Effects of 3-Chloromethylthiochromone-1,1-dioxide on Nucleic Acid, Protein, and Aerobic and Anaerobic Metabolism of Ehrlich Ascites Tumor Cells

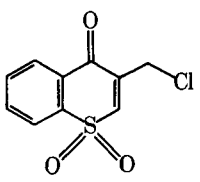
MARK H. HOLSHOUSER *, LARRY J. LOEFFLER, and IRIS H. HALL ˣ

Received April 22, 1981, from the *Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.* Accepted for publication October 6, 1981. *Present address: Division of Medicinal Chemistry, School of Pharmacy, Northeast Louisiana University, Monroe, LA 71209.

Abstract □ 3-Chloromethylthiochromone-1,1-dioxide was observed to be a potent inhibitor of Ehrlich ascites carcinoma growth and a moderate inhibitor of P-388 lymphocytic leukemia growth at 10 mg/kg/day. Preliminary *in vitro* studies showed that the agents significantly inhibited RNA and DNA synthesis in Ehrlich ascites cells. *In vivo* studies after dosing on Days 6, 7, and 8 demonstrated the same reductions in nucleic acid synthesis and a moderate reduction in protein synthesis. The primary site of nucleic acid synthesis, which was blocked by 3-chloromethylthiochromone, was at orotidine monophosphate decarboxylase in the primidine pathway. Other enzymes, in anaerobic and aerobic glycolysis, which were blocked include hexokinase, phosphofructokinase, succinic and α-ketoglutarate dehydrogenases, as well as States 3 and 4 of oxidative phosphorylation.

Keyphrases □ 3-Chloromethylthiochromone-1,1-dioxide—effect on nucleic acid, protein, aerobic and anaerobic metabolism of Ehrlich ascites tumor cells □ Metabolism—aerobic and anaerobic, effects of 3-chloromethylthiochromone-1,1-dioxide on nucleic acid, protein, Ehrlich ascites tumor cells □ RNA—effects of 3-chloromethylthiochromone-1,1-dioxide, Ehrlich ascites tumor cells □ DNA—effects of 3-chloromethylthiochromone-1,1-dioxide, Ehrlich ascites tumor cells

The antitumor activity of compounds containing the 1,4-naphthoquinone ring system has been reported previously. For example, dichloroallylawsone is currently in clinical trials in the United States (1–8). An investigation of sulfone analogs of naphthoquinones was undertaken recently (9), resulting in a series of substituted thiochromones and thiochroman-4-ones and their 1,1-dioxides. The synthesis and physical characteristics were reported previously (9). Certain of these agents were found to be active against the growth of Ehrlich ascites carcinoma and moderately active against P-388 lymphocytic leukemia growth in mice (9). The most potent compound, 3-chloromethylthiochromone-1,1-dioxide (I), afforded 100%



I

Scheme I

inhibition of Ehrlich ascites growth at 10-mg/kg/day dose ip (9). Previously, it has been postulated that certain 1,4-naphthoquinones require bioactivation by reduction of the quinone and subsequent displacement of a leaving group to form the potential alkylating agent (10). However, replacement of one of the naphthoquinone carbonyls with a sulfone group would alter the electronic character as well as the reduction potential of the molecules. The mechanism of action on cellular metabolism of the sulfone analogs may be different from the naphthoquinone; thus, a study of the effects of I on Ehrlich ascites cell metabolism was undertaken and those results are reported.

EXPERIMENTAL

Male CF_1 mice (~30 g) were implanted intraperitoneally on day 0 with 2×10^6 Ehrlich ascites tumor cells. For the anticarcinoma screens, animals were dosed intraperitoneally with compounds from Days 1 to 9. Animals were sacrificed on day 10; the volume of ascites fluid and the packed cell volume (ascitocrit) were determined (11). For the P-388 lymphocytic leukemia screen, BDF_1 male mice (~25 g) were implanted with 10^6 P-388 cells on Day 0. Test compounds were administered from Day 1 to 9 intraperitoneally. The day of death was recorded for both control and treated mice (12).

The metabolic *in vivo* studies were performed after inoculating CF_1 mice (~25 g) with 2×10^6 Ehrlich ascites tumor cells on Day 0. On Days 6, 7, and 8, a 10 mg/kg dose ip of I was administered. On Day 9, the mice were sacrificed and the ascites fluid was removed for *in vivo* biochemical assay. The number of tumor cells per milliliter and the 0.4% trypan blue uptake were determined with a hemocytometer. *In vitro* studies were conducted on Day-9 ascites cells with the drug present at 0.036–0.36 m*M* concentrations.

In vivo incorporation of thymidine into DNA was determined by the method of Chae *et al.* (13). One hour prior to the animal sacrifice on Day 9, 10 μCi of [6-^{3}H]thymidine[1] (24.7 Ci/mmole) was injected intraperitoneally. The DNA was isolated and the tritium content was determined in toluene–octoxynol (2:1), 0.4% of 2,5-diphenyloxazole, and 0.01% of 1,4-bis[2-(5-oxazolyl)]benzene scintillation fluid, and corrected for quenching. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as a standard (13).

