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IMMUNOSUPPRESSIVE PROPERTIES OF CHLOROQUINOXALINE SULFONAMIDE*

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Abstract—Chloroquinoxaline sulfonamide (CQS), a sulfanilamide derivative with antitumor activity, was found to be toxic to lymphoid tissue during preclinical studies. The mechanism of this toxicity appears to involve profound inhibition of lymphocyte activation. Incubation of human peripheral blood mononuclear cells (PBMNCs) with CQS decreased cellular incorporation of thymidine and deoxyuridine in a dose-dependent manner. Analysis of cell cycle distribution by flow cytometry indicated that CQS blocked movement out of the G_0/G_1 phase. Drug-treated cells were smaller and expressed fewer receptors for interleukin-2 (IL-2) and transferrin than untreated mitogen-stimulated lymphocytes. These observations support the notion that CQS has cell cycle specificity in regulating lymphocyte proliferation. As little as 10 μM CQS markedly inhibited both human lymphocyte and murine CTLL cell replication in response to IL-2 containing growth factors. However, CQS did not block secretion of IL-2 into culture supernatant fractions by human PBMNCs. Finally, COS inhibited *in vitro* production of immunoglobulins G and M by mitogen-stimulated lymphocytes, primarily by causing cytotoxicity. In all of these drug effects, CQS was approximately one to two logs more potent than the parent compound, sulfaquinoxaline (SQ). These studies indicate that CQS inhibits essential basic processes in human lymphocytes. This agent may find use as an immunosuppressive drug.

Chloroquinoxaline sulfonamide (CQS‡, NSC 339004) is the first sulfanilamide derivative to be considered seriously for clinical trials as a cancer chemotherapeutic agent [1]. CQS is active against human and murine tumors *in vitro* and shows therapeutic activity in human tumor xenograft models *in vivo* [1]. Pharmacokinetic and toxicologic studies indicate that relatively high concentrations of the drug can be achieved *in vivo* with little overt toxicity [2, 3]. Although its mechanism of action is unknown, CQS was found in our laboratory to inhibit the proliferation of murine melanoma cells by arresting the cell cycle in G_0/G_1 [4]. This observation suggested to us that CQS may have immunosuppressive properties, since several agents which inhibit lymphocyte function also act by delaying progression in this phase of the cell cycle [5, 6]. In addition, CQS was noted to be toxic to lymphoid tissue in dogs and rats during preclinical studies [2]. Consequently, we investigated the influence of CQS on lymphocyte metabolism and proliferation. Our studies indicated that CQS profoundly inhibits lymphocyte activation at drug concentrations which are pharmacologically relevant. This inhibitory effect resulted in impaired proliferation in response to mitogen stimulation and in cytotoxicity which drastically reduced *in vitro* immunoglobulin (Ig) production.

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‡ Abbreviations: CQS, chloroquinoxaline sulfonamide; SQ, sulfaquinoxaline; PBMNCs, peripheral blood mononuclear cells; Ig, immunoglobulin; [^{3}H]TdR, tritiated thymidine; [^{3}H]dU, tritiated deoxyuridine; DMEM, Dulbecco's Modified Eagle's Medium; PHA, phytohemagglutinin; PBS, phosphate-buffered saline; PWM, pokeweed mitogen; IL-2, interleukin-2; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

MATERIALS AND METHODS

Isolation of mononuclear cells. Human peripheral blood mononuclear cells (PBMNCs) were isolated from healthy volunteers by Ficoll–Hypaque density gradient centrifugation (Histopaque-1077; Sigma Diagnostics). Cells were collected from the interface, washed three times with Dulbecco's Modified Eagle's Medium (DMEM) (Whittaker M.A. Bioproducts, Walkersville, MD), supplemented with 2% glutamine, 1% penicillin–streptomycin, and 10% dialyzed horse serum (all obtained from Gibco Laboratories, Grand Island, NY). For assays of immunoglobulin synthesis, PBMNCs were cultured in RPMI 1640 containing penicillin–streptomycin and 10% fetal bovine serum (Gibco Laboratories). Cell counts were performed with a Coulter Counter model ZBI, and viability was assessed by trypan blue exclusion.

