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CELLULAR PHARMACOLOGY OF CHLOROQUINOXALINE SULFONAMIDE AND A RELATED COMPOUND IN MURINE B16 MELANOMA CELLS*

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Abstract—Chloroquinoxaline sulfonamide (CQS), a chlorinated derivative of sulfaquinoxaline (SQ), inhibited proliferation of murine B16 melanoma cells, but only when relatively high drug concentrations (1 mM) were used. The inhibition of cell growth by CQS was at least partially reversible by incubation in drug-free medium. Incubation of melanoma cells with CQS was associated with an arrest of the cell cycle in G_0/G_1 as measured by flow cytometry. The drug slightly decreased uptake of radiolabeled deoxyuridine and thymidine after 24- and 48-hr incubation periods but increased nucleoside incorporation at 72 hr. No evidence of intercalation with DNA was found. Because SQ previously was reported to inhibit an aspect of folate metabolism, we investigated the possibility that CQS limits tumor cell growth by altering folate homeostasis. This appears unlikely, however, in view of the following observations: (1) the cytotoxic effects of CQS could not be reversed by folinic acid; (2) deoxyuridine suppression of thymidine incorporation was not affected by CQS treatment; (3) CQS did not inhibit dihydrofolate reductase from mammalian or bacterial sources; and (4) CQS toxicity in mice was not reduced by folinic acid. Experiments performed with analogues modified in the quinoxaline and *para*-amino phenyl functions indicated that tumor cell inhibition did not require preservation of the conventional sulfonamide structure.

Sulfaquinoxaline (SQ‡) is a sulfonamide which was first synthesized more than 40 years ago. Subsequently, it has been used as an antiparasitic agent and as an antimicrobial in veterinary medicine. This compound is not known to have antineoplastic activity. Therefore, it was surprising to find that the closely related substance, chloroquinoxaline sulfonamide (CQS; NSC 339004), formed by the addition of a single chloro group to SQ, is active against human and murine tumors. For example, in the human tumor colony forming assay, CQS in a concentration of 10 μg/ml (30 μM) produced response rates of 27, 43 and 50% for melanoma, ovarian and lung carcinomas, respectively, with an overall response rate of 29% [1]. Activity was also observed against human tumor xenograft models *in vivo*. It is noteworthy that higher drug concentrations were required to inhibit murine tumor cells *in vitro*.

Since CQS is structurally dissimilar from other cancer chemotherapeutic agents, its mechanism of action is not readily apparent and may, in fact, be novel. We investigated several aspects of its cellular pharmacology to elucidate its mechanism of action. We were particularly interested in considering the possibility that CQS inhibits tumor cell growth by altering folate homeostasis, because SQ previously has been reported to inhibit an aspect of folate metabolism [2].

MATERIALS AND METHODS

Cell culture. Murine B16 melanoma cells (F10 strain) were obtained from the DCT Tumor Repository, NCI, Frederick Cancer Research Facility, Frederick, MD. The cells were grown in Dulbecco's modified Eagle's medium (M.A. Bioproducts, Walkersville, MD). In some experiments, "folate-free" medium, also obtained from M.A. Bioproducts, was supplemented with 4 μg/ml of either folic acid or folinic acid (Sigma). To these media were added 10% dialyzed horse serum, 2% glutamine and 1% penicillin-streptomycin (all obtained from Gibco Laboratories, Grand Island, NY). Cell suspensions in 13 ml of medium were placed in T 75 flasks (Corning) and incubated at 37° with 5% CO_2. Cell counts were performed manually, and viability was assessed by trypan blue exclusion. Cell cultures were divided by treating for 3 min with 2 ml of Versene, 1:5000, washing with medium, and inoculating a new flask with 2×10^5 cells.

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‡ Abbreviations: SQ, sulfaquinoxaline; CQS, chloroquinoxaline sulfonamide; and DHFR, dihydrofolate reductase.

Deoxyuridine suppression test. After Versene treatment and washing with medium, melanoma cells were resuspended in appropriate medium at a concentration of 1×10^6 cells/ml. One milliliter of suspension was placed in 12×75 mm tubes, to which was added deoxyuridine (Sigma) in phosphate-buffered saline or buffer alone. After 1 hr at 37°, 0.1 μCi of [^{3}H]thymidine (Research Products International Corp., Mt. Prospect, IL) was added, and the mixture was incubated for an additional 3 hr. Radioactivity was then determined in the trichloroacetic acid precipitates. Results are expressed as thymidine incorporation in samples with deoxyuridine divided by incorporation by control cells in deoxyuridine-free cultures × 100. All determinations were done in triplicate [3].