In vivo uridine incorporation of Ehrlich ascites cells into RNA was determined using 10 μCi of [5,6-^{3}H]uridine (24 Ci/mmole) injected into the animal 1 hr prior to sacrifice. The method of Wilson *et al.* (14) was used to extract RNA. Using yeast RNA as a standard, the RNA was quantitated by the orcinol reaction (14). *In vivo* leucine incorporation into protein was determined by the method of Sartorelli (15) using 10 μCi of [4,5-^{3}H]leucine (24.7 Ci/mmole) injected into the animal 1 hr prior to sacrifice. Extracted protein was determined by the Lowry (16) procedure using bovine albumin as the standard.

In vitro incorporation of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine[1] was determined using 10^6 Ehrlich ascites day-9 cells, 1 μCi of labeled precursor, 650 μl of minimum essential medium, and 100 ml of drug (0.36 m*M*) (17). The tubes were incubated at 37° for 60 min and inactivated by trichloroacetic acid. The acid insoluble labeled DNA was filtered on glass filter disks[2] and RNA and protein were filtered on nitrocellulose filters[3] by vacuum suction. Results were expressed as disintegrations per minute of incorporated precursor per hour per 10^6 cells.

In vivo nuclear DNA polymerase activity was determined on isolated Ehrlich ascites nuclei (18) after 3 days of dosing. The incubation medium was that of Sawada *et al.* (19) except that [^{3}H]deoxythymidine triphosphate (14 Ci/mmole) was used. The insoluble nucleic acids were collected on glass fiber filters[2] and counted.

In vivo nuclear RNA polymerase activities of Ehrlich ascites cells were determined with isolated nuclei (18). Messenger, ribosomal, and transfer RNA polymerases were isolated by using different concentrations of

[1] All isotopes were purchased from New England Nuclear and substrates and cofactors were purchased from Sigma or Calbiochem.
[2] Whatman GF/F.
[3] HA 0.45 μm, Millipore Corp.

0022-3549/82/0800-0857$01.00/0

Table I—Incorporation of Radiolabeled Precursors into Ehrlich Ascites Cells *In Vitro* During 1-hr Incubation with 3-Chloromethylthiochromone-1,1-dioxide

	Control	Control, %
[^{3}H]Thymidine Incorporation into DNA	100 ± 2[a]	17 ± 2[d]
[^{3}H]Uridine Incorporation into RNA	100 ± 5[b]	22 ± 4[d]
[^{3}H]Leucine Incorporation into Protein	100 ± 9[c]	88 ± 10

[a] 21,052 dpm/mg of DNA. [b] 37,651 dpm/mg of RNA. [c] 18,264 dpm/mg of protein. [d] $p \leq 0.001$.

ammonium sulfate in magnesium chloride (20). The incubation medium was that of Anderson *et al.* (20, 21) using [^{3}H]uridine triphosphate (23.2 Ci/mmole). Insoluble RNA was collected on nitrocellulose filters[3] and counted.

In vivo deoxythymidine and deoxythymidylate monophosphate and diphosphate kinase activities of Ehrlich ascites cells were measured spectrophotometrically at 340 nm for 20 min using 0.1 μmole of reduced nicotinamide adenine dinucleotide (22).

In vivo carbamyl phosphate synthetase activity of Ehrlich ascites cells was determined, using the reaction medium of Kalman *et al.* (23), in the presence of ornithine and ornithine transcarbamylase. Citrulline, formed from ornithine, was measured at 490 nm by the method of Archibald (24). *In vivo* aspartate transcarbamylase activity was assayed using the incubation medium of Kalman *et al.* (23). Colorimetric determination of carbamyl aspartate was carried out by the procedure of Koritz and Cohen (25). *In vivo* orotidine monophosphate decarboxylase activity was assayed by the method of Appel (26) using 0.1 μCi of [^{14}C]orotidine monophosphate (34.9 mCi/mmole). The [^{14}C]carbon dioxide generated in 15 min was trapped in potassium hydroxide[4] and counted. *In vitro* studies of orotidine monophosphate decarboxylase activity were conducted over the range of 0.036–0.36 mmole concentration of drug for 15 min. *In vivo* thymidylate synthetase activity of Ehrlich ascites cells was determined using a postmitochondrial (9000×*g* for 10 min) supernate and 5 μCi of [5-^{3}H]deoxyuridine monophosphate (11 Ci/mmole) according to the method of Kampf *et al.* (27). [^{14}C]Formate incorporation into purine of Ehrlich ascites cells was determined by the method of Spassova *et al.* (28) using 0.5 μCi of [^{14}C]formic acid (4.95 mCi/mmole). Purine was separated on silica gel TLC plates eluted with *n*-butanol–acetic acid–water (4:1:5). After identifying R_f values consistent with the standards, adenine and guanine, the plates were scraped and the radioactive content determined. Phosphoribosyl-1-pyrophosphate amidotransferase activity of Ehrlich ascites cells was determined on a supernate at 600×*g* for 10 min, measuring the reduction of 0.6 μmole of oxidized nicotinamide adenine dinucleotide at 340 nm for 30 min (29).

In vivo inosinic acid dehydrogenase activity of Ehrlich ascites cells was determined spectrophotometrically at 340 nm for 30 min using a supernatant fraction (600×*g* for 10 min). The assay medium was that of Magasanik (30) which contained nicotinamide adenine dinucleotide. *In vivo* dihydrofolate reductase activity of Ehrlich ascites cells was determined at 340 nm for 30 min as the oxidation of reduced nicotinamide adenine dinucleotide phosphate (31).

