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Cytotoxicity of 2-ethenyl-2,3-dihydrophthalazine-1,4-diones in murine and human tumor cultured cells

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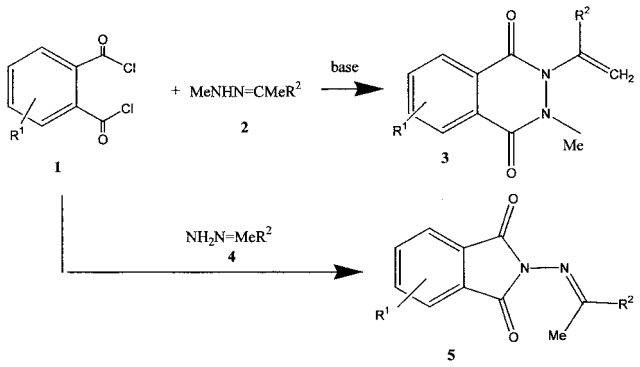
2-Ethenyl-2,3-dihydrophthalazine-1,4-diones were successfully synthesized and proved to be effective cytotoxic agents against the growth of suspended murine and human leukemias and lymphomas. Selected compounds were also active in human HeLa uterine carcinoma, suspended effusion breast MCF-7 and glioma HS683 screens. These agents suppressed P388 lymphocytic leukemia DNA synthesis after 60 min at 100 μM. Their target appeared to be the *de novo* synthesis pathway with significant inhibition of the activities of both regulatory enzymes of the pathway, i.e. PRPP-amide transferase and IMP dehydrogenase resulting in a reduction in the d[NTP] pool levels for DNA incorporation. The compounds did not affect de novo pyrimidine synthesis and its regulatory enzymes. Very minor reduction by the agents was noted for the nucleoside kinases and the DNA and RNA polymerase activities within 60 min. DNA was not a target of the agents in that there was no alkylation of the nucleotide bases, intercalation between base pairs or cross-linking of the DNA strands; however, the agents did cause P388 DNA strand scission after 24 h at 100 μM.

1. Introduction

Previously, it was demonstrated that 2,3-dihydrophthalazine-1,4-diones possess potent cytotoxicity activity against the growth of human $Tmolt_3$ leukemia and human HeLa-S^3 uterine suspended cells and KB nasopharynx carcinoma and colon adenocarcinoma SW480 cultured solid tumor cells [1]. A few of the derivatives were active against bronchogenic lung, osteosarcoma and glioma growth. 2,3-Dihydrophthalazine-1,4-dione was active at 8 mg/kg *in vivo* against the growth L1210 lymphoid and P388 lymphocytic leukemias, as well as Ehrlich ascites carcinoma and Lewis lung in mice. A mode of action study in L1210 lymphoid leukemia cells with the 2,3-dihydrophtalazine-1,4-diones showed that DNA and RNA syntheses were inhibited at 100 μM after 60 min due to the agents blocking *de novo* purine synthesis at the regulatory sites of PRPP-amido transferase and IMP dehydrogenase which resulted in reductions of the deoxyribonucleotide pools after 60 min [1]. In the present study a novel synthetic modification has been made to this class of compounds, and the cytotoxicity and mode of action in P388 lymphocytic leukemic cells of the new derivatives has been evaluated.

Scheme



	R1	R2
3a	H	Ph
3b	H	3,4-$(MeO)_2$Ph
3c	H	3-MeOPh
3d	H	4-MeOPh
3e	6-/7-Me	4-MeOPh
3f	H	4-ClPh
5	H	4-MeOPh

2. Investigations and results

2.1. Synthesis

We have previously reported that disubstituted malonyl chlorides reacted with aliphatic oximes in the presence of triethylamine to yield 2-alkenyl-3,5-isoxazolidinediones [2]. In order to determine if aliphatic hydrazones would react with phthaloyl chlorides in a similar manner to form the previously unreported 2-alkenylphthalazine-1,4-diones, 4′-methoxyacetophenone hydrazone (**4**) ($R^2 = 4-MeOPh$) was reacted with phthaloyl chloride (**1**) ($R^1 = H$) in the presence of triethylamine. The product was 2-(1-(4-methoxyphenyl)ethylideneamino)-1,3-isoindolinedione (**5**) (Scheme). Therefore, closure to the five-membered rather than the six-membered ring was favored. In order to prevent five-membered ring formation one of the hydrazone hydrogens was substituted with a methyl group. Reaction of *N*-methylacetophenone hydrazones (**2**) with compounds **1** led to the formation of 2-ethenyl-3-methyl-2,3-dihydrophthalazine-1,4-diones **3a–f** (Table 1). The ^{1}H NMR spectra of **3** showed the presence of the two vinyl hydrogens which exhibited little ($J = \sim 1$ Hz) or no geminal coupling at δ 5.58–5.72 and 6.12–6.27, respectively, and the methyl hydrogens at ring position-2 at δ 3.47–3.50. Compound **3e** was presumably formed as a mixture of the 6-methyl and 7-methyl substituted isomers. However, it was isolated in low yield by column chromatography, and the NMR spectra are consistent with the presence of only one isomer. It is not obvious from the NMR spectra, which of the two isomers was isolated [3].