Cell cycle distribution. Melanoma cells (1×10^4) were stained with propidium iodide (50 μg/ml in 10% horse serum containing 1 mg/ml RNAase and 0.025% NP_{40}). Nuclear fluorescence intensity was determined with an Ortho Cytofluorograf 50 H/H using 488 nm argon ion laser excitation.

Electric cell volume. Mean channel volume was measured on a Coulter Counter model ZBI and channelyzer 256 (Coulter Electronics, Inc., Hialeah, FL). The mean channel volume was converted into mean volume by calibrating the machine with microspheres of 9.77, 14.75 and 20.01 μm in diameter (Epics Division of Coulter Corp., Hialeah, FL).

Radiolabeled nucleoside incorporation. A 1-ml suspension of cells, containing approximately 1×10^6 cells/ml, was incubated in 12×75 mm tubes with either 0.4 μCi [^{3}H]deoxyuridine (21.4 Ci/mmol) (DuPont, New Research Products, Boston, MA) or 0.4 μCi [^{3}H]thymidine for 4 hr. Radioactivity was determined in trichloroacetic acid precipitates [4].

Cloning efficiency. Tumor cells were plated at a density of 200 cells/plate in 60 mm culture dishes containing standard culture medium. After 1 week, the plates were stained with crystal violet, and the colonies were counted. Results are expressed as a percentage of colonies formed per cells plated.

Assays of DHFR activity. The possibility of inhibition of DHFR by CQS was investigated by measuring enzyme activity spectroscopically (340 nm) with NADPH (100 μM) and dihydrofolate (66 μM) in phosphate buffer (pH 7.0, 0.1 M); DHFR preparations used were from both beef liver (Sigma) and *Lactobacillus casei* (New England Enzyme Center), and assays were carried out as described previously [5].

An exploratory toxicological study. An exploratory study of leucovorin modulation of CQS toxicity in mice was carried out in a manner comparable to that used to demonstrate leucovorin protection from methotrexate toxicity [6]. CQS (as the *N*-methylglucamine salt) was administered intraperitoneally at three dose levels (600, 800 and 1000 mg/kg) to groups of six mice (BDF_1; 27–29 g), and leucovorin (Lederle, 100 mg/kg) was injected intraperitoneally at 4 and 24 hr after CQS treatment.

DNA binding evaluation. To examine the interaction of CQS with DNA the absorption spectrum of CQS (10 μM in pH 7.0 phosphate buffer) was recorded in the 240–360 nm range. Successive increments (5–10 μl) of a solution of calf thymus DNA (Sigma, Type 1) in phosphate buffer (pH 7.0 containing 2 mg of DNA in 50 ml of buffer) were added, and the absorption spectrum was recorded after each addition of DNA.

HPLC assay for CQS. Separations were performed using an Alltech Econosphere C_{18} column (5 μm particle size; 4.6 mm × 250 mm) protected by a guard column (Alltech C_{18} Adsorbosphere: 4.6 mm × 10 mm). The mobile phase was 70% HPLC grade water containing potassium dihydrogen phosphate (50 mM) and heptane sulfonic acid (10 mM) and 30% acetonitrile; triethylamine (0.02%) was added to the mobile phase and the final pH of the mobile phase was 6.1. The flow rate was 1 ml/min; detection was accomplished at 254 nm. Sample size injected into the chromatographic system was 20 μl. The internal standard for pharmacokinetic studies was acetophenone (10^{-5} M in injected solution); the internal standard had a retention time of approximately 13–15 min, whereas CQS appeared at 11–12 min. Sample preparation: 10 μl of cell preparation (supernatant solution or homogenate) with 10 μl of internal standard was diluted with 980 μl of methyl alcohol/water (50:50). Twenty microliters was injected into the chromatographic system. A Spectra-Physics 4200 system was used for all assays.

