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The anti-neoplastic activity of 2,3-dihydrophthalazine-1,4-dione and *N*-butyl-2,3-dihydrophthalazine-1,4-dione in human and murine tumor cells

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2,3-Dihydrophthalazine-1,4-dione derivatives demonstrated potent cytotoxicity against the growth of murine leukemia cells and human single cell suspension, i.e. $Tmolt_3$ leukemia and HeLa-S^3, as well as colon adenocarcinoma and KB nasopharynx. However, only select compounds demonstrated activity against bronchogenic lung, osteosarcoma and glioma growth. 2,3-Dihydrophthalazine-1,4-dione was active *in vivo* against L1210 leukemia, Lewis lung and Ehrlich ascites carcinoma growth. In L1210 cells the agents inhibited both DNA and RNA synthesis, and a few of the compounds were capable of inhibiting protein synthesis at 3 times their ED_{50} values. When 2,3-dihydrophthalazine-1,4-dione and *N*-butyl-2,3-dihydrophthalazine-1,4-dione were examined for their mode of action in the L1210 lymphoid leukemia cells, the sites of inhibition by the agents appear to be the *de novo* purine pathway at the enzymes IMP dehydrogenase and PRPP amido transferase. IMP dehydrogenase activity was inhibited at least 45% by 45 min at 100 μM concentration of drugs whereas the remaining enzymes that were affected by the drugs were not inhibited as early. Secondary sites were dihydrofolate reductase and thymidylate synthetase. The d(NTP) levels were also reduced specifically dATP and dCTP levels.

Key words: Anti-neoplastic activity, 2,3-dihydrophthalazine-1,4-dione, *N*-butyl-2,3-dihydrophthalazine-1,4-dione.

Introduction

2,3-Dihydrophthalazine-1,4-diones have been reported to have therapeutic activity, e.g. hypolipidemic, lowering both serum cholesterol and triglyceride levels and elevating HDL-cholesterol levels in rats.[1-5] We have noted a strong correlation between those agents which have hypolipidemic activity and anti-neoplastic activity.[6,7] Mevinolin (lovastatin) and compactin, which are potent HMG CoA reductase inhibitors,[8-15] have been shown to also inhibit DNA synthesis in cultured cells.[10,14] Sesquiterpene lactones,[6] boron derivatives of purine and pyrimidines, heterocyclic amines and trimethylamine carboxyborones,[7] all have demonstrated similar cross-over pharmacological activities. Since the basic structure of 2,3-dihydrophthalazine-1,4-dione is roughly the same size as a purine, we initiated studies to determine if these agents have cytotoxicity against the growth of murine and human cultural tumor cells.

Materials and methods

Source of compounds

2,3-Dihydrophthalazine-1,4-dione derivatives (Figure 1) were synthesized as previously outlined in the literature.[16] The physical and chemical characteristics were identical to those previously reported. 2,3-Dihydrophthalazine-1,4-dione was purchased from Aldrich Chemical Co. All isotopes were purchased from New England Nuclear, Dupont (Boston, MA) unless otherwise noted. Radioactivity was determined in Fisher Scintiverse fluid using a Packard scintillation beta counter corrected for quenching. Substrates and cofactors were purchased from Sigma Chemical Co. (St Louis, MO).

Cytotoxic activity

Compounds **1–28** were tested for cytotoxic activity by preparing a 1mM solution of the drug in 0.05%

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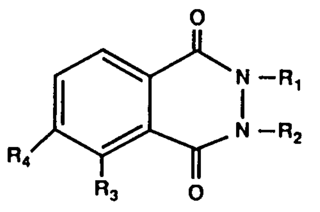
Figure 1 Structure of 2,3.dihydrophthalazine-1,4-dione.



Compound	R_1	R_2	R_3	R_4
1	–H	–H	–H	–H
2	$-CH_3$	–H	–H	–H
3	$-CH_3$	$-CH_3$	–H	–H
4	$-C_2H_5$	–H	–H	–H
5	$-C_2H_5$	$-C_2H_5$	–H	–H
6	$-nC_3H_7$	–H	–H	–H
7	$-nC_3H_7$	$-nC_3H_7$	–H	–H
8	$-nC_4H_9$	–H	–H	–H
9	$-nC_4H_9$	nC_4H_9	–H	–H
10	$-nC_5H_{11}$	–H	–H	–H
11	$-nC_5H_{11}$	nC_5H_{11}	–H	–H
12	–H	–H	–Cl	–H
13	–H	–H	–H	–Cl
14	–H	–H	$-CH_3$	–H
15	–H	–H	–H	$-CH_3$
16	–H	–H	$-OCH_3$	–H
17	–H	–H	–H	$-OCH_3$
18	$-CH_2CH_2COOH$	–H	–H	–H
19	$-CH_2COOC_2H_5$	–H	–H	–H
20	$-CH_2CH_2COCH_3$	–H	–H	–H
21	$-C_6H_5$	–H	–H	–H
22	$-oClC_6H_4$	–H	–H	–H
23	$-mClC_6H_4$	–H	–H	–H
24	$-pClC_6H_4$	–H	–H	–H
25	$-oCH_3C_6H_4$	–H	–H	–H
26	$-mCH_3C_6H_4$	–H	–H	–H
27	$-pCH_3C_6H_4$	–H	–H	–H