2.2. Cytotoxicity

The substituted 2,3-dihydrophthalazine-1,4-diones demonstrated good cytotoxicity against the growth of suspended tumor cells. Compounds **3d**, **3e**, **3f** and **5** were active in the murine L1210 lymphocytic leukemia screen, and all of

Table 1: Cytotoxicity of 2,3-dihydrophthalazine-1,4-diones in suspended murine and human tumors

	Murine	Murine	Rat	Human				
Compd.	L1210	P388	UMR 106	$Tmolt_4$	HuT-78	THP-1	Hela-S^3	$Tmolt_3$	HL-60
3a	4.22	0.88	7.56	4.22	2.00	3.37	3.67	3.87	4.67
3b	4.36	1.83	8.22	3.16	1.63	2.95	5.89	4.29	2.81
3c	4.97	1.34	7.19	4.97	2.75	3.03	5.35	2.79	3.84
3d	2.74	3.65	6.47	2.41	3.49	2.05	2.44	3.66	3.78
3e	1.37	2.71	5.66	2.41	2.48	2.20	3.05	2.51	3.83
3f	3.72	1.61	7.31	3.45	2.25	2.40	5.11	3.70	4.70
5	1.78	2.43	6.69	1.50	2.84	2.00	1.79	5.53	2.14
std agents									
6-MP	2.43	2.04	9.13	1.62	1.63	3.34	2.12	1.62	3.35
Ara-C	2.43	0.79	0.86	2.36	2.50	2.54	2.13	2.67	4.00
Hydroxyurea	2.67	1.30	2.87	4.47	3.87		1.96		
5-FU	1.41	0.99	5.52	2.14	1.50	0.49	2.21	2.87	
VP-16	1.83	0.99	3.57	1.92	1.13	3.27	1.89	1.00	4.43

$ED_{50} \leq 4$ μg/ml required for significant activity
N = 4
ED_{50} = μg/ml

Table 2: Cytotoxicity of 2,3-dihydrophthalazine-1,4-diones in solid human tumors

Compd.	Solid Hela	KB Nasoph arynx	Melanoma SK-2	SW 480 colon	HCT-8 ileum	Lung MB 9812	Lung A549	Breast MCF-7	Renal clear cell	Skin A431	Saos2 Bone	Glioma HS683
3a	3.88	7.62	12.24	1.70	4.85	7.24	5.20	3.10	10.72	7.83	7.56	4.45
3b	6.92	6.33	4.84	5.22	16.66	6.08	9.84	2.85	8.53	7.11	7.13	2.48
3c	7.28	8.25	6.69	9.07	5.26	5.82	9.58	2.75	10.27	8.15	7.19	3.31
3d	3.92	9.10	7.25	7.43	6.28	3.48	3.35	4.47	8.40	1.47	6.47	7.55
3e	6.77	6.03	6.60	5.13	4.41	5.03	4.84	5.23	7.56	4.80	5.66	4.44
3f	9.19	6.33	5.47	2.69	7.17	6.02	9.26	2.96	10.25	6.98	8.31	1.87
5	8.10	3.10	9.25	5.79	3.14	6.48	5.48					8.39
Std												
6-MP	5.61	5.74	3.42	3.61	1.15	4.29	4.71	8.84	9.35	3.41	5.07	4.46
Ara-C	4.74	2.84	0.92	3.42	2.54	6.16	5.62	12.45	1.30	0.92	8.90	1.88
Hydroxy urea	8.12	5.27	3.21	7.33	1.77	7.18	8.89			3.21	–	2.27
5-FU	4.11	1.25	0.61	2.47	1.12	5.07	3.58	6.82	5.29	0.61	8.73	1.28
VP-16	3.05	3.32	0.71	0.93	3.78	3.50	4.74	11.00	7.01	0.71	8.61	2.44