Chemicals. CQS was obtained from the National Cancer Institute, Bethesda, MD. SQ was purchased from the Aldrich Chemical Co., Milwaukee, WI. The other quinoxaline derivatives were provided by Dr. Allen Oliff, Merck & Co., West Point, PA.

RESULTS

B16 murine melanoma cells (F10 strain) were incubated with increasing concentrations of SQ and CQS. As shown in Fig. 1, both drugs were only inhibitory at the highest concentration tested, 1 mM. After 3 days in culture, cell number in SQ-containing medium was reduced approximately 40% compared to parallel untreated control cells, whereas CQS in the same concentration reduced viable cell number by 74%. At concentrations of 100 and 10 μM, cell number was within 2 standard deviations of control for both drugs after 24 and 48 hr of incubation. However, following 72 hr of culture, viable cell number was increased modestly by exposure to 100 μM and 10 μM SQ and by 100 μM CQS.

We next investigated the effect of duration of drug exposure on cell growth and cloning efficiency, using a 1 mM concentration. A representative experiment is shown in Fig. 2. Incubation of melanoma cells with CQS for as short a time period as 4 hr resulted in a reduced cell number at 96 hr. This effect became more pronounced with progressively longer exposure periods. However, it can be seen from Fig. 2 that most of this effect was due to an early inhibition of proliferation. After the drug was removed and the cells were washed, proliferation resumed at approximately the same rate as in control cells. If CQS were not removed, as in the 72-hr samples, inhibition of cellular proliferation persisted. These observations suggest that the inhibitory effects of CQS on cell growth are at least partially reversible. Similar studies were performed with SQ. Very modest inhibition of cellular proliferation was noted with drug

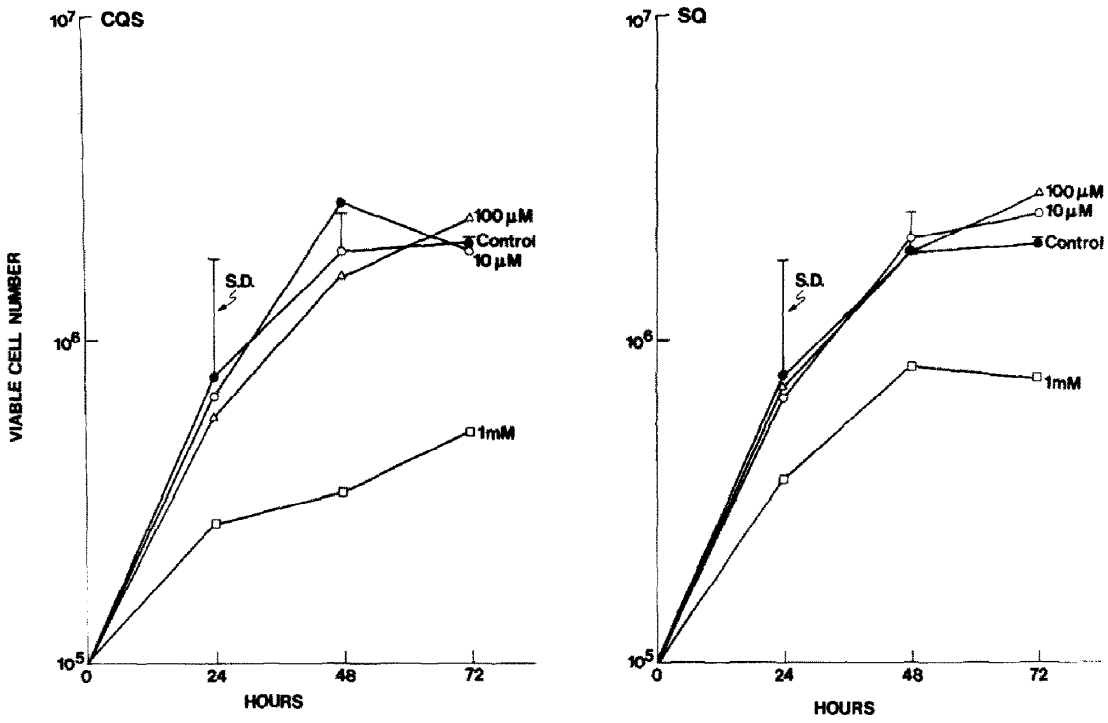


Fig. 1. Effect of drug concentration on murine melanoma cell proliferation in culture. Tumor cells were incubated with a final drug concentration of 1 mM (□), 100 μM (△), or 10 μM (○), and cell numbers at 24-hr intervals were compared to parallel cultures of untreated control (●) cells. Each point represents the mean of three separate experiments, each performed in duplicate. Brackets indicate one standard deviation of the mean for control cultures. Left panel: CQS-treated cells. Right panel: SQ-treated cells.