Tween $80/H_2O$ by homogenization. The drug solutions were sterilized by passing them through a 45 μM acrodisc. The following cell lines were maintained by the literature techniques: murine L1210 lymphoid leukemia,[17] P388 lymphocytic leukemia, human $Tmolt_3$ acute lymphoblastic T cell leukemia, colorectal adenocarcinoma SW480, lung bronchogenic MB-9812, osteosarcoma TE418, KB epidermoid nasopharynx, HeLa-S^3 suspended cervical carcinoma and glioma EH 188 MG. The protocol used to assess cytotoxicity was that of Geran *et al.*[17] Standards were determined in each cell line. Values are expressed for the drug's cytotoxicity as ED_{50} (μg/ml), i.e. the concentration which inhibits 50% of the cell growth determined by the trypan blue exclusion technique. Solid tumor cytotoxicity was determined by the method of Leibovitz *et al.*[18] A value of less than 4 μg/ml is necessary for significant activity according to the NCI protocol.[17]

Anti-neoplastic activity

In vivo activity of 2,3-dihydrophthalazine-1,4-dione (**1**) was determined against L1210 lymphoid leukemia and P388 lymphocytic leukemia in DBA/2 male mice (weight around 30 g), against Lewis Lung in $C_{57}B1/6$ male mice (around 30 g)[17] and Ehrlich ascites carcinoma in CF_1 male mice (around 25 g).[7]

Mode of action study

Incorporation of labeled precursors into [^{3}H]DNA, [^{3}H]RNA and [^{3}H]protein for 10^6 L1210 cells was determined by the method of Liao *et al.*[19] The concentrations of drugs employed were 25, 50 and 100 μM to determine inhibition of DNA, RNA and protein synthesis of L1210 cells at 60 min. Inhibition of various enzyme activities were carried out by first preparing the appropriate L1210 cell homogenate or subcellular fraction, then adding the drug to be tested during the enzyme assay. For the concentration response studies, the inhibition of enzyme activity was determined at 25, 50 and 100 μM for 60 min. Kinetic studies of selected enzyme activities were conducted at 100 μM of **1** and **6** for 15, 30, 45 and 60 min incubations.

Enzyme assays

DNA polymerase α activity was determined in a cytoplasmic extract by the method of Eichler *et al.*[20] Nuclear DNA polymerase was determined by isolating nuclei.[21] The polymerase assay for α and β was that of Sawada *et al.*[22] with [^{3}H]TTP. Messenger, ribosomal and transfer RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate[23,24] and the individual RNA polymerase activities were determined using [^{3}H]UTP. Ribonucleotide reductase activity was measured with [^{14}C]CDP with and without dithioerythritol.[25] The deoxyribonucleotides [^{14}C]dCDP were separated from the ribonucleotides by TLC on PEI plates. Thymidine, TMP and TDP kinase activities were measured using [^{3}H]thymidine and the reaction medium of Maley and Ochoa.[26] Carbamyl phosphate synthetase activity was determined by the method of Kalman *et al.*[27] and citrulline was determined colorimetrically.[28] Aspartate transcarbamylase activity was determined by the method of Kalman *et al.*[27] and carbamyl aspartate was determined colorimetrically. Thymidylate synthetase activity was analyzed by the method of Kampf *et al.*[30] The ^3H_2O measured was proportional to the amount of TMP formed from [^{3}H]dUMP. Dihydrofolate reductase activity was determined by the spectrophotometric method of Ho *et al.*[31] PRPP amidotransferase activity was determined by the method of Spassova *et al.*[32] and IMP dehydrogenase activity was determined with [^{14}C]IMP (Amersham, Arlington Heights, IL) where XMP was separated on PEI plates (Fisher Scientific) by TLC.[33] Protein was determined for all of the enzymatic assays by the Lowry technique.[34]

Deoxyribonucleoside triphosphates were extracted by the method of Bagnara and Finch.[35] Deoxyribonucleoside triphosphates were determined by the method of Hunting and Henderson[36] with calf thymus DNA, *Escherichia coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of [^{3}H]methyl-dTTP or 5-[^{3}H]-dCTP.

The effects of compounds **1** and **6** on DNA strand scission was determined by the *in vitro* method of Suzuki *et al.*,[37] Pera *et al.*[38] and Woynarowski *et al.*[39] L1210 lymphoid leukemia cells were incubated with 10 μCi thymidine (methyl-^{3}H, 84.0 Ci/mmol) for 24 h at 37°C. After harvesting the L1210 cells (10^6), the cells were centrifuged at 600 × *g* for 10 min in PBS, washed and suspended in 1 ml of PBS. Lysis

Table 1. The cytotoxicity of 2,3-dihydrophthalazine-1,4-dione derivatives on murine and human tissue cultured cells (ED_{50} values = μg/ml)