In vivo ribonucleotide reductase activity of Ehrlich ascites cells was determined by the method of Moore and Hurlbert (32) using [5-^{3}H]cytidine-5′-diphosphate (25 Ci/mmole). Ribose and deoxyribose nucleotides were separated on polyethyleneiminecellulose F plastic precoated TLC plates eluted with 4% boric acid–4 *M* LiCl (4:3) and scraped at the R_f values consistent with the standard deoxycytidine diphosphate.

In vivo phosphorylation of histones of Ehrlich ascites cells was determined by injecting 10 μCi of [γ-^{32}P]ATP (18.8 Ci/mmole) into mice 1 hr prior to sacrifice. The nuclei were isolated (17) and the histone chromatin proteins were extracted by the method of Raineri *et al.* (33). *In vivo* nonhistone protein phosphorylation dependent on nuclear protein kinase was determined using 2 nmoles of [γ-^{32}P]adenosine triphosphate (27 Ci/mmole) and isolated nuclei chromatin protein was collected on nitrocellulose filters (34). *In vivo* cyclic 3′,5′-adenosine monophosphate levels of Ehrlich ascites cells were determined by the radioimmunoassay method of Steiner *et al.* (35) using a commercial kit[5].

In vivo deoxyribonuclease (36), ribonuclease, and cathepsin (37) lysosomal enzymatic activities of Ehrlich ascites cells were determined using DNA, RNA, and azocasein as substrates, respectively. Bound acid hydrolytic enzymes were released using the detergent, octoxynol-100[6], pretreatment. Both free and total enzyme activities were observed by measuring nucleotides and aromatic peptide fragments which were determined at 260 and 280 nm, respectively. UV binding studies (38) were conducted *in vitro* with drug (0.36 m*M*) and DNA (35 μg/ml) in phosphate buffer, pH 7.2, over the 200–340 nm range for 24 hr at room temperature.

Table II—*In Vivo* Effects of 3-Chloromethylthiochromone-1,1-dioxide on Nucleic Acid and Protein Metabolism of Ehrlich Ascites Cells[a]

Parameter	Control, % Control	Control, % Treated
[^{3}H]Thymidine incorporation into DNA	100 ± 16	23 ± 15[b]
[^{3}H]Uridine incorporation into RNA	100 ± 9	23 ± 5[b]
[^{3}H]Leucine incorporation into protein	100 ± 13	50 ± 5[b]
Number of cells per milliliter of ascites fluid	100 ± 9	28 ± 8[b]
DNA polymerase	100 ± 10	94 ± 14
Messenger RNA polymerase	100 ± 13	93 ± 10
Ribosomal RNA polymerase	100 ± 6	91 ± 22
Transfer RNA polymerase	100 ± 14	125 ± 17
Thymidine kinase	100 ± 3	94 ± 10
Thymidylate monophosphate kinase	100 ± 8	103 ± 6
Thymidylate diphosphate kinase	100 ± 12	84 ± 5
Carbamyl phosphate synthetase	100 ± 7	145 ± 15[b]
Aspartate transcarbamylase	100 ± 9	116 ± 5
Orotidine monophosphate decarboxylase	100 ± 9	25 ± 4[b]
Thymidylate synthetase	100 ± 10	104 ± 11
[^{14}C]Formate incorporation into purine	100 ± 9	91 ± 7
Phosphoribosyl pyrophosphate amidotransferase	100 ± 8	93 ± 7
Inosinic acid dehydrogenase	100 ± 16	137 ± 6
Dihydrofolate reductase	100 ± 17	119 ± 33
Ribonucleotide reductase	100 ± 12	99 ± 10
Phosphorylation of histone proteins	100 ± 18	82 ± 3
Phosphorylation of nonhistone proteins	100 ± 10	183 ± 15[b]
Cyclic adenosine monophosphate levels	100 ± 6	148 ± 8[b]
Deoxyribonuclease	100 ± 11	118 ± 3
Ribonuclease	100 ± 4	109 ± 3
Cathepsin	100 ± 19	97± 13

[a] $n = 6$. [b] $p \leq 0.001$.

An *in vitro* method (39) was used to determine if the drug was an initiation or an elongation protein synthesis inhibitor of Ehrlich ascites cells by comparison with the standards, pyrocatechol violet and emetine, using 1 μCi of [^{3}H]leucine (24.7 Ci/mmole). The reaction medium was spotted on filter paper disks[7], which were treated for 10 min in boiling 5% trichloroacetic, for 10 min in cold 5% trichloroacetic; and washed with cold 5% trichloroacetic acid, ether–ethanol (1:1) ether; and counted (32).

In vitro and *in vivo* oxidative phosphorylation studies (40) were conducted on Ehrlich ascites cells and the liver homogenates[8] of tumor-bearing mice with the substrates, α-ketoglutarate or succinate. Basal oxygen consumption (State 4) was determined with an oxygen electrode connected to an oxygraph. Adenosine diphosphate was then added to obtain State 3 or stimulated respiration. The number of microlites of oxygen per hour per milligram of protein for States 3 and 4 were calculated. The following glycolytic and Krebs cycle enzymatic activities were determined spectrophotometrically: hexokinase (41), phosphofructokinase (42), lactic dehydrogenase (43), succinic dehydrogenase (44), α-ketoglutarate dehydrogenase, and malic dehydrogenase (45). To be consistent in the expression of data for the spectral enzyme studies, a unit of enzyme activity is defined as that quantity of activity giving a change in absorbance of 0.001/min under the condition of the assay. Protein for the enzymatic assay was determined by the Lowry technique (16).