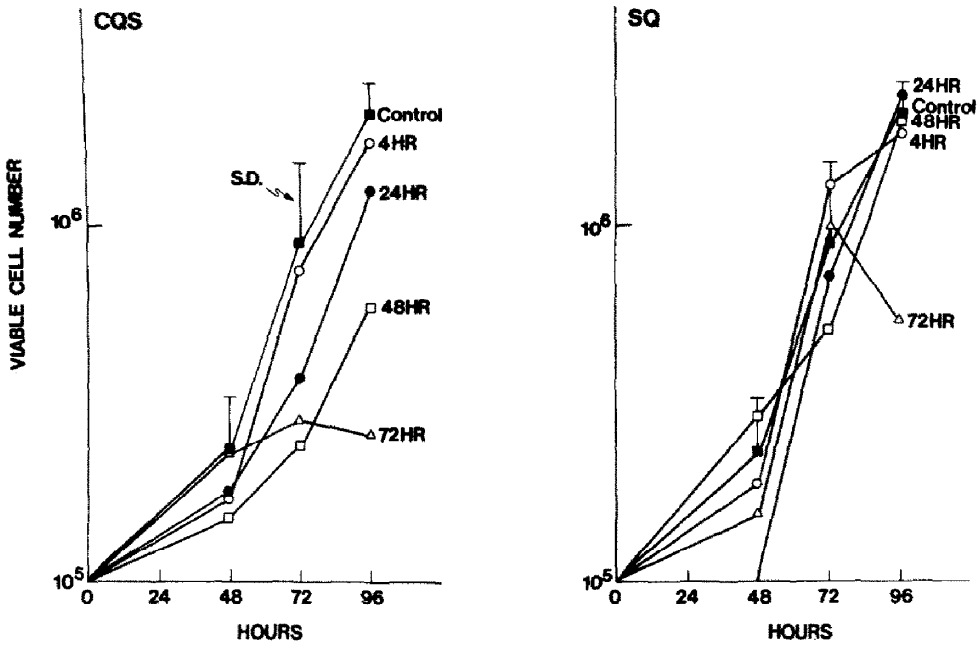


Fig. 2. Effect of duration of drug exposure on melanoma cell proliferation in culture. Tumor cells were incubated for 4 (○), 24 (●), 48 (□) or 72 (△) hr with the indicated drug in a final concentration of 1 mM. The drug was added 24 hr after the initiation of the cell culture and was washed away after the indicated time periods. The cells were then bathed in fresh, drug-free medium and incubated for a total of 96 hr. Cell counts at 24-hr intervals were compared with cell numbers obtained from five untreated control cultures (■). Brackets indicate one standard deviation of the mean for the control cultures. Left panel: CQS-treated cells. Right panel: SQ-treated cells.

exposure times of 4, 24 and 48 hr, while continuous treatment for 72 hr produced a clear reduction in cell number (Fig. 2).

These results were confirmed in replicate experiments. Data for three to nine separate experiments are compiled in Fig. 3 and show that, while drug effects were detectable after shorter exposure times, toxicity was greatest if the drug were present throughout the incubation period. This exposure time need not be 72 hr. For example, we found in twenty experiments that, following a 24-hr incubation cell number was reduced in CQS-treated cultures by 51.2 ± 16.7% and by 36.4 ± 18.7% in SQ-treated cultures compared to untreated control cultures.

The effects of treatment of melanoma cells for various time periods with 1 mM concentrations of CQS and SQ on colony formation are shown in Table 1. Incubation periods longer than 4 hr with CQS produced a progressive decrease in cloning efficiency. However, the greatest effect was seen when the drug was present throughout the 7-day period of colony culture. SQ slightly enhanced colony formation unless cells were exposed to the agent for a prolonged period of time. Like CQS, SQ was most inhibitory when it was present continuously during the 1-week culture period.