Compound	Murine		$Tmolt_3$	HeLa-S^3	Human colon	KB	Lung	Osteosarcoma	Glioma
	P388	L1210							
1		1.23	2.68	2.63	2.78	4.38	7.62		5.79
2	6.45	4.10	2.84	3.58	2.13	3.60	7.19		5.73
3	4.55	3.78	2.90	2.06	3.94	3.60	7.60		6.91
4	4.06	3.57	0.94	2.70	1.87	2.73	5.72	6.18	3.43
5	4.33	1.19	0.99	1.60	2.30	3.47	7.70	1.87	3.20
6	5.25	3.08	1.83	2.91	1.34	2.30	7.17	7.08	3.24
7	4.62	2.52	3.06	3.15	3.11	3.29	7.96	3.70	3.73
8	4.93	2.33	2.46	2.16	2.44	2.36	7.86	7.54	9.59
9	5.36	2.43	2.70	1.89	1.84	4.34	5.73	5.26	5.77
10	4.78	2.56	2.04	2.40	4.76	4.71	6.16		7.69
11	4.58	2.59	1-74	2.75	2.73	5.46	7.82	4.09	5.00
12	4.74	4.46	5.30	3.88	4.14	3.41	3.72	2.83	3.60
13	4.36	5.04	3.06	2.89	3.06		7.70	7.43	2.89
14	4.61	3.12	3.16	2.72		2.67	6.56	3.89	3.25
15	3.32	4.22	3.06	2.06	4.07	3.10	7.67	3.22	2.52
16	4.62	3.05	2.48		5.67	2.79	3.06	2.35	3.25
17	4.03	3.74	3.45	3.15	4.07	2.67		7.98	
18	5.73	1.35	1.30	3.61	1.81			7.98	9.29
19	3.66	3.53	2.90	1.94	5.67	2.79	7.85	7.78	5.12
20	4.51	2.88	4.24	3.47	6.89	6.65		8.32	2.96
21	4.10	2.07	1.88	2.06	2.75	3.66	6.20	5.97	4.40
22	2.95	2.31	2.73	1.78	2.59	5.14	6.16	7.88	4.57
23	4.07	2.40	1.71	1.98	2.33	4.96	5.37	7.36	3.80
24	6.25	1.46	1.57	3.41	2.43	6.82	6.38	8.09	6.33
25	4.25	2.23	3.66	1.78	4.15	2.85	7.81	6.57	4.64
26	1.70	2.19	1.41	3.34	6.07	2.67	7.88	5.42	5.12
27		3.21	2.06	2.12	4.13	2.35	7.21	8.11	5.12
28		2.96			5.92	4.38	1.95		
Standard									
29 5FU	3.72	1.41	2.14	2.47	3.09	1.25	5.64		1.28
30 araC	4.06	2.76	2.67	2.13	3.42	2.54	4.60		1.88
31 Hydrourea		2.67	3.18	1.96	4.76	5.29	7.33	7.57	2.27
32 Cycloleucine		3.08	2.38	2.38	3.81	5.71	4.36	6.18	5.89

buffer (0.5 ml of 0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X-100 and 2.5% sucrose) was layered onto a 5–20% alkaline–sucrose gradient (5ml of 0.3 M NaOH, 0.7 M KCl and 0.01 M EDTA) followed by 0.2 ml cell preparation. After incubating for 2.5 h at room temperature, the gradient was centrifuged at 12000 r.p.m. at 20°C for 60 min (Beckman rotor SW60). Fractions (0.2 ml) were collected from the bottom of the gradient, neutralized with 0.2 ml of 0.3 N HCl and the radioactivity was measured. Thermal calf thymus DNA denaturation studies and DNA viscosity studies were conducted after incubation of compounds **1** or **6** at 100 μM at 37°C for 24 h.[40]

Table 2. The effects of 2,3-dihydrophthalazine-1,4-dione derivatives on L1210 lymphoid leukemia cells on DNA, RNA and protein synthesis after 24 h incubation at the ED_{50} value of the drug

Compound (*N* = 5)	Percent of control ($X \pm$ SD)		
	DNA	RNA	Protein
Control	100 ± 6[a]	100 ± 7[b]	100 ± 5[c]
1	37 ± 3*	38 ± 6*	80 ± 6
2	50 ± 4*	77 ± 7*	114 ± 7
3	78 ± 7*	71 ± 6*	166 ± 8*
4	44 ± 5*	88 ± 5	142 ± 7*
5	44 ± 3*	36 ± 4*	28 ± 3*
6	47 ± 5*	124 ± 7	174 ± 6*
7	52 ± 6*	134 ± 8*	71 ± 5*
8	52 ± 6*	90 ± 5	68 ± 6*
9	54 ± 5*	53 ± 4	37 ± 4*
10	65 ± 5*	124 ± 5	63 ± 5*
11	50 ± 3*	145 ± 8*	85 ± 6
12	84 ± 5	37 ± 3*	59 ± 5*
13	96 ± 6	51 ± 4*	72 ± 5*
14	85 ± 7	36 ± 3*	55 ± 5*
15	76 ± 6*	59 ± 4*	82 ± 6
16	58 ± 6*	61 ± 4*	52 ± 4*
17	54 ± 5*	56 ± 5*	38 ± 4*
18	42 ± 4*	38 ± 4*	63 ± 5*
19	61 ± 5*	71 ± 5*	109 ± 5
20	49 ± 4*	36 ± 4*	68 ± 6*
21	58 ± 6*	36 ± 5*	72 ± 5*
22	47 ± 6*	30 ± 4*	89 ± 5
23	55 ± 5*	187 ± 8*	73 ± 4*
24	51 ± 6*	27 ± 3*	59 ± 4*
25	47 ± 4*	78 ± 5*	89 ± 4
26	46 ± 3*	159 ± 6*	73 ± 5*
27	43 ± 5*	26 ± 4*	108 ± 6

[a] 118342 d.p.m./10^6 cells; [b] 2512 d.p.m./10^6 cells; [c] 4203 d.p.m./10^6 cells.
* $p \leq 0.001$; Student's *t*-test.