Measurements of [^{3}H]*TdR and* [^{3}H]*dU incorporation.* Cell suspensions containing 1×10^6 cells/ml (100 μl) were plated in quadruplicate in microtitration multi-well plates (Nunclon 96-well plates). For controls without PHA, 100 μl DMEM was added to each well. For mitogen-stimulated cells, 20 μl of a 0.025% PHA solution (Bacto Phytohemagglutinin P; DIFCO, Detroit, MI) was added to each well. Drug (CQS or SQ) was added in a volume of 10 μl. Enough medium was added to each well to bring the total volume to 200 μl. At 18–24 hr prior to harvest,

10 μl of either 0.4 μCi [^{3}H]deoxyuridine (21.4 Ci/mmol) (DuPont, NEN Products, Boston, MA) or 0.4 μCi [*methyl*-^{3}H]thymidine (20 Ci/mmol; CEA, France) was added to all wells. After 72-hr incubations, cells were harvested with a mini-MASH (M.A. Bioproducts) cell harvester, and radioactivity was determined.

Flow cytometry analysis. Measurements of cell cycle distribution were performed as previously described [7]. After 72 hr of incubation in 12 × 75 mm culture tubes (American Scientific Products, McGaw Park, IL) containing 1 ml of culture medium, 1×10^6 cells were centrifuged and suspended in a hypotonic 0.1% sodium citrate solution containing 50 μg/ml propidium iodide (Sigma Chemical Co., St Louis, MO), 500 μg/ml RNAase (Sigma), 0.05% Nonidet P-40 nonionic detergent (Sigma), and 10% horse serum. Tubes were incubated in a 37° water bath for 30 min prior to analysis, using a 50-H/H Cytofluorograf flow cytometer, interfaced with an Ortho 2150 data handling system (Ortho Diagnostics Systems, Inc., Westwood, MA). Excitation was by argon laser, operating in the output stabilized mode at an output of 200 mW, wavelength 488 nm. Red fluorescence was defined by a 560 nm short pass dichroic mirror (Omega Optics Inc., Brattleboro, VT) and a 630 nm long pass filter (Schott). By correlating the integrated area with a peak of each fluorescence pulse in a two-parameter cytogram, a rectilinear region could be set which excluded signals due to nuclear doublets from further analysis. Fluorescence of cells falling in this defined region was then presented as a one-parameter histogram of red fluorescence. Typically, 50,000 events were acquired per histogram.

Electric cell volume. Mean channel volume was determined with a channelyzer 256 (Coulter Electronics, Inc., Hialeah, FL). The mean channel volume was converted to mean volume by calibrating the instrument with microspheres of 9.77, 14.75 and 20.01 μm in diameter (Epics Division of the Coulter Corp., Hialeah, FL).

Indirect immunofluorescence using anti-TAC and OKT9 monoclonal antibodies. Following 72 hr of incubation, tubes containing 1×10^6 cells were centrifuged, and all but 50 μl of the supernatant fluid was removed. Ten microliters of anti-TAC antibody (provided by Dr T. Waldmann, NIH, Bethesda, MD) or 5 μl of OKT9 (Ortho Diagnostics, Raritan, NJ) and 40 μl PBS with 0.1% azide were added to some tubes. After a 30-min incubation at 4°, cells were washed once with cold PBS with 0.1% azide and again with cold medium. After centrifugation, all but 50 μl of the supernatant fluid was removed. Ten microliters of goat antimouse Ig (FITC conjugated IgG) (1:50 concentration) was added to all tubes. Following a 30-min incubation at 4°, the cells were washed twice with PBS with azide, centrifuged again, and resuspended in 1 ml PBS. The percent positive cells was determined by flow cytometry; the number of cells reacting with non-specific antibody was subtracted from the number reactive with specific antibody.

Preparation of T-cell growth factor. T-cell growth factor was produced by collecting the supernatant solution from human PBMNCs after culture for 4 days at a concentration of 10^6 cells/ml in RPMI 1640 medium containing 2 mM glutamine, 2% human serum and recombinant IL-2 (Hoffmann-LaRoche, Nutley, NJ) at a final concentration of 1500 units/ml.