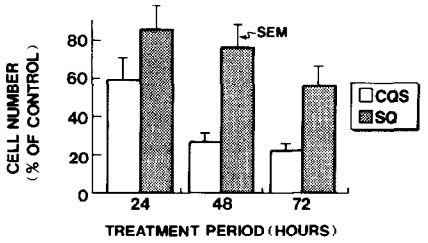


Fig. 3. Relationship between tumor cell number and duration of drug exposure. Experiments were performed as described in the legend to Fig. 2. Treatment period refers to duration of drug exposure. Cell number is expressed as percentage of concurrent untreated control cultures. The height of the bars represents the mean of multiple experiments (for 24 hr, N = 7; for 48 hr, N = 3; for 72 hr, N = 9); brackets indicate standard error of the mean. The number of viable cells in control cultures at 24, 48 and 72 hr was: $2.2 \pm 0.4 \times 10^6$, $3.3 \pm 0.8 \times 10^6$, and $3.1 \pm 0.7 \times 10^6$ respectively (mean ± SEM).

Because CQS is a planar heteroaromatic compound, in principle capable of ionic interactions with macromolecules, we considered the possibility that the drug might interact with DNA and, in this way, perturb cell growth patterns. We observed no hypochromic and bathochromic alterations of the absorption spectrum of CQS in the 240–360 nm range containing the drug's most characteristic absorption peaks, produced by addition of calf thymus DNA (data not shown). Such observations indicate that CQS does not interact with DNA in a manner comparable to that described for other heteroaromatic compounds with intercalating capability [7].

Radiolabeled nucleoside incorporation was determined in melanoma cells following incubations for 4, 24 and 72 hr with 1 mM concentrations of CQS and SQ. As shown in Fig. 4, there was only a slight inhibition of deoxyuridine and thymidine incorporation after 4- and 24-hr incubations with CQS, whereas a 72-hr incubation was associated with increased uptake of both nucleosides. SQ stimulated uptake of deoxyuridine and thymidine at all three time periods. These drug-induced changes in nucleoside incorporation did not correlate in any simple way with drug effects on either cell growth or colony formation.

The effects of 24-hr incubations with either 1 mM CQS or SQ on cell size and DNA distribution were determined. Both drug treatments were associated with a slight increase in cell size: 2431 ± 317 fl for CQS and 2502 ± 306 fl for SQ compared to untreated

Table 1. Effect of drug treatment on B16 melanoma colony formation

Treatment period (hr)	Cloning efficiency (% of control) Drug treatment CQS	SQ	N‡
1	94.7 ± 34.8	121.7 ± 38.1	3
4	107.3 ± 67.8	122.3 ± 51.1	3
24	84.8 ± 33.1	97.8 ± 37.4	9
48	71.7 ± 28.2	87.5 ± 58.0	6
72	61.5 ± 16.6	90.5 ± 59.0	8
168	28.8 ± 20.2	75.7 ± 35.8	10†, 6

Some cells were treated with a 1 mM concentration of the indicated drug for various time periods (1–72 hr), were washed three times with medium, and then 200 cells were placed in 60-mm culture plates. Following 1 week of incubation at 37° with 5% CO_2, the number of colonies was enumerated as described in Materials and Methods. Cloning efficiency of drug-treated cells was compared with parallel cultures of untreated control cells, expressed as percent of control. Other cells were similarly seeded at 200 cells/plate but the indicated drug at a final concentration of 1 mM was added directly to the culture plate, where it remained throughout the 168-hr (7-day) incubation period. Mean cloning efficiencies in control cultures at 1, 4, 24, 48, 72 and 168 hr were 54.3, 47.7, 54.8, 63.7, 51.4 and 54.3% respectively. Values are means ± SD.

* Number of experiments, each performed in triplicate.

† For CQS, N = 10; for SQ, N = 6.