Results

The 2,3-dihydrophthalazine-1,4-diones demonstrated cytotoxicity in a number of the tissue culture lines (Table 1). Most of the compounds were not active against P388 lymphocytic leukemia with the exception of **19**, **22** and **26**. However, when these compounds were tested in the L1210 lymphoid leukemia screen most of the compounds were active with the exception of **2**, **13** and **15**. Compounds **1**, **5**, **18** and **24** all resulted in ED_{50} values of less than 2.00 μg/ml in the human lines. The compounds were active against $Tmolt_3$ leukemia growth with the exception of **12** and **20**. Compounds **4** and **5** resulted in ED_{50} values less than 1.0, and compounds **6**, **11**, **18**, **21**, **23**, **24** and **20** gave values less than 2.0. Hela-S^3 uterine cancer growth was inhibited by all 28 compounds, i.e. all values were less than 4 μg/ml. Compounds **5**, **9**, **19**, **22**, **23** and **25** resulted in ED_{50} values less than 2.0 mg/ml. Adenocarcinoma colon growth was inhibited by all of the agents except **10**, **12**, **15**, **16**, **17**, **19**, **20**, **26**, **27** and **28**. Compounds **4**, **6**, **9** and **18** resulted in ED_{50} values less than 2.0 μg/ml. KB nasopharynx carcinoma growth was suppressed by most of the agents except **1**, **9**, **10**, **11**, **20**, **22**, **24** and **28**. Bronchogenic lung cancer growth was not particularly effected by most of the 2,3-dihydrophthalazine-1,4-dione derivatives. Exceptions to this were **12**, **16** and **28**. Osteogenic bone sarcoma growth was inhibited by **5**, **7**, **12**, **14**, **15** and **16**. Brain glioma growth was inhibited by **4**, **5**, **6**, **7**, **12**, **13**, **14**, **15**, **16**, **20** and **23**.

2,3-Dihydrophthalazine-1,4-dione at 8 mg/kg/day inhibited Ehrlich ascites carcinoma growth 84%. In the Lewis Lung carcinoma screen at 8 mg/kg/day a T/C% = 140 was obtained. In the L1210 lymphoid leukemia screen T/C% = 140, 172, 167 and 136 were obtained at 10, 20, 30 and 40 mg/kg/day, respectively. In the P388 screen, 50 mg/kg/day afforded a T/C% = 127.

The compounds, except **12**, **13** and **14**, were effective at their ED_{50} values in inhibiting [^{3}H]thymidine incorporating with L1210 DNA after 24 h (Table 2). A parallel existed between the ED_{50} value and the ability to inhibit DNA synthesis of L1210 cells. Compounds **1**, **5**, **12**, **14**, **18**, **20**, **21**, **22**, **24** and **27** at their ED_{50} values significantly suppressed RNA synthesis by greater than 60%, all of which had ED_{50} less than 4 μg/ml except **14**. Compounds **5**, **9** and **17** significantly suppressed protein synthesis by greater than 60%, all of which had an ED_{50} less than 4 μg/ml.

Compounds **1** and **6** were selected because they both were active against L1210 lymphoid growth

Table 3. The effects of 2,3-dihydrophthalazine-1,4-dione derivatives on nucleic acid metabolism of L1210 lymphoid leukemia cells