Measurement of IL-2-stimulated lymphocyte proliferation. Human PBMNCs were isolated by density gradient centrifugation and suspended at a concentration of 10^6 cells/ml in RPMI 1640 containing 20% HL 1 (Ventrex Laboratories, Portland, ME) and 5% fetal calf serum (Hazelton Dutchland, Denver, PA) in T25 culture flasks (Corning). PHA-P was added to a final concentration of 1 μg/ml, and the cell suspension was incubated for 40 hr. The cells were then harvested, counted, and added in a concentration of 10^4 cells/2 ml to each well of 24-well plates (Corning) in RPMI containing 25% T-cell growth factor, 0.5 μg/ml PHA-P, 20% HL 1, and 5% fetal calf serum. Each well also contained 2.5×10^5 irradiated (9000 rad) TK 6 cells/ml (provided by Dr R. Albertini, University of Vermont). After 24 hr in culture, drug was added in a 20 μl volume. Daily counts were performed beginning 5 days later.

Rat growth factor preparation. Spleens were removed from female Sprague–Dawley rats and coarsely minced. The cells were washed twice with PBS and then resuspended in culture medium (RPMI 1640 containing 2% fetal calf serum, 0.05 mM 2-mercaptoethanol, penicillin/streptomycin, 2% glutamine and 5 μg/ml concanavalin A) at a concentration of 10^6 cells/ml. The cell suspension was incubated in T75 flasks for 42 hr. The supernatant fraction was collected by centrifugation and sterilized through a 0.22 μm membrane.

IL-2-assay. This assay was performed as previously described [8], using a modification of the procedure of Gillis *et al.* [9]. CTLL-2 cells (American Type Culture Collection, Rockville, MD) are IL-2 dependent. Rat growth factor was used at a dilution of 1:5 as a known source of IL-2 to provide optimal incorporation of [^{3}H]TdR. CTLL cells were grown from a concentration of 10^4/ml to approximately $1.0–2.0 \times 10^5$/ml over a 3-day period in medium (RPMI 1640 with 5% fetal calf serum, 15 mM Hepes, 2 mM glutamine, 2 mM pyruvate, 0.05 mM 2-mercaptoethanol, and penicillin/streptomycin) containing 20% rat growth factor. They were then washed and resuspended in medium without growth factor and incubated for an additional 4 hr. Cells were then added to flat-bottom microtiter plates (Cell Wells, Corning, NY) in a concentration of 10^4 cells/well in medium containing rat growth factor. Drug in various concentrations was added to some wells. After 24 hr of culture, [^{3}H]TdR was added, and the plates were incubated for an additional 4 hr and then harvested. In other experiments, supernatant fractions from human PBMNC cultures were added in a volume of 100 μl. Cultures were performed in triplicate.

Immunoglobulin synthesis. Mitogen-induced Ig synthesis was performed as previously described [7]. Pokeweed mitogen (PWM), diluted 1:200, was added to 10^6 PBMNCs cultured in 2 ml of complete medium. An enzyme-linked immunosorbent assay

Table 1. Tritiated nucleoside incorporation by PBMNCs after incubation with CQS or SQ for 72 hr

Treatment	[^{3}H]TdR Incorporation (cpm)		[^{3}H]dU Incorporation (cpm)	
	+PHA	No PHA	+PHA	No PHA
None	51,800 ± 12,400*	1,500 ± 1,100	29,600 ± 5,500	180 ± 70
CQS				
10 μM	41,400 ± 12,500	1,400 ± 1,100	19,800 ± 3,200†	130 ± 50
100 μM	15,600 ± 4,400†	680 ± 310	5,100 ± 870+	70 ± 30†
1 mM	1,250 ± 680†	320 ± 180	400 ± 180†	20 ± 10†
SQ				
10 μM	55,000 ± 12,800	490 ± 140	33,500 ± 7,000†	240 ± 80†
100 μM	57,900 ± 14,500	470 ± 130	32,900 ± 7,200†	210 ± 50
1 mM	32,700 ± 8,300†	200 ± 90	14,200 ± 2,700†	140 ± 50†

* Mean ± SE for seven experiments, each performed in at least triplicate.
† Significantly different ($P < 0.05$ or greater) from untreated control cells in parallel cultures by Student's paired *t*-test.