Probable (p) significant differences were determined by Student's t test. Data are expressed in Tables I–VI as percent of the control with standard deviation (n equals the number of animals per group).

RESULTS

Compound I was shown to have 100% inhibition of Ehrlich ascites tumor growth at a 10 mg/kg ip dose. Doses of 20 and 30 mg/kg were found to be less active, *i.e.*, 25 and 18% inhibition, respectively. In the P-388 lymphocytic leukemia screen, I at 10 mg/kg/day intraperitoneally was marginally active at T/C% = 130 (Table VII). After dosing with the agent on days 6, 7, and 8, the cell count per milliliter of Ehrlich ascites fluid was reduced from 226 × 10^6 to 63 × 10^6. The *in vitro* incorporation studies

[4] Hyamine Hydroxide, New England Nuclear.
[5] Becton Dickinson radioimmunoassay kit, Iodine 125.
[6] Research Products International Corp.
[7] Whatman number 3.
[8] Homogenates (10%) in 0.25 *M* sucrose + 0.001 *M* ethylenediaminetetraacetic acid.

Table III—The *In Vitro* Effects of 3-Chloromethylthiochromone-1,1-dioxide on Orotidine Monophosphate Decarboxylase Activity in Ehrlich Ascites Cells

Control Treated *in vitro* concentration, m*M*	Control, %
	100 ± 8[a]
.036	86 ± 7
.090	84 ± 5
.180	68 ± 7
.360	44 ± 6

[a] $p \leq 0.001$.

(Table I) demonstrated, using 10^6 cells collected from 9-day Ehrlich ascites animals, that thymidine incorporation into DNA was reduced 83%, and uridine incorporation into RNA was reduced 78%. The *in vivo* thymidine incorporation into DNA for the control was 95,970 dpm/mg, which was reduced 77% by drug administration for 3 days. *In vivo* uridine incorporation into RNA for the untreated animals was 198,200 dpm/mg of RNA, which was reduced 77% by drug therapy. *In vivo* leucine incorporation into protein for the control was 37,946 dpm/mg of protein, which was reduced 50% in the treated animals.

The DNA and RNA polymerase activities were not altered by drug administration. The control values were 45,734 dpm/mg of nuclear protein for DNA polymerase, 6434 dpm/mg for mRNA polymerase, 7340 dpm/mg for rRNA polymerase, and 8254 dpm/mg of nuclear protein for tRNA polymerase. The activity for thymidine kinase was 8.90 enzyme units/mg of protein, thymidine monophosphate kinase was 8.65 enzyme units/mg of protein, and thymidine diphosphate kinase activity was 5.60 enzyme units/mg of protein. Drug treatment did not alter the activities of the kinases except for the diphosphate which was inhibited by 16%. Carbamyl phosphate synthetase activity for the control resulted in the formation of 0.117 mg of citrulline formed/hr/mg of protein, and aspartate transcarbamylase activity resulted in 0.81 mg of carbamyl aspartate formed per hour per milligram of protein. Drug administration resulted in an increase of 45% in carbamyl phosphate synthetase and 16% in aspartate transcarbamylase activities.

In vivo orotidylic monophosphate decarboxylase activity for the control was 26,298 dpm of [^{14}C]carbon dioxide produced/hr/mg of protein, which was reduced 75% by 3 days of drug treatment. *In vitro* orotidylic monophosphate decarboxylase activity was reduced significantly: 56%, by 0.36 m*M* concentration of drug (Table V). *In vivo* thymidylate synthetase activity for normal animals was 103,308 dpm of [^{3}H]thymidine formed hr/mg of protein, which was unchanged by drug therapy. *In vivo* [^{14}C]-formate incorporation into purines for the control was 10,788 dpm of purines/hr/mg of protein. *In vivo* phosphoribosyl pyrophosphate amidotransferase activity for normal cells was 376 enzyme units/mg of protein. *In vivo* inosinic monophosphate dehydrogenase activity resulted in 2.06 enzyme units/mg of protein. Drug treatment resulted in no change in amidotransferase activity but caused a 37% increase in the dehydrogenase activity.

In vivo dihydrofolate reductase activity was 8.56 enzyme units/mg of protein, which was increased 19% by drug treatment. *In vivo* ribonucleotide reductase activity was 155,065 dpm of [^{3}H]deoxycytidine diphosphate formed/hr/mg of protein. This was unaffected by drug treatment. *In vivo* histone phosphorylation for the control was 2954 dpm of [γ-^{32}P]incorporated into histones/hr/mg of nuclear protein, inhibited 18% by drug administration. Nonhistone phosphorylation resulted in 9807 dpm of [γ-^{32}P]incorporated into protein/hr/mg of chromatin protein, elevated 83% by drug therapy. *In vivo* cyclic adenosine monophosphate levels for the control were 3.6 pmole/10^6 cells elevated by 48% after drug administration.