Assay ($N = 6$)	Control	Percent of control (X ± SD)							
		1				**6**			
		10 μM	25 μM	50 μM	100 μM	10 μM	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5[a]	73 ± 3*	70 ± 4*	69 ± 3*	45 ± 4*	73 ± 4	72 ± 3	71 ± 6*	56 ± 5*
RNA synthesis	100 ± 7[b]	101 ± 6	70 ± 5*	69 ± 4*	51 ± 3*	88 ± 5	79 ± 6	54 ± 4*	46 ± 4*
Protein synthesis	100 ± 6[c]	91 ± 7	88 ± 6	91 ± 7	104 ± 9	121 ± 7	186 ± 8	211 ± 9	202 ± 8
DNA polymerase α	100 ± 5[d]	80 ± 6	73 ± 5*	69 ± 5*	63 ± 4*	98 ± 6	90 ± 5	89 ± 7	82 ± 6
mRNA polymerase	100 ± 8[e]	91 ± 7	85 ± 7	78 ± 6*	67 ± 6	100 ± 7	92 ± 6	91 ± 5	156 ± 8*
rRNA polymerase	100 ± 7[f]	88 ± 7	82 ± 6	80 ± 6	78 ± 5*	103 ± 10	97 ± 9	83 ± 7	75 ± 7*
tRNA polymerase	100 ± 9[g]	101 ± 8	98 ± 7	95 ± 6	93 ± 7	212 ± 8	269 ± 9	409 ± 10*	495 ± 9*
Ribonucleoside reductase	100 ± 5[h]	55 ± 4*	110 ± 7	119 ± 8	145 ± 6*	96 ± 5	107 ± 7	109 ± 6	122 ± 8
[^{14}C]Glycine into purine	100 ± 7[i]	105 ± 7	98 ± 6	80 ± 7	53 ± 5*	106 ± 6	71 ± 5	70 ± 5*	56 ± 6*
IMP dehydrogenose	100 ± 6[j]	65 ± 5*	61 ± 4*	48 ± 4*	30 ± 4*	101 ± 7	95 ± 6	79 ± 6*	78 ± 5*
PRP amidotransferase	100 ± 5[k]	161 ± 8*	108 ± 6	69 ± 5*	66 ± 5*	93 ± 6	92 ± 7	89 ± 5	86 ± 6
[^{14}C]Formate into pyrimidine	100 ± 6[l]	125 ± 7	140 ± 6*	102 ± 7	99 ± 8	255 ± 11	90 ± 9	61 ± 5*	55 ± 6*
Carbamyl phosphate synthetase	100 ± 7[m]	117 ± 8	112 ± 6	103 ± 5	118 ± 7	98 ± 9	92 ± 9	88 ± 5	79 ± 6*
Aspartate transcarbamylase	100 ± 6[n]	108 ± 5	90 ± 8	89 ± 6	84 ± 6	198 ± 11*	124 ± 8	95 ± 5	73 ± 5*
OMP decarboxylase	100 ± 7[o]	128 ± 7	110 ± 9	106 ± 7	105 ± 7	135 ± 6	127 ± 9	126 ± 6	114 ± 6
Dihydrofolate reductase	100 ± 5[p]	120 ± 6	119 ± 10	107 ± 6	65 ± 5*	98 ± 7	75 ± 6	69 ± 5*	69 ± 5*
Thymidylate synthetase	100 ± 6[q]	72 ± 5*	71 ± 6*	63 ± 6*	60 ± 5*	119 ± 6	99 ± 7	91 ± 4	44 ± 4*
Thymidine kinase	100 ± 6[r]	88 ± 6	78 ± 7*	68 ± 6*	67 ± 5*	124 ± 5	114 ± 6	96 ± 5	87 ± 5
TMP kinase	100 ± 7[s]	84 ± 6	61 ± 5*	47 ± 5*	25 ± 3*	119 ± 5	99 ± 4	91 ± 5	42 ± 4*
TDP kinase	100 ± 7[t]	71 ± 5*	56 ± 5*	38 ± 4*	24 ± 3*	105 ± 6	90 ± 6	86 ± 6	22 ± 5*
dATP	100 ± 6[u]	—	—	—	33 ± 4*	—	—	—	58 ± 5*
dGTP	100 ± 5[v]	—	—	—	78 ± 5*	—	—	—	115 ± 6
dCTP	100 ± 5[w]	—	—	—	26 ± 3*	—	—	—	69 ± 4*
dTTP	100 ± 6[x]	—	—	—	87 ± 6	—	—	—	137 ± 7

Control values for 10^6 cells/60 min incubation

[a] 29801 d.p.m.; [b] 29196 d.p.m.; [c] 6564 d.p.m.; [d] 5318 d.p.m.; [e] 1338 d.p.m.; [f] 58 d.p.m.; [g] 36 d.p.m.; [h] 92975 d.p.m.; [i] 92251 d.p.m.; [j] 20875 d.p.m.; [k] 0.055 OD units: [l] 13680 d.p.m.; [m] 0.426 μmol citulline; [n] 0.66 mol. *n*-carbamyl aspartate; [o] 44743 d.p.m.; [p] 0.133 OD units; [q] 51560 d.p.m.; [r] 4562 d.p.m.; [s] 666 d.p.m.; [t] 275 d.p.m.; [u] 32.39 pmol; [v] 23.79 pmol; [w] 86.29 pmol; [x] 22.04 pmol.

* $p < 0.001$; Student's *t*-test.

but were on opposite ends of the spectrum of L1210 cytotoxicity activity. When the L1210 cells were incubated with compound **1** or **6** for 60 min at 10, 25, 50 and 100 μM, the DNA and RNA synthesis was inhibited in a concentration dependent manner (Table 3). Protein synthesis of L1210 cells was actually stimulated for the first 60 min. DNA polymerase α activity was inhibited moderately with compound **1** by 37% and **6** by 18% at 100 μM. mRNA and rRNA polymerase activities were inhibited by compound **1**. Compound **6** reduced rRNA polymerase activity but stimulated mRNA and tRNA polymerase activities after 60 min. Ribonucleoside reductase activity was inhibited by **1** at 10 μM but higher concentrations caused stimulation of the activity as did compound **6** at 100 uM. *De novo* synthesis of purines as measured by [^{14}C]glycine incorporation into purines was significantly inhibited by compound **1** and **6** with greater than 45% reduction at 100 μM. IMP dehydrogenase activity was markedly inhibited by **1** in a concentration-dependent manner. With compound **6**, moderate inhibition of IMP dehydrogenase activity L1210 was observed. PRPP amido-transferase activity was not inhibited significantly by **6** but with compound **1** first there was stimulation followed later by inhibition. Dihydrofolate reductase activity was inhibited significantly by compound **6** in a concentration dependent manner but was not significantly affected by **1**. *De novo* synthesis of pyrimidine measured as formate incorporation into pyrimidines was not affected by compound **1** but was by **6**. Carbamyl phosphate synthetase activity was inhibited marginally, i.e. 19%, by **6** but not by **1**. Aspartate transcarbamylase activity was moderately inhibited by **1** by 16% and **6** by 27% at 100 μM. OMP decarboxylase activity was not inhibited by either drug. Thymidylate synthetase activity was inhibited 28% after 60 min by **1** and 56% by **6**. Thymidine kinase activity was not significantly inhibited by the agents but TMP and TDP kinase activities were inhibited in a concentration-dependent manner by both agents. When deoxyribonucleotide levels were examined in L1210 cells after incubation for 60 min, dATP and dCTP levels were significantly reduced by compound **1** and **6**. The magnitude of reduction was more with **1** compared to **6**. dGTP levels were reduced by **1** but not **6** and dTTP levels were not significantly reduced by either compound.