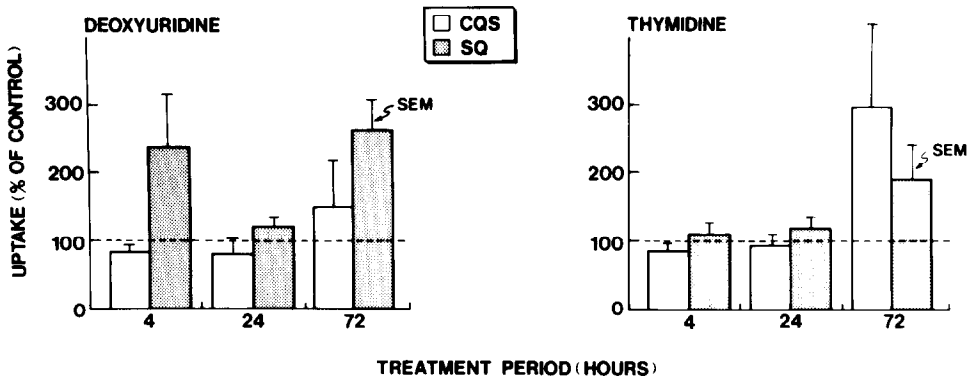


Fig. 4. Radiolabeled nucleoside incorporation after drug treatment. Treatment period refers to duration of exposure to drug in a 1 mM concentration. Uptake is expressed as a percentage of radioactivity incorporated by an equal number of untreated control cells in parallel cultures (indicated by broken line). For [^{3}H]deoxyuridine, at 4, 24 and 72 hr, the cpm were: 33,102; 14,180; and 35,636 in control cultures. For [^{3}H]thymidine, the cpm in control cultures were: 36,450; 31,830; and 22,346 at the same time points. The height of the bars represents the mean of separate experiments, each performed in triplicate. Brackets indicate the standard error of the mean. Left panel: [^{3}H]deoxyuridine incorporation. Right panel: [^{3}H]thymidine incorporation.

control cells (2224 ± 248 fl) in five experiments. However, these differences were not statistically significant when tested by Student's *t*-test. Incubation of melanoma cells with CQS led to an arrest of the cell cycle in G_0/G_1. For six experiments, the DNA distribution after cell cycle analysis for control cells was: 53.4 ± 7.0% G_0/G_1; 37.8 ± 11.0% S; and 8.8 ± 6.4% G_2/M, whereas for CQS-treated cells the percentages were: 66.5 ± 7.9% G_0/G_1; 25.6 ± 8.9% S; and 7.5 ± 2.7% G_2/M. The differences between control and CQS-treated cells were significant at the 0.005 level for G_0/G_1 and at 0.002 for S by the paired *t*-test. Under similar incubation conditions SQ had essentially no effect on DNA distribution: 53.0 ± 7.5% G_0/G_1; 36.4 ± 10.2% S, and 10.6 ± 5.4% G_2/M. None of these changes induced by SQ was statistically significant.

We were interested in determining whether there was any evidence to support the possibility that these sulfonamide compounds were acting as antifolates to inhibit tumor cell growth. Melanoma cells were incubated for 24 or 72 hr with 1 mM CQS or SQ in culture medium containing either folic acid or folinic acid. Cell number was determined, and then the cells were washed, resuspended in the same medium without CQS or SQ, and placed into culture for an additional 7 days. Afterward colony formation was determined. As shown in Table 2, the inhibitory effects of CQS and SQ on growth were not ameliorated by folinic acid. Interestingly, folinic acid supplemented medium consistently enhanced the cloning efficiency of the murine melanoma cells compared to cells grown in the standard folic acid containing medium. Nevertheless, the cloning efficiency of CQS- and SQ-treated cells was not improved significantly when compared to that of control cells in the same medium.

The weak inhibitory activity reported by Poe and his colleagues [2, 8] for SQ against DHFR from mammalian and bacterial sources suggested the desirability of assessing the ability of CQS to inhibit DHFR. We were unable to detect inhibition by CQS of DHFR from either beef liver or *L. casei* at concentrations as high as 2 mM, even when CQS was preincubated with the enzyme reaction system for 10 min prior to addition of dihydrofolate (data not shown). However, it should be noted that the DHFRs studied were like, but not identical to, the DHFR in murine melanoma cells.

Incubation of melanoma cells with 1 mM concentrations of CQS or SQ for 24 hr did not impair significantly deoxyuridine suppression of thymidine incorporation. This test is a sensitive index of intracellular folate activity [4]. Radiolabeled thymidine incorporation was suppressed to 5.8 ± 3.5% of control by 1 μM deoxyuridine and to 28.7 ± 7.7% of control by 0.1 μM deoxyuridine in six experiments. The respective percentages for SQ-treated cells were 4.7 ± 3.6% and 21.3 ± 14.9%. CQS-treated cells showed less suppression: 21.3 ± 27.6% for 1 μM deoxyuridine and 42.2 ± 22% for 0.1 μM deoxyuridine. However, these differences were not statistically significant, using the paired *t*-test.