The UV studies with I and DNA demonstrated an elevation of UV peaks at 222 and 240 nm, and a hyperchromic shift at 260 nm, indicating possible binding between the drug and nucleic acids. The effects on glycolytic and Krebs cycle enzymatic activities of I were dramatic. Hexokinase activity for untreated 9-day Ehrlich ascites cells was 195 enzyme units/mg of protein, which was inhibited 84% by drug administration (Table IV). Phosphofructokinase activity was determined for the control as 176 enzyme units/mg of protein, which was reduced 54% by the drug. Malic dehydrogenase activity for the control was 80 enzyme units/mg of protein and lactic dehydrogenase activity was 623 enzyme units/mg of protein. Treatment with I had little effect on these two dehydrogenase activities, causing 14 and 9% inhibition, respectively. Succinic dehydrogenase activity for the control was 69 enzyme units/mg of protein which was reduced 52% by drug treatment. α-Ketoglutarate dehydrogenase activity was measured as 1.68 enzyme units/mg of protein for the control cells, which was reduced 76% by drug therapy for 3 days.

Table IV—*In Vivo* Effects of 3-Chloromethylthiochromone-1,1-dioxide on Glycolytic and Krebs Cycle Enzyme Activities of Ehrlich Ascites Cells of CF_1 Mice [a]

Parameter	Control	Treated
Hexokinase	100 ± 9	14 ± 1[b]
Phosphofructokinase	100 ± 3	46 ± 3[b]
Lactic dehydrogenase	100 ± 9	91 ± 20
Malic dehydrogenase	100 ± 20	86 ± 19
Succinic dehydrogenase	100 ± 16	48 ± 3[b]
α-Ketoglutarate dehydrogenase	100 ± 11	24 ± 7[b]

[a] $n = 6$. [b] $p \leq 0.001$.

Table V—*In Vitro* Effects of 3-Chloromethylthiochromone-1,1-dioxide on Oxidative Phosphorylation Processes of 9-Day Ehrlich Ascites Cells [a]

Parameter	Control, % Control	Control, % Treated
Succinate —state 4	100 ± 7[b]	70 ± 7[f]
—state 3	100 ± 4[c]	59 ± 5[f]
α-Ketoglutarate—state 4	100 ± 5[d]	57 ± 10[f]
—state 3	100 ± 11[e]	49 ± 7[f]

[a] $n = 6$. [b] 5.27 μl O_2 consumed/hr/mg protein. [c] 8.73 μl O_2 consumed/hr/mg protein. [d] 3.57 μl O_2 consumed/hr/mg protein. [e] 5.16 μl O_2 consumed/hr/mg protein. [f] $p \leq 0.001$.

Both basal and adenosine diphosphate-stimulated respiration rates were reduced 75% by drug therapy *in vivo*, whereas *in vitro* studies at 0.36 m*M* concentration of drug demonstrated 30 and 41% inhibition, respectively, using succinate as substrate (Tables V and VI). The basal- and adenosine diphosphate-stimulated respiration rates using α-ketoglutarate as substrate were inhibited 82 and 86%, respectively. In the *in vitro* studies with α-ketoglutarate as substrate, I demonstrated 43 and 51% inhibition for States 4 and 3, respectively. The *in vivo* effects of the drug on liver respiration rates of tumor-bearing mice were not as severe as those observed in the tumor cells, and only minimal changes were observed (Table VI).

Figure 1 demonstrates that I, when incubated with endogenous mRNA and essential amino acids, follows a pattern of inhibition of protein synthesis, which resembles the initiation inhibitor, pyrocatechol violet, as opposed to the elongation inhibitor, emetine. The degree of inhibition of protein synthesis was relatively moderate.

DISCUSSION

A series of potential bioreductive alkylating agents have been synthesized (7, 8), specifically α-substituted naphthoquinones, quinoline-5,8-quinones, and naphthazarines which contain halomethyl, acetoxymethyl, carbamoylmethyl, and carbophenoxymethyl groups alpha to a carbonyl group as the alkylating moieties. A number of these agents were observed to be active in the sarcoma 180 and adenocarcinoma 755 ascites screens. A rough correlation between antitumor activity in the sarcoma 180 screen and polarographic reduction potential ($E_{1/2}$) of the quinone moiety was demonstrated. Those compounds that possessed the most negative half-wave reduction potential, *i.e.*, those compounds that were difficult to reduce, demonstrated the highest activity against sarcoma 180 tumor growth. Lin *et al.* (7, 8) suggested that those quinone deriva-

Table VI—*In Vivo* Effects of 3-Chloromethylthiochromone-1,1-dioxide on Oxidative Phosphorylation of CF_1 Mice [a]

Parameter	Control, % Control	Control, % Treated
Ehrlich Ascites Cells		
Succinate—state 4	100 ± 3	15 ± 3[b]
—state 3	100 ± 4	15 ± 2[b]
α-Ketoglutarate—state 4	100 ± 5	18 ± 7[b]
—state 3	100 ● 11	14 ± 2[b]
Liver from Tumor-Bearing Mice		
Succinate—state 4	100 ± 14	99 ± 9
—state 3	100 ± 6	86 ± 13
α-Ketoglutarate—state 4	100 ± 8	104 ± 17
—state 3	100 ± 12	120 ± 10[c]

[a] $n = 6$. [b] $p \leq 0.001$. [c] $p \leq 0.005$.