Kinetic studies with enzymes which were inhibited by the 2,3-dihydrophthalazine-1,4-diones at 100 μM were performed (Table 4). With compound **1**, IMP dehydrogenase activity was inhibited 53% and compound **6** by 36% by 15 min. Dihydrofolate reductase activity was inhibited over time reaching 31–32% inhibition by 60 min. Thymidylate synthetase activity was inhibited 42% but 60 min incubation was required. DNA polymerase α activity was preferentially inhibited by **1** compared to **6** affording greater than 30% in 45 min. With compound **6**, dihydrofolate reductase and IMP dehydrogenase activities were inhibited significantly after 15 min; however, greater than 45 min was required to cause at least 50% inhibition. Thymidylate synthetase, DNA polymerase and carbamyl phosphate synthetase activities were not inhibited significantly until 45 min, and they had not achieved 50% inhibition even at 60 min.

Calf thymus DNA demonstrated no observable alteration of UV absorption after 24 h incubation with compounds **1** and **6**. There were no observable effects on thermal denaturation or DNA viscosity of calf thymus DNA after incubating with compounds **1** or **6** for 24 h. L1210 DNA strand scission studies after incubation with compounds **1** or **6** showed a shift of the DNA in the gradient (Figure 2).

[^{14}C]2,3-Dihydrophthalazine-1,4-dione uptake in L1210 cells was shown to be rapid with 32% of the drug present within the cells after 30 min. The binding of ^{14}C drug to DNA was 4.58%, RNA was

Table 4. The kinetic effects of compounds 1 and 6 on rate limiting L1210 enzyme activities at 100 μM concentration

	Percent of control ($X \pm$ SD)							
	1				**6**			
	15 min	30 min	45 min	60 min	15 min	30 min	45 min	60 min
DNA polymerase α	109 ± 9	92 ± 7	69 ± 4*	63 ± 5*	102 ± 6	88 ± 5	70 ± 4*	83 ± 6*
IMP dehydrogenase	84 ± 6	68 ± 6*	55 ± 7*	47 ± 4*	78 ± 7*	72 ± 6*	69 ± 5*	64 ± 5*
Dihydrofolate reductase	97 ± 7	86 ± 5	74 ± 6*	68 ± 6*	78 ± 6*	73 ± 6	68 ± 5*	69 ± 4*
Carbamyl phosphate synthetase	—	—	—	—	115 ± 8	93 ± 7	78 ± 6*	79 ± 6
Thymidylate synthatase	120 ± 8	103 ± 6	76 ± 6*	58 ± 5*	120 ± 6	103 ± 8	76 ± 7*	58 ± 5*

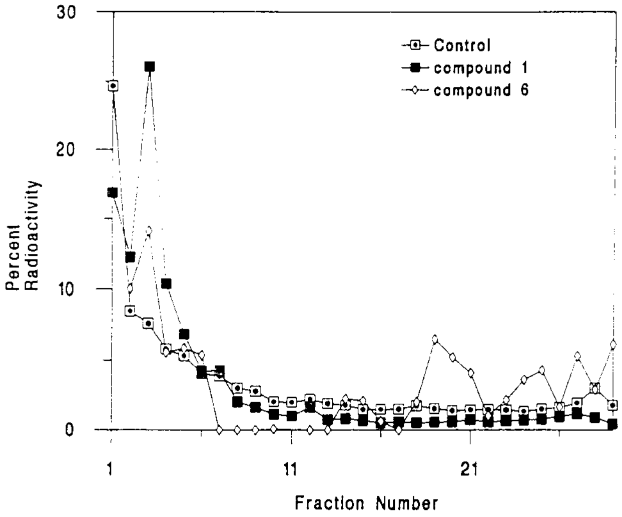


Figure 2. DNA strand scission in L1210 cells; fraction number increases from bottom to top of gradient.

19.0% and protein was 7.79%, demonstrating some differential binding to the macromolecules in L-1210 cells.