$ED_{50} \leq 4$ μg/ml required for significant activity
N = 4
ED_{50} = μg/ml

the compounds were active against the growth of murine P388 lymphocytic leukemia. In human the $Tmolt_4$ T cell leukemia screen compounds **3b**, **3d**, **3e**, **3f** and **5** were active. In the $Tmolt_3$ T cell leukemia screen compounds **3a**, **3c**, **3d**, **3e**, and **3f** were active, and in the HL-60 leukemia screen compounds **3b**, **3c**, **3d**, **3e** and **5** were active. In the HuT-78 lymphoma and the THP-1 acute monocytic leukemia screens all of the compounds demonstrated significant activity. In the HeLa-S^3 suspended uterine carcinoma screen compounds **3a**, **3d**, **3e** and **5** were significantly active (Table 1).

In the cultures derived from solid cancers the 2,3-dihydrophthalazine-1,4-diones demonstrated more selectivity. Compounds **3a** and **3d** were active in the solid HeLa uterine carcinoma screen. Compounds **3a** and **3f** demonstrated activity in colon adenocarcinoma SW480 screen. Compound **3d** was the only derivative to suppress lung MB-9812 and lung A549 growth. MCK-7 breast carcinoma growth was reduced by compounds **3a**, **3b**, **3c** and **3f**. Skin epidermoid A431 growth was inhibited by compound **3d** and glioma growth was reduced by compounds **3b**, **3c** and **3f**. Compound **5** was active against the growth of human KB nasopharynx and HCT-8 ileum adenocarcinoma. None of the tested compounds were active against human melanoma Sk-2, osteosarcoma Saos-2, or clear cell renal carcinoma growth (Table 2).

2.3. Pharmacology

Mode of action studies in P388 lymphocytic leukemia cells showed that compounds **3a**, **3b**, **3c** and **3f** at 100 μM suppressed DNA synthesis greater than 80% after 60 min. Compound **5** only caused 37% reduction of DNA synthesis and a 57% reduction of RNA synthesis. The inhibition of RNA and protein synthesis was only marginal after 60 min with compounds **3a**, **3b**, **3c**, and **3f**. DNA α polymerase activity was reduced only by compound **3b** by 40% after 60 min at 100 μM. mRNA polymerase activity was reduced 29% to 44%, r-RNA polymerase activity was inhibited 33% to 61% and t-RNA polymerase activity was suppressed 24% to 47% by compounds **3a**, **3b**, **3c**, **3f** and **5** at 100 μM over 60 min. Ribonucleotide reductase activity was not reduced significantly by the compounds with the exception of compound **5** which caused 63% reduction. Dihydrofolate reductase activity was suppressed 44% to 67% by compounds **3a**, **3b**, **3c** and **3f** at 100 μM over 60 min. *De novo* purine synthesis was suppressed 35 to 72%, while the regulatory enzymes PRPP-amido-transfer-

Table 3: Effects of compound 3a in P388 lymphocytic leukemia metabolism after 60 min incubation

(N = 6) Assay	Percent of control (X ± S.D.)			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5[a]	83 ± 4	77 ± 5*	16 ± 4*
RNA synthesis	100 ± 6[b]	140 + 6*	109 ± %	84 ± 5
Protein synthesis	100 + 5[c]	96 ± 5	80 ± 4*	72 ± 4*
DNA polymerase α	100 ± 6[d]	129 ± 5*	108 ± 5	87 ± 5
mRNA polymerase	100 ± 7[e]	68 ± 5*	63 ± 4*	56 ± 3*
rRNA polymerase	100 ± 4[f]	68 ± 5*	44 ± 3*	39 ± 3*
tRNA polymerase	100 ± 7[g]	93 ± 5	90 ± 5	76 ± 5*
Ribonucleotide reductase	100 ± 5[h]	97 ± 5	92 ± 4	88 ± 4
Dihydrofolate reductase	100 ± 5[i]	81 ± 4*	78 ± 5*	47 ± 3*
Purine *de novo* synthesis	100 ± 5[j]	97 + 5	74 ± 4	65 ± 3
PRPP amido transferase	100 ± 6[k]	99 ± 5	95 ± 6	86 ± 4
IMP dehydrogenase	100 ± 5[l]	67 ± 4*	67 ± 3	53 + 4*
Pyrimidine *de novo* synthesis	100 ± 5[m]	101 ± 5	96 ± 4	93 ± 5
Carbamyl phosphate synthetase	100 ± 8[n]	116 ± 6	102 ± 4	100 ± 4
Aspartate transcarbamylase	100 ± 6[o]	102 ± 4	101 ± 5	100 ± 4
Thymidylate synthetase	100 ± 5[p]	126 ± 6*	119 ± 5	89 ± 4
Thymidine kinase	100 ± 6[q]	55 ± 4*	49 ± 3*	48 ± 3*
Thymidine monophosphate kinase	100 ± 7[r]	72 ± 5*	70 ± 3*	61 ± 4*
Thymidine diphosphate kinase	100 ± 6[s]	94 ± 5	74 ± 3*	73 ± 2*
d[ATP]	100 ± 5[t]			76 ± 3*
d[GTP]	100 ± 6[u]			72 ± 5*
d[CTP]	100 ± 5[v]			105 ± 4
d[TTP]	100 ± 4[w]			77 ± 5*