Table 2. Cell cycle distribution and volume of human lymphocytes after treatment with CQS or SQ

Treatment	Cell cycle distribution			Volume (fl)
	$G_0 + G_1$	S	$G_2 + M$	
None	95.5 ± 1.3*	1.7 ± 0.5	1.0 ± 0.3	205 ± 10
PHA	60.9 ± 3.1	27.6 ± 3.8	10.2 ± 1.2	557 ± 83
CQS				
10 μM	60.8 ± 3.4	28.7 ± 4.2	9.3 ± 2.3	572 ± 100
100 μM	71.8 ± 4.7	19.2 ± 4.7	7.5 ± 2.4	451 ± 42
1 mM	90.5 ± 2.1	4.9 ± 1.3	3.2 ± 0.7	300 ± 33
SQ				
10 μM	58.2 ± 2.6	28.2 ± 3.9	12.3 ± 1.2	543 ± 76
100 μM	60.1 ± 2.1	27.0 ± 3.4	10.8 ± 1.4	569 ± 94
1 mM	63.6 ± 4.2	26.6 ± 4.5	8.5 ± 1.9	498 ± 45

* Mean ± SE for seven (cell cycle distribution) or five (volume) experiments.

(ELISA) was used to measure the IgM and IgG in the culture supernatant fractions after 12 days in culture.

Chemicals. CQS was obtained from the National Cancer Institute, Bethesda, MD. SQ was purchased from the Aldrich Chemical Co., Milwaukee, WI.

RESULTS

As shown in Table 1, incubation of mitogen-stimulated PBMNCs from normal human volunteers with increasing concentrations of CQS progressively inhibited incorporation of tritiated thymidine ([^{3}H]TdR) and tritiated deoxyuridine ([^{3}H]dU). At the highest concentration tested (1 mM), nucleoside uptake was diminished by >95%, but even at a concentration of 10 μM, CQS measurably reduced incorporation of both thymidine and deoxyuridine. The related compound, SQ, had less effect in equimolar concentrations. For example, a 1 mM concentration inhibited uptake of these nucleosides by only 40–50%. Both drugs were toxic to unstimulated PBMNCs in culture (Table 1), although this inhibition was statistically significant only at the highest concentrations tested.

The effects of these sulfonamides on cell cycle distribution were measured by flow cytometry. As expected, mitogen stimulation of lymphocytes initiated DNA synthesis and progression into the S and G_2 + M phases of the cell cycle (Table 2). At 100 μM and 1 mM concentrations, CQS appeared to block movement out of the G_0/G_1 phase. In contrast, SQ had no important effect on cell cycle distribution at any of the concentrations tested. The results obtained with CQS suggested that the drug might be active at an early stage of lymphocyte activation. Morphologic observation of Wright stained cells supported this possibility, in that CQS-treated cells were smaller and resembled inter-mitotic lymphocytes rather than typical stimulated lymphocytes (not shown). This impression was confirmed by measuring cell volumes (Table 2). PHA-stimulated lymphocytes after 72 hr in culture were more than twice as large as inter-mitotic cells. Exposure of lymphocytes to CQS in the same concentrations which altered cell cycle distribution was associated with a reduction in cell volume, whereas SQ had little effect (Table 2).

We next measured drug effects on early manifestations of lymphocyte activation. Incubation of mitogen-stimulated cells with CQS (1 mM) caused a 50% reduction in the expression of receptors for interleukin-2 (IL-2) and transferrin, as detected by

Table 3. Percentage of TAC and OKT9 positive cells in cultures following incubation with CQS or SQ

Treatment	TAC+ cells (%)	OKT9+ cells (%)
None	1.7 ± 0.6*	0.6 ± 0.4
PHA	15.9 ± 4.8	46.2 ± 10.9
CQS		
10 μM	17.8 ± 9.5	39.9 ± 10.3
100 μM	16.0 ± 4.5	40.9 ± 3.6
1 mM	7.7 ± 2.2	21.8 ± 6.5
SQ		
10 μM	17.7 ± 8.1	44.4 ± 5.0
100 μM	25.6 ± 6.1	51.0 ± 7.8
1 mM	17.1 ± 8.5	35.3 ± 9.4

* Mean ± SE for five (TAC) or three (OKT9) experiments.

the monoclonal antibodies TAC and OKT9 respectively (Table 3). Similar exposure to SQ had essentially no effect on the expression of these membrane receptors.