Discussion

The 2,3-dihydrophthalazine-1,4-dione derivatives proved to be potent anti-neoplastic and cytotoxic agents. The derivatives demonstrated some selectivity with selected agents demonstrating potent activity against the growth of certain tumors. The compounds were generally active against single cell suspensions, e.g. L1210 lymphoid, Tmolt$_3$ leukemia cells and HeLa-S^3 uterine tumor (Table 1). However, the growth of colon adenocarcinoma, KB nasopharynx carcinoma and brain glioma tumors derived from solid tumors were also effectively inhibited by the derivatives. With a few exceptions, the derivatives were not effective against lung bronchogenic and osteosarcoma tumors. When selected derivatives were examined for their effects on L1210 DNA and RNA metabolism the agents were specific of sites in the *de novo* purine pathway. The observed inhibition afforded by the 2,3-dihydrophthalazine-1,4-diones of IMP dehydrogenase and PRPP amido transferase activities was sufficient to account for the observed inhibition of DNA synthesis of L1210 cells. A secondary site of the drugs may be DNA polymerase and mRNA polymerase activities. It should be noted that whereas the effects of the drugs at these sites may contribute to the overall inhibition of DNA synthesis, the degree of inhibition is not of a magnitude to explain the observed inhibition of DNA or RNA synthesis. Likewise the inhibition of either dihydrofolate reductase or thymidylate synthetase activity was significant but it alone would not explain the total inhibition of DNA synthesis from the lack of one carbon transfer in purine or pyrimidine synthesis. All of these effects of the drugs probably add to the overall reduction of DNA and RNA synthesis which eventually brings about cell death. The sites of inhibition by the drugs in the purine pathway are important because the inhibition is reflected both in the DNA and RNA synthesis. Furthermore, these enzymes were inhibited the earliest at a fixed concentration of compound **1** and **6**. An additional site which may be of some importance was the observation that L1210 cell DNA strand scission was present after 24 h incubation with the drugs (Figure 2). The fragmentation of the DNA may be due to the drugs actually being incorporated into DNA, since the molecular structure is very similar in size to a purine ring. Once in the DNA molecule, the drug may cause an unstable situation and the DNA breaks. Since there was no evidence of drug binding, intercalation or cross-linking of the DNA strands based on the *in vitro* studies with calf thymus DNA, the incorporation of the drug into DNA appears a plausible explanation of the DNA strand scission.

In conclusion, the 2,3-dihydrophthalazine-1,4-diones offer a new class of anti-neoplastic agents which function at feasible sites in the tumor cell metabolism. Previous studies have demonstrated that these derivatives were not toxic in rodents at higher doses than required for anti-neoplastic activity.[5] Thus, further evaluation of these derivatives is warranted.

References

1. Murthy ARK, Wong OT, Reynolds DJ, *et al.* Synthesis and hypolipidemic activity of 3-imino-1-oxoisoinodolines in rodents. *Pharmac Res* 1987; **4**: 21–7.
2. Hall IH, Oswald CB, Wyrick SC, *et al.* Disposition of hypolipidaemic agents-2,3-dihydrophthalazine-1,4-dione in Sprague Dawley rats. *J Pharm Pharmacol* 1989; **42**: 394–7.
3. Wong OT, Williams WL Jr, Oswald BS, *et al.* Effects of cyclic imides on lipoprotein receptor binding and degradation of rat and human cells and effects of regulatory enzymes of lipid metabolism. *Res Commun Chem Pathol Pharmac* (in press).
4. Hall IH, Reynold DJ, Wong OT, *et al.* Effects of cyclic imides on lipid adsorption from the intestine and on bile lipid and bile acids on Sprague Dawley rats. *Pharm Res* (submitted).
5. Hall IH, Wong OT, Reynolds DJ, *et al.* Effects of 2,3-dihydrophthalazine-1,4-dione on Sprague Dawley rat

lipid metabolism and serum lipoproteins. *Biomed Biochem Acta* 1988; **47**: 423–33.