* $P \leq 0.01$; control values based on 10^6 P388 cells
[a] 26152 dpm; [b] 4851 dpm; [c] 7461 dpm; [d] 47804 dpm; [e] 4239 dpm; [f] 1502 dpm; [g] 6400 dpm; [h] 2744 dpm; [i] 0.868 OD units; [j] 92551 dpm [k] 0.121 OD units; [l] 76058; [m] 19758 dpm; [n] 0.3902 mol citrulline; [o] 1.064 mol N-carbamyl aspartate; [p] 18463 dpm; [q] 1317 dpm; [r] 1179 dpm; [s] 1891 dpm; [t] 6.17 pmol; [u] 5.27 pmol; [v] 6.87 pmol; [w] 6.94 pmol

ase activity was reduced 14% to 85%, and IMP dehydrogenase activity was reduced 42% to 59% by the five compounds at 100 μM over 60 min.

De novo pyrimidine synthesis as well as its regulatory enzymes carbamyl phosphate synthetase, aspartate transcarbamylase or thymidylate synthetase were not affected by the compounds. Thymidine kinase activity was inhibited 30% to 66% by the five compounds, TMP kinase activity was reduced only by compounds **3a** and **5** by 39–42%, and TDP kinase activity was suppressed 24% to 55% by the five compounds at 100 μM over 60 min. d[ATP] pools were lowered 24% to 41%, d[GTP] pools were suppressed 24% to 32%, and d[TTP] pools were reduced 16% to 51% by the compounds at 100 μM over 60 min.

2.4. DNA studies

ct-DNA studies indicated after 24 h incubation with compounds at 100 μM that there was no interaction of the agents with the nucleotide bases of DNA to cause a hyper-

Table 4: Effects of compound 3b in P388 lymphocytic leukemia metabolism after 60 min incubation

(N = 6) Assay	Percent of control (X ± S.D.)			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	78 ± 4*	74 ± 3*	10 ± 3*
RNA synthesis	100 ± 6	121 ± 5	108 ± 5	84 ± 5
Protein synthesis	100 + 5	68 ± 4*	67 ± 3*	63 ± 3*
DNA polymerase α	100 ± 6	84 ± 4	67 ± 4*	60 ± 3*
mRNA polymerase	100 ± 7	78 ± 4*	62 ± 3*	60 ± 3*
rRNA polymerase	100 ± 4	98 ± 6	62 ± 5*	53 ± 3*
tRNA polymerase	100 ± 7	116 ± 5	102 ± 4	76 ± 3*
Ribonucleotide reductase	100 ± 5	97 ± 5	95 ± 5	91 ± 5
Dihydrofolate reductase	100 ± 5	91 ± 6	59 ± 5*	33 ± 3*
Purine *de novo* synthesis	100 ± 5	97 ± 5	87 ± 4	55 ± 3*
PRPP amido transferase	100 ± 6	81 ± 4*	62 ± 3*	62 ± 2*
IMP dehydrogenase	100 ± 5	100 ± 6*	75 ± 4	49 ± 3*
Pyrimidine de novo synthesis	100 ± 5	104 ± 6	102 ± 4	99 ± 6
Carbamyl phosphate synthetase	100 ± 8	103 ± 4	99 ± 4	98 ± 4
Aspartate transcarbamylase	100 ± 6	102 ± 5	102 ± 4	101 ± 4
Thymidylate synthetase	100 ± 5	117 ± 4	98 ± 4	79 ± 3*
Thymidine kinase	100 ± 6	71 ± 4*	65 ± 4*	59 ± 3*
Thymidine monophosphate kinase	100 ± 7	125 ± 6*	102 ± 6	93 ± 5
Thymidine diphosphate kinase	100 ± 6	101 ± 5	97 ± 6	74 ± 3*
d[ATP]	100 ± 5			67 ± 4*
d[GTP]	100 ± 6			71 ± 4*
d[CTP]	100 ± 5			94 ± 6
d[TTP]	100 ± 4			74 ± 3*