CQS toxicity in mice was not reduced by leucovorin in dosages which protect against methotrexate toxicity. Five of six animals receiving CQS at 1000 mg/kg died within 24 hr of drug administration and the remaining animal died on day 2. No change in the pattern of toxicity was observed with leucovorin treatment. Two of six animals receiving CQS alone at 800 mg/kg died within 24 hr and all animals were dead within 72 hr. Again no protection or "rescue" was observed when animals were treated with leucovorin after CQS. Only one of six animals receiving CQS at 600 mg/kg died within 72 hr and no further deaths occurred; the same pattern was observed in animals receiving CQS at 600 mg/kg and leucovorin. Assessment of body weight changes showed no advantage for leucovorin treatment. Our observations indicate that the acute toxicity of CQS is different from that of a representative folate antagonist (methotrexate), both in time course and in reversibility by leucovorin. It seems likely that CQS toxicity in mice is not of the "antifolate" type.

Table 2. Effects of culture in folinic acid-supplemented medium on proliferation and colony formation by drug-treated murine melanoma cells

	Viable cells at 72-hr incubation					
	Folic acid*			Folinic acid		
	Count ($\times 10^6$)	% Control	N†	Count ($\times 10^6$)	% Control	N
24-hr Exposure						
No drug	2.5 ± 0.5	—	3	2.1 ± 0.4	—	3
CQS	1.1 ± 0.2	45	3	1.1 ± 0.1	50	3
SQ	1.7 ± 0.4	70	3	1.8 ± 0.9	61	3
72-hr Exposure						
No drug	3.0 ± 0.9	—	3	2.4 ± 0.2	—	3
CQS	1.0 ± 0.4	33	3	0.9 ± 0.2	37	3
SQ	1.9 ± 1.1	60	3	2.1 ± 0.5	88	3

	Colony formation					
	Folic acid			Folinic acid		
	Cloning efficiency (%)	% Control	N	Cloning efficiency (%)	% Control	N
24-hr Exposure						
No drug	18.4 ± 11.9	—	3	72.3 ± 12.1	—	3
CQS	14.7 ± 8.5	82	3	21.3 ± 9.3	31	3
SQ	26.3 ± 19.4	151	3	45.3 ± 45.6	64	3
72-hr Exposure						
No drug	18 ± 10.6	—	3	31.7 ± 19.9	—	3
CQS	10.7 ± 6.4	59	3	14.7 ± 8.5	65	3
SQ	17 ± 11.3	93	3	43.7 ± 31.1	138	3
7-day Exposure						
No drug	56.5 ± 7.0	—	4	69.3 ± 33.3	—	4
CQS	15.5 ± 9.2	27	4	15.0 ± 9.2	22	4
SQ	40.8 ± 21.4	72	4	76.8 ± 33	111	4

Melanoma cells were cultured for 24 or 72 hr with a 1 mM final concentration of the indicated drug or with added saline. The cells treated for 24 hr were washed three times with fresh medium and divided into aliquots for assays of cell proliferation and colony formation. All samples were counted for viable cells at 72 hr as indicated in Materials and Methods. Data are expressed (mean ± SD) as viable cell count and as percentage of cells in drug-treated plates compared to parallel cultures without drug in the same medium. Colony formation was assayed by adding cells after 24 or 72 hr of drug treatment to 60 mm plates in a concentration of 200 cells/plate. Alternatively, drug or saline was added to some plates of cells directly. After a 7-day incubation in the indicated medium, the cell colonies were stained and cloning efficiency was determined. The absolute number of colonies counted was double the cloning efficiency.

* Culture medium was supplemented with 5 mM folic acid or folinic acid.

† Number of experiments; each determination was performed in triplicate.