6. Hall IH, Lee KH, Starnes CO, *et al.* Antihyperlipidemia activity of sesquiterpene lactones and related compounds. *J Pharmac Sci* 1980; **69**: 694–7.
7. Das MK, Maiti PK, Roy S, *et al.* The relationship of hypolipidemic and antineoplastic activities of tricyclohexyl and triphenyl phosphine borave, carboxyboranes, cyanoboranes and related derivatives. *Arch Pharmaz* 1992; 325.
8. Kancko I, Hazama-Shimada Y, Siperstein MD. Inhibitory effects on lipid metabolism in cultured cells of ML-236B, a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase. *Eur J Biochem* 1978; **87**: 313–21.
9. Siperstein MO. Role of cholesterogenesis and isoprenoid synthesis in DNA replication and cell growth. *Lipid Res* 1984; **25**: 1462–8.
10. Larson RA, Yachnin S. Mevalonic acid induces DNA synthesis in chronic lymphocytic leukemia cells. *Blood* 1984; **64**: 257–62.
11. Chen HW, Kaudutch AA, Waymouth C. Inhibition of cell growth by oxygenated derivatives of cholesterol. *Nature* 1974; **251**: 419–21.
12. Brown MS, Goldstein JC. Suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and inhibition of growth of human fibroblasts by 7-ketocholesterol. *J Biol Chem* 1974; **249**: 7306–64.
13. Quesney-Huneeus W, Wiley ?, Siperstein D. Essential role for mevalonate synthesis in DNA replication. *Proc Natl Acad Sci USA* 1979; **76**: 5056–60.
14. Perkin SL, Lodia SF, Stubbs JB. Linkage of the isprenoid biosynthetic pathway with induction of DNA synthesis in mouse lymphocytes. Effects of Compaction on mitogen-induced lymphocytes in serum-free medium. *Biochem Biophys Acta* 1982; **711**: 83–9.
15. Quesney-Huneeus W, Galick A, Sispersteim MD, *et al.* The dual role of mevalonate in the cell cycle. *J Biol Chem* 1983; **258**: 378–85.
16. Murthy ARK, Hall, IH, Chapman Jr JM, *et al.* The hypolipidemic activity of a series of 2,3-dihydrophthalazine-1,4-dione derivatives in rodents. *Pharmac Res* 1986; **3**: 93–101.
17. Geran RJ, Greenburgh NH, Macdonald MM, *et al.* Protocol for screening chemical agents and natural products against animal tumor and other biological systems. *Cancer Chemother* 1972; **3**: 9–24.
18. Leibovitz AL, Stinson JC, McComb III WB, *et al.* Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* 1976; **36**: 4562–9.
19. Liao L, Kupchan SM, Horwitz SB. Mode of action of the antitumor compound bruceantin, an inhibitor of protein synthesis. *Mol Pharmacol* 1976; **12**: 167–76.
20. Eichler DC, Fisher PA, Korn D. Effect of calcium on the recovery distribution of DNA polymerase alpha from cultured human cells. *J Biol Chem* 1977; **252**: 4011–4.
21. Mamaril FP, Dobrjasky A, Green S. A rapid method for isolation of nuclei of Ehrlich ascites tumor cells. *Cancer Res* 1970; **30**: 352–6.
22. Sawada H, Tatsumi K, Sadada M, *et al.* Effect of neocarzinostatin of DNA syntheses in L1210 cells. *Cancer Res* 1974; **34**: 3341–6.
23. Hall IH, Carlson GL, Abernathy GS, *et al.* Cycloalkanones 3 antifertility activity. *J Med Chem* 1974; **17**: 1253–7.
24. Anderson KM, Mendelson IS, Guzik G. Solubilized DNA-dependent nuclear RNA polymerases from the mammary glands of late-pregnant rats. *Biochim Biophys Acta* 1975; **383**: 56–66.
25. Moore EC, Hurlbert RB. Regulation of mammalian deoxyribonucleotide biosynthesis of nucleotides or activators and inhibitors. *J Biol Chem* 1966; **241**: 4802–9.
26. Maley F, Ochoa S. Enzymatic phosphorylation of deoxycytidylic acid. *J Biol Chem* 1958; **233**: 1538–43.
27. Kalman SM, Duffield PH, Brzozwski TJ. Purification and properties of a bacterial carbamyl phosphate synthetase. *J Biol Chem* 1966; **241**: 1871–7.
28. Archibald RM. Determination of citrulline and allontoin and demonstration of citrulline in blood plasma. *J Biol Chem* 1944; **156**: 121–142.
29. Koritz SB, Gohen PP. Colorimetric determination of carbamyl amino acid and related compounds. *J Biol Chem* 1954; **209**: 145–50.
30. Kampf A, Barfknecht RL, Schaffer PJ, *et al.* Synthetic inhibitors of *Escherichia coli* calf thymus and Ehrlich ascites tumor thymidylate synthetase. *J Med Chem* 1976; **19**: 903–8.
31. Ho YK, Hakala T, Zakrzewski SF. 5-(1-Adamantyl) pyrimidines as inhibitors of folate metabolism. *Cancer Res* 1972; **32**: 1023–8.
32. Spassova MK, Russev GC, Goovinsky EV. Some pyrazoles as inhibitors of purine biosynthesis *de novo*. *Biochem Pharmacol* 1976; **25**: 923–4.
33. Becker JH, Lohr GW. Inosine 5′-phosphate dehydrogenase activity in normal and leukemic blood cells. *Klin Wochenschr* 1979; **57**: 1109–15.
34. Lowry OH, Rosebrough J, Farr AL, *et al.* Protein measurement with folin phenol reagent. *J Biol Chem* 1951; **193**: 265.
35. Bagnara AS, Finch LR. Quantitative extraction and estimation of intracellular nucleoside triphosphates in *Escherichia coli*. *Anal Biochem* 1972; **45**: 24.
36. Hunting D, Henderson JF. Determination of deoxyribonucleoside triphosphates using DNA polymerase alpha A critical evaluation. *Can J Biochem* 1982; **59**: 723–7.
37. Suzuki H, Nishimura T, Muto SK, *et al.* Mechanism of action of macromycin: DNA strand scission inhibition of DNA synthesis and mitosis. *J Antibiot* 1978; **32**: 875–83.
38. Pera MF Jr, Rawlings CJ, Shackleton J, *et al.* Quantitative aspects of the formation and loss of DNA interstrand crosslinks in chinese hamster cells following treatment with *cis*-diamminedichloro-platinum II (*cis*-platin). *Biochim Biophys Acta* 1981; **655**: 152–66.
39. Woynarowski JW, Beerman TA, Konopa J. Induction of deoxyribonucleic acid damage in HeLa-S^3 by cytotoxic and antitumour sesquiterpene lactones. *Biochem Pharmacol* 1981; **30**: 3005–7.
40. Zhao Y, Hall IH, Oswald CB, *et al.* Antimalerial agents III. Mechanism of action of artesunate against *Phasmadium berghie* infection. *Chem Pharmac Bull* 1987; **35**: 2052–61.

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