [5], the concentration of free (active) drug is <1 μM. These data imply that *in vivo* cytotoxicity of ISCU may most likely be a consequence of the proliferation-dependent mechanism in tumor cells. The mechanism causing proliferation-dependent cell killing, rather than that occurring after short exposure to high concentrations of ISCU, may be more informative in understanding these novel antitumor agents.

Acknowledgements—We thank Gerald B. Grindey for providing radiolabeled ISCU, and George B. Boder for

discussions on apoptosis caused by ISCU. 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).

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