Table VII—The Antineoplastic Activity of 3-Chloromethylthiochromone-1,1-dioxide in the Ehrlich Ascites and P-388 Lymphocytic Leukemia Screens

Compound	Ehrlich Ascites Tumor Screen (CF_1 Male Mice) Dose, mg/kg	Survival After 9 Days	Ascrit	Ascites Volume	Inhibition, %[a]
Control (0.05% polysorbate 80–H_2O)	—	8/8	33.6 ± 8.7	1.8 ± 1.02	—
3-Chloromethylthiochromone-1,1-dioxide	5	8/8	22.0	2.45	57.1
	10	8/8	0.0	0.0	100.0
	20	8/8	35.7	0.88	75.1
	30	8/8	11.3	2.02	81.8
Mercaptopurine (standard)		6/6	3.0	0.1	99

Compound[b]	P-388 Lymphocytic Leukemia (BDF_1 Male Mice) Dose, mg/kg	Average Days Survived	T/C %[c]
Control (0.05% polysorbate-80/H_2O)	—	9.5	100
3-Chloromethylthiochromone-1,1-dioxide	10	12.4	130
	15	9.8	103
	30	2.9	toxic
Fluorouracil (standard)	25	17.6	186

[a] Greater than 80% inhibition is required for significant activity. [b] $n = 6$. [c] T/C % > 125 is required for significant activity.

tives, when reduced, suffered loss of a leaving group to form a quinone methide, a potential alkylating agent which could alkylate bionucleophiles. It was shown that such an active species could be trapped *in vitro* by an amine function or be allowed to dimerize.

The 3-substituted thiochromone-1,1-dioxides were synthesized as novel analogs of naphthoquinones. Isosteric replacement of one carbonyl with a sulfone group alters the electronic character and ease of reduction. However, preliminary experiments indicated that no apparent correlation existed between half-wave reduction potential and antineoplastic activity in the Ehrlich ascites screen. The thiochromone-1,1-dioxides may be acting as direct alkylating agents *via* a Michael-type addition or a carbamoylation reaction with some biological nucleophile or a direct displacement of the leaving group. For example, high reactivity of I with a variety of alcohols and amines would support this view.

In the naphthoquinone class, lapachol and dichloroallylawsone are the most potent antineoplastic agents (5). Lapachol is currently marketed in Brazil and dichloroallylawsone[9] [2-(3,3-dichloroallyl)-3-hydroxy-1-4-naphthoquinone] is in clinical trials in the United States (15). Bennett *et al.* (46) observed, in cultured L-1210 lymphoid leukemia cells, that dichloroallylawsone interfered with L-1210 cell respiration and inhibited pyrimidine biosynthesis at the dihydro-orotate dehydrogenase step. Thus, it is interesting to observe that the major effect of I is on basal (State 4) and adenosine diphosphate-stimulated (State 3) respiration in Ehrlich ascites cells. Drastic reduction in respiration of Ehrlich ascites cells was observed with succinate as substrate, which requires a flavin adenine nucleotide linked dehydrogenase, as well as α-ketoglutarate, which requires a nicotinamide adenine dinucleotide linked dehydrogenase, both *in vivo* and *in vitro*.

No effects were demonstrated on the respiration of livers of tumor-bearing mice, indicating that the drug has some tissue specificity. However, when the Krebs cycle dehydrogenase activities of Ehrlich ascites cells were measured, it was observed that both succinic dehydrogenase and α-ketoglutarate dehydrogenase activities were inhibited by this agent. Furthermore, activities of the regulatory enzymes of anaerobic glycolysis, hexokinase and phosphofructokinase, were also reduced significantly by I. These studies would indicate that after drug treatment, there existed a reduction in energy available from mitochondrial oxidative phosphorylation, as well as glycolytic substrate phosphorylation, for the synthetic process of rapidly proliferating tumor cells.

Initial studies indicated that I was a moderate inhibitor of protein

[9] NSC 126771.

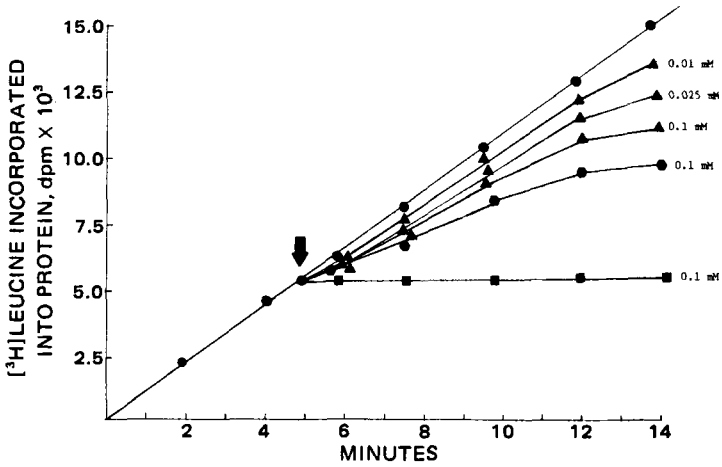


Figure 1—*Effect of 3-chloromethylthiochromone-1,1-dioxide on the initiation and elongation of protein molecular synthesis. Key: (●), control; (▲), drug, 0.01 mM, 0.025 mM, 0.1 mM; (●), pyrocatechol violet, 0.1 mM; (■), emetine, 0.1 mM; ↓, addition of drug.*

synthesis, probably blocking the initiation process of translation. Moderate elevation of cyclic adenosine monophosphate levels of the Ehrlich ascites cells was also observed after drug therapy. Elevated levels are linked with reversal of tumor morphology to a more normal state, reduction of cell proliferation, and enhanced cellular differentiation (47–49).