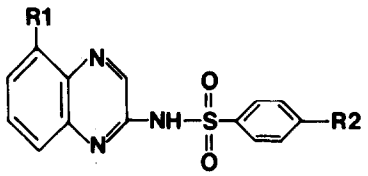
To determine if tumor cell inhibition requires preservation of the conventional sulfonamide structure, melanoma cells were incubated with congeners of CQS. These compounds are modified in the quinoxaline and *para*-amino phenyl functions. Tumor cells were incubated with a 1 mM concentration of each drug for 72 hr, and cytotoxicity was expressed as percentage of viable cells present in simultaneous untreated control cultures. As shown in Table 3, cytotoxic activity was maintained despite major modification of the *para*-amino group at R2. Studies with a second group of compounds suggest that activity was preserved after substitution at the R1 position.

Finally, we looked for evidence of drug metabolism by the melanoma cells during the culture period. Tumor cells at two concentrations (1×10^6/ml and 2×10^6/ml) were incubated with 1 mM CQS for 1, 24, 48 and 72 hr. The cells were collected by centrifugation, washed three times with drug-free medium, pelleted and sonicated. The cell homogenate and the suspending medium from the culture period were then analyzed by HPLC. CQS was easily detectable in the suspending medium and faintly detectable in the cell homogenate at all time periods. No metabolites were found under the conditions of these experiments (data not shown).

DISCUSSION

Our results indicate that CQS and, to a lesser extent, SQ were active agents against murine B16 melanoma cells, but only in relatively high concentrations. These concentrations are achievable in rats and mice with relatively little overt toxicity, although it should be stressed that the slope of the dose–response curve for toxicity appears to be steep since other investigators have observed lethality at

Table 3. Relative cytotoxicities of quinoxaline sulfonamides on murine melanoma cells



Compound	R1	R2	Viable Cells (% Control)
SQ		NH_2	41
CQS	Cl	NH_2	7
1		NO_2	0
2		$NH\text{-}CO\text{-}CH_3$	0
3		$NH\text{-}CH_2\text{-}OSO_3H$	0
4		$NH\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}COOH$	57
5	CH_3	NH_2	2
6	Br	NH_2	2

Cytotoxicity, expressed as a percentage of viable cell number in simultaneous control cultures without added drug, was determined at the end of a 72-hr incubation period. All drug treatments were performed with 1 mM drug concentrations. Control cultures contained $3.28 \pm 0.91 \times 10^6$ viable cells (mean ± SD) in eight experiments.

400 mg/kg in mice [9]. Dogs are even more sensitive to CQS, suffering lethal effects at doses of 12 mg/kg [9]. Whether the rodent or canine toxicity profiles are more applicable to humans will be determined in phase I trials. Human tumor cells *in vitro* are approximately a log more sensitive to CQS than murine melanoma cells [1]. It may be possible, therefore, to deliver tumoricidal dosages to human patients with acceptable toxicity if human susceptibility to the toxic effects of the drug lies between the two species.

Despite its sulfonamide structure, CQS does not appear to act by interfering with folate function. Typical folate antagonists, such as methotrexate and trimetrexate, cause arrest of the cell cycle in G_0/G_1 and S phase, inhibit deoxyuridine incorporation into DNA but preserve utilization of thymidine by the salvage pathway, impair deoxyuridine suppression of thymidine uptake, and inhibit dihydrofolate reductase activity. Their toxic effects are ameliorated by folinic acid (leucovorin) [6, 10–13]. In contrast, in the present study incubation of tumor cells with CQS was associated with accumulation of DNA in G_0/G_1. There was only a slight reduction of incorporation of both deoxyuridine and thymidine into DNA after 24- and 48-hr incubation periods, and actual enhancement of nucleoside incorporation at 72 hr. Deoxyuridine suppression of thymidine uptake was normal in CQS-treated cells, and no evidence of inhibition of dihydrofolate reductase from mammalian or bacterial sources was detectable. Neither the cytotoxic effects of CQS *in vitro* nor its lethal effects *in vivo* were reduced by folinic acid in dosages which protect against methotrexate toxicity. Finally, tumor cell inhibition did not require preservation of the conventional sulfonamide structure.

The anti-tumor effects of CQS do not appear to depend upon direct interaction with DNA, since we found no evidence of intercalation. And, as pointed out above, there is little to indicate that the drug interferes with DNA metabolism. CQS has a high avidity for protein [14]; therefore, the cell membrane or intracellular proteins may be important sites of drug action. We conclude that the mechanism of action of CQS involves cellular functions other than folate or DNA metabolism and requires further investigation.

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