Since CQS-treated cells had fewer detectable receptors for IL-2, it was of interest to determine whether their response to this lymphokine was impaired. As shown in Fig. 1, as little as 1 μM CQS reduced human lymphocyte proliferation in response to growth factor, which contains IL-2, and concentrations of 10 μM or higher completely inhibited replication. In contrast, SQ had no effect on IL-2-dependent lymphocyte proliferation unless a 1 mM concentration was used.

The inhibitory effect of CQS on IL-2-dependent lymphocyte proliferation was confirmed in another experimental system. CTLL cells are a murine lymphocyte-derived cytotoxic T-cell line which only replicates in the presence of exogenous IL-2. Incubation of these cells with an optimal amount of rat growth factor containing IL-2 predictably caused cell division, which was assayed by tritiated thymidine incorporation (Table 4). When CQS was added to the culture medium in increasing concentrations, thymidine incorporation was progressively reduced, indicating substantial inhibition of CTLL proliferation by the drug.

The same experimental system was used to determine whether CQS inhibited IL-2 production *in vitro*. Human PBMNCs were incubated for 24 hr with PHA and increasing concentrations of CQS. The cell-free supernatant fractions were then collected and added to cultures of CTLL without exogenous IL-2. The CTLL cells incubated with drug-free supernatant fraction incorporated approximately 100 times as much tritiated thymidine as CTLL cells in medium alone (103 + 54 cpm), indicating the presence of measurable amounts of IL-2 in these supernatant fractions. CTLL thymidine uptake in cultures with drug-containing supernatant fractions was approximately the same as when drug was added directly to medium containing optimal amounts of IL-2 (Table 4). For example, when CTLL cells were incubated in a final concentration of 100 μM CQS, thymidine uptake was 39% of incorporation by cells in drug-free medium. Addition of supernatant fluid from human PBMNCs following incubation with 100 μM CQS resulted in thymidine uptake by CTLL cells which was 33% of that by cells in drug-free supernatant fluid. We conclude, therefore, that CQS does not substantially interfere with production of IL-2 by human lymphocytes.

Finally, we measured the effect of CQS on *in vitro* immunoglobulin production. As shown in Table 5, CQS in concentrations of 10 μM or greater markedly inhibited secretion of IgG and IgM into the suspending medium. This inhibitory effect appeared to be due in large part to drug-induced cytotoxicity, since the number of viable cells in culture was concomitantly reduced at these same concentrations (Table 5). Immunoglobulin production by PWM-stimulated PBMNCs was not affected by SQ unless concentrations in excess of 100 μM were used (data not shown).

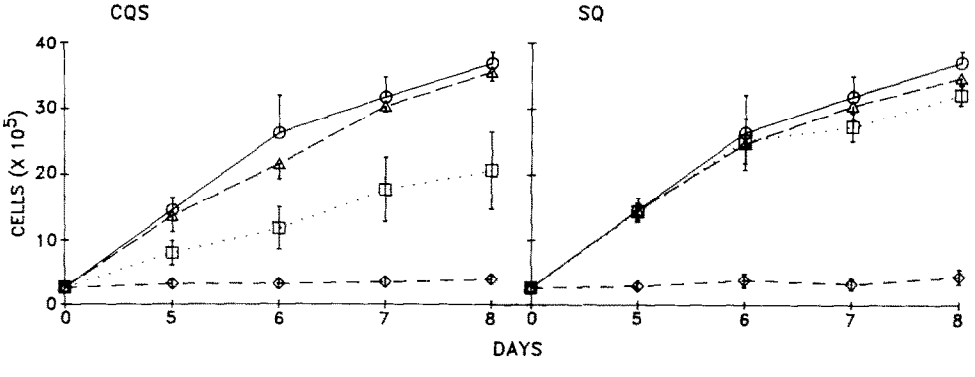

Fig. 1. Effect of the sulfanilamide derivatives CQS (left) and SQ (right) on IL-2-augmented lymphocyte proliferation. The total number of cells present in each culture well was enumerated on the days indicated. Data are expressed as cells/ml. Control cultures (no drug) are represented by circles and solid lines. For CQS, the drug concentrations used were: 0.1 μM (triangles), 1 μM (squares), and 10 μM (diamonds). The SQ concentrations were: 10 μM (triangles), 100 μM (squares), and 1 mM (diamonds). Brackets indicate standard error of the mean.