The inhibition of DNA and RNA synthesis appears to be caused by a lack of pyrimidine due to the fact that I blocks orotidine monophosphate decarboxylase activity, depleting uridylate monophosphate for the conversion to thymidylate monophosphate and cytidylate monophosphate. The decarboxylase enzyme was inhibited both *in vivo* and *in vitro* and is the same site of pyrimidine synthesis inhibition, which has been implicated for the standard clinical antineoplastic agent, azauridine-5′-phosphate (50).

Thus, this sulfone analog of 1,4-naphthoquinones has similar effects on tumor cell metabolism, *i.e.*, inhibition of pyrimidine synthesis and oxidative phosphorylation, as those reported for 1,4-naphthoquinones (46) despite the fact that the sulfone analog does not have the same electronic characteristics or reduction capability as the 1,4-naphthoquinones. Compound I does not suppress the oxidative phosphorylation process exclusively, but its effects are extended to the actual dehydrogenase and kinase enzymes which regulate anaerobic and aerobic glycolysis, indicating that the agent is not simply blocking the electron transport system by acting as an electron acceptor as would be expected for a quinone.

REFERENCES

(1) J. S. Driscoll, G. F. Hazard, Jr., H. B. Wood, Jr., and A. Goldin, *Cancer Chemother. Rep.*, **4**, 1 (1974).

(2) U. Sankawa, Y. Ebizuka, T. Miyazaki, Y. Isomura, H. Otsuka, S. Shubata, M. Inomata, and F. Fukuoka, *Chem. Pharm. Bull.*, **25**, 2392 (1977).

(3) T. H. Porter, G. S. Tsai, T. Kishi, H. Kishi, and K. Folkers, *Acta Pharm. Suec.*, **15**, 97 (1978).

(4) S. M. Tiffany, D. G. Graham, F. S. Vogel, M. W. Cass, and P. W. Jeffs, *Cancer Res.*, **38**, 3230 (1978).

(5) M. da Consolacao, F. Linardi, M. M. de Oliveria, and M. R. P. Sampaio, *J. Med. Chem.*, **18**, 1159 (1975).

(6) E. M. McKelvey, M. Lomedico, K. Lu, M. Chadwick, and T. L. Loo, *Clin. Pharmacol. Ther.*, **25**, 586 (1979).

(7) A. J. Lin, R. S. Pardini, L. A. Cosby, B. J. Lillis, C. W. Shansky, and A. C. Sartorelli, *J. Med. Chem.*, **16**, 1268 (1973).

(8) A. J. Lin, B. J. Lillis, and A. C. Sartorelli, *ibid.*, **18**, 917 (1975).

(9) M. H. Holshouser, L. J. Loeffler, and I. H. Hall, *ibid.*, **24**, 853 (1981).

(10) A. J. Lin and A. C. Sartorelli, *J. Org. Chem.*, **38**, 813 (1973).

(11) C. Piantadosi, C. S. Kim, and J. L. Irvin, *J. Pharm. Sci.*, **58**, 921 (1969).

(12) R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. Schumacher, and B. Abbott, *Cancer Chemother. Rep., Part 3*, (2)**3**, 9 (1972).

(13) C. B. Chae, A. Williams, H. Krasny, J. L. Irvin, and C. Piantadosi,

Cancer Res., **30**, 2652 (1970).
(14) R. G. Wilson, R. H. Bodner, and G. E. MacHorter, *Biochim. Biophys. Acta*, **378**, 260 (1975).
(15) A. C. Sartorelli, *Biochem. Biophys. Res. Commun.*, **27**, 26 (1967).
(16) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(17) I. H. Hall, K. H. Lee, S. A. ElGebaly, Y. Imakura, and R. Y. Wu, *J. Pharm. Sci.*, **68**, 883 (1979).
(18) W. C. Hymer and E. L. Kuff, *J. Histochem. Cytochem.*, **12**, 359 (1964).
(19) H. Sawada, K. Tatsumi, M. Sasada, S. Shirakawa, T. Nakumura, and G. Wakisaka, *Cancer Res.*, **34**, 3341 (1974).
(20) I. H. Hall, G. L. Carlson, G. S. Abernethy, and C. Piantadosi, *J. Med. Chem.*, **17**, 1253 (1974).
(21) K. M. Anderson, I. S. Mendelson, and G. Guzik, *Biochim. Biophys. Acta*, **383**, 56 (1975).
(22) F. Maley and S. Ochoa, *J. Biol. Chem.*, **233**, 1538 (1958).
(23) S. M. Kalman, P. H. Duffield, and T. Brzozouski, *J. Biol. Chem.*, **241**, 1871 (1966).
(24) R. M. Archibald, *J. Biol. Chem.*, **156**, 121 (1944).
(25) S. B. Koritz and P. P. Cohen, *ibid.*, **209**, 145 (1954).
(26) S. H. Appel, *ibid.*, **243**, 3929 (1968).
(27) A. Kampf, R. L. Barfknecht, P. J. Schaffer, S. Osaki, and M. P. Mertes, *J. Med. Chem.*, **19**, 903 (1976).
(28) M. K. Spassova, G. C. Russell, and E. V. Golovinsky, *Biochem. Pharmacol.*, **25**, 923 (1976).
(29) J. B. Wyngaarden and D. M. Ashton, *J. Biol. Chem.*, **234**, 1492 (1959).
(30) B. Magasanik, *Methods Enzymol.*, **6**, 106 (1963).
(31) M. K. Ho, T. Hakalo, and S. F. Zakrzewski, *Cancer Res.*, **32**, 1023 (1972).
(32) E. G. Moore and R. B. Hurlbert, *J. Biol. Chem.*, **241**, 4802 (1966).
(33) A. Raineri, R. C. Simsiman, and R. K. Boutwell, *Cancer Res.*, **33**, 134 (1973).
(34) Y. M. Kish and L. J. Kleinsmith, *Methods Enzymol.*, **40**, 201 (1975).
(35) A. L. Steiner, D. M. Kipnis, R. Utiger, and C. Parker, *Proc. Natl. Acad. Sci., USA*, **64**, 367 (1969).
(36) I. H. Hall, K. S. Ishaq, and C. Piantadosi, *J. Pharm. Sci.*, **63**, 625 (1974).
(37) Y. S. Cho-Chung and P. M. Gullino, *J. Biol. Chem.*, **248**, 4743 (1973).
(38) I. H. Hall, K. H. Lee, E. C. Mar, and C. O. Starnes, *J. Med. Chem.*, **20**, 333 (1977).
(39) L. L. Liao, S. M. Kupchan, and S. B. Horwitz, *Mol. Pharmacol.*, **12**, 167 (1976).
(40) I. H. Hall, K. H. Lee, C. O. Starnes, S. A. ElGebaly, T. Ibuka, Y. S. Wu, T. Kimura, and M. Haruna, *J. Pharm. Sci.*, **67**, 1235 (1978).
(41) D. G. Walker and J. J. Parry, *Methods Enzymol.*, **9**, 381 (1966).
(42) T. E. Masour, *ibid.*, **9**, 430 (1966).
(43) M. K. Schwartz and O. Bodansky, *ibid.*, **9**, 294 (1966).
(44) D. V. DerVartanian and C. Veerger, *Biochim. Biophys. Acta*, **92**, 233 (1964).
(45) S. Ochoa, A. H. Mehler, and A. Kornberg, *J. Biol. Chem.*, **174**, 979 (1948).
(46) L. L. Bennett, D. Smithers, L. M. Rose, D. J. Adamson, and H. J. Thomas, *Cancer Res.*, **39**, 4868 (1979).
(47) V. Stefanovich, *Res. Commun. Chem. Pathol. Pharmacol.*, **7**, 573 (1974).
(48) T. Posternak, *Annu. Rev. Pharmacol.*, **14**, 23 (1974).
(49) Y. S. Cho-Chung and P. M. Gullino, *Science*, **183**, 87 (1974).
(50) W. B. Pratt and R. W. Ruddon, "The Anticancer Drugs," Oxford University Press, New York, N.Y., 1979, p. 124.

An *In Vitro* Model for the Study of Antibacterial Dosage Regimen Design

ROGER D. TOOTHAKER *§, **PETER G. WELLING** *x, and **WILLIAM A. CRAIG** ‡

Received August 7, 1981, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706 and the ‡Veterans Administration Hospital, Madison, WI 53705. Accepted for publication November 16, 1981. §Present address: School of Pharmacy, University of Washington, Seattle, WA 98195.

Abstract □ A model was developed that is capable of simulating antibacterial agent concentration *versus* time profiles commonly observed following intravenous and intramuscular bolus injections, intravenous infusions, and oral doses, administered as single or multiple doses. The model consisted of two physical compartments separated by a membrane of a commercial hemodialyzer. The 1.08 m^2 membrane surface area allowed rapid transmembrane passage of drugs and other small molecules, while membrane pore size prevented bacterial passage. These characteristics allowed bacteria in one of the two compartments of the model to be exposed to time-variant drug concentrations without affecting the number or concentration of bacteria. The model was used to study the effects of a multiple intravenous bolus dosage regimen of ampicillin on *Escherichia coli* ATCC 12407.

Keyphrases □ Penicillin—*in vitro* model for the study of antibacterial dosage regimen design □ Models—*in vitro*, study of antibacterial dosage regimen design □ Antibacterials—*in vitro* model, study of dosage regimen design

The antibacterial agent concentration profiles to which bacteria are exposed *in vivo* vary with the method of drug administration. Continuous intravenous infusion yields constant plasma and tissue drug concentrations once steady state is achieved, while the short elimination half-life of most antimicrobial agents results in rapid decreases in plasma and interstitial fluid drug concentrations (1) following bolus intravenous, intramuscular, or oral doses.

Considerable progress has been made during the past 15 years in determining the mechanism of action of β-lactam antibiotics (2, 3). However, the relative therapeutic effectiveness of intermittent and continuous dosage regimens for these compounds is uncertain (4, 5). Attempted correlations of therapeutic effectiveness with various pharmacokinetic parameters, such as maximum plasma concentration (6), the time period during which drug levels exceed the minimum inhibitory concentration (7), area under the plasma level curve (8), intensity factor (9), and the degree of serum protein binding (10), have been difficult due to the many interactions between the drug, bacteria, infection site, and host. Information ob-

0022-3549/82/0800-0861$01.00/0