Table 4. Effect of CQS on IL-2-dependent CTLL cell proliferation and on IL-2 secretion into supernatant fractions by human PBMNCs

CQS concn	CTLL cells + Rat growth factor			CTLL cells + Supernatant		
	[^{3}H]TdR (cpm)	% Control	N*	[^{3}H]TdR (cpm)	% Control	N
0	14,900 ± 1,600†		9	16,200 ± 3,800		7
100 nM	11,600 ± 1,600	78	7	13,100 ± 2,500	81	7
1 μM	11,000 ± 1,600	74	7	13,500 ± 3,800	83	7
10 μM	9,100 ± 1,200	61	9	8,800 ± 2,800	54	7
100 μM	5,800 ± 1,500	39	9	5,400 ± 2,000	33	7
1 mM	600 ± 330	4	9	70 ± 35	0.4	7

* Number of experiments.
† Mean ± SE.

Table 5. Effect of CQS on viable cell number and *in vitro* immunoglobulin production by human PBMNCs following stimulation by PWM

CQS concn	Viable cells/ml ($\times 10^5$)	IgG (ng/ml)	IgM (ng/ml)
0	8.3 ± 1.4*	3300 ± 560†	3500 ± 1600†
100 nM	6.4 ± 2.1	4600 ± 1600	3700 ± 1400
1 μM	4.0 ± 1.3	2600 ± 2000	1800 ± 1000
10 μM	2.1 ± 0.7	180 ± 70	260 ± 150
100 μM	1.0 ± 0.7	<100	160 ± 160
1 mM	1.4 ± 1.2	120 ± 40	110 ± 110

* Mean ± SE, N = 3.
† Means ± SE for four experiments, each performed in duplicate.

DISCUSSION

These studies indicate that the addition of a single chloro group to sulfaquinoxaline markedly enhances its activity as an inhibitor of lymphocyte proliferation. CQS was approximately two logs more potent than SQ at inhibiting thymidine and deoxyuridine incorporation by PHA-stimulated lymphocytes and IL-2-augmented proliferation of activated T-cells. The inhibitory effects were found to be dose dependent. The inhibition described here was obtained with concentrations of CQS which have been readily attained during pre-clinical pharmacokinetic and toxicologic studies of the drug with relatively little toxicity [2, 3].

The antiproliferative action of CQS appears to block transition from G_1 to S. CQS-treated cells accumulated in the G_0/G_1 compartment when DNA distribution was measured by flow cytometry. Cellular morphology resembled inter-mitotic rather than blastic lymphocytes, and mean cell volume was reduced compared to untreated mitogen-activated cells. Moreover, expression of both IL-2 receptor, an early G_1 event, and transferrin receptor, a late G_1 event, are inhibited by CQS [5]. Thus, this chlorinated sulfonamide has cell cycle specificity in regulating lymphocyte proliferation.

Whether the reduction of IL-2 receptors on PBMNCs by CQS is a secondary phenomenon or accounts, at least in part, for the inhibition of IL-2 driven proliferation is unclear at present. IL-2 receptor density helps determine the onset of cell cycle progression [10]. It has been suggested that a threshold of triggered IL-2 receptors must be reached to initiate DNA replication [10]. CQS, by interacting with or reducing expression of IL-2 receptors, might render the cells less competent to receive the IL-2 "signal" to proceed to S phase.

CQS does not appear to inhibit production of IL-2 by lectin-activated lymphocytes. Supernatant fractions from PHA-stimulated PBMNCs contained measurable amounts of IL-2 when assayed with CTLL cells. This conclusion must be considered tentative because CTLL cells are themselves inhibited by CQS, which confounds interpretation of the assay for IL-2. More precise assessment of the effect of CQS on IL-2 production must await the availability of assays which are not dependent on cell proliferation.

While the elucidation of the mechanism of action of CQS will require further studies, it is clear that the drug inhibits essential basic processes in lymphocytes. Micromolar concentrations of the drug are capable of blocking proliferation and *in vitro* immunoglobulin production. These effects will need to be considered during clinical trials of CQS as a cancer chemotherapeutic agent. The possibility exists that this drug may have efficacy as an immunosuppressive agent.

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