Table 5: Effects of compound 3c in P388 lymphocytic leukemia metabolism after 60 min incubation

(N = 6) Assay	Percent of control (X ± S.D.)			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	84 ± 6	83 ± 4	9 ± 2*
RNA synthesis	100 ± 6	72 ± 3*	72 ± 4*	71 ± 3*
Protein synthesis	100 + 5	73 ± 4*	71 ± 4*	70 ± 3*
DNA polymerase α	100 ± 6	119 ± 6	112 ± 5	95 ± 5
mRNA polymerase	100 ± 7	81 ± 5	74 ± 4*	66 ± 3*
rRNA polymerase	100 ± 4	92 ± 6	71 ± 5*	54 ± 4*
tRNA polymerase	100 ± 7	90 ± 5	70 ± 4*	63 ± 4*
Ribonucleotide reductase	100 ± 5	92 ± 4*	84 ± 3	79 ± 4*
Dihydrofolate reductase	100 ± 5	60 ± 3*	57 ± 4*	38 ± 3*
Purine de novo synthesis	100 ± 5	92 ± 3	81 ± 4	51 ± 4*
PRPP amido transferase	100 ± 6	123 ± 5	79 ± 3*	68 ± 3*
IMP dehydrogenase	100 ± 5	102 ± 6	79 ± 4*	50 ± 3*
Pyrimidine ne novo synthesis	100 ± 5	106 ± 6	106 ± 5	99 ± 6
Carbamyl phosphate synthetase	100 ± 8	108 ± 5	102 ± 4	99 ± 5
Aspartate transcarbamylase	100 ± 6	103 ± 4	102 ± 4	101 ± 5
Thymidilate synthetase	100 ± 5	108 ± 3	86 ± 5	82 ± 4
Thymidine kinase	100 ± 6	75 ± 4*	46 ± 3*	34 ± 3*
Thymidine monophosphate kinase	100 ± 7	113 ± 5	86 ± 5	85 ± 5
Thymidine diphosphate kinase	100 ± 6	115 ± 5	102 ± 4	71 ± 3*
d[ATP]	100 ± 5			71 ± 5*
d[GTP]	100 ± 6			68 ± 3*
d[CTP]	100 ± 5			111 ± 4
d[TTP]	100 ± 4			84 ± 3

Table 6: Effects of compound 3f in P388 lymphocytic leukemia metabolism after 60 min incubation

(N = 6) Assay	Percent of control (X ± S.D.)			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	94 ± 5	79 ± 4*	16 ± 3*
RNA synthesis	100 ± 6	145 ± 6*	139 ± 5*	117 ± 5
Protein synthesis	100 + 5	78 ± 4*	74 ± 3*	68 ± 4*
DNA polymerase α	100 ± 6	113 ± 6	113 ± 5	97 ± 5
mRNA polymerase	100 ± 7	90 ± 5	83 ± 4	71 ± 4*
rRNA polymerase	100 ± 4	132 ± 5*	128 ± 5*	67 ± 4*
tRNA polymerase	100 ± 7	91 ± 6	87 ± 5	53 ± 4*
Ribonucleotide reductase	100 ± 5	97 ± 5	88 ± 4	81 ± 5
Dihydrofolate reductase	100 ± 5	96 ± 4	94 ± 4	56 ± 3*
Purine de novo synthesis	100 ± 5	87 ± 4	76 ± 4*	43 ± 3*
PRPP amido transferase	100 ± 6	104 ± 6	74 ± 5*	54 ± 3*
IMP dehydrogenase	100 ± 5	67 ± 5*	48 ± 3*	41 ± 3*
Pyrimidine de novo synthesis	100 ± 5	96 ± 5	96 ± 6	92 ± 4
Carbamyl phosphate synthetase	100 ± 8	106 ± 6	104 ± 5	95 ± 4
Aspartate transcarbamylase	100 ± 6	101 ± 5	100 ± 3	93 ± 4
Thimydylate synthetase	100 ± 5	110 ± 5	96 ± 6	91 ± 4
Thymidine kinase	100 ± 6	96 ± 6	75 ± 4*	70 ± 3*
Thymidine monophosphate kinase	100 ± 7	112 ± 5	111 ± 4	98 ± 5
Thymidine diphosphate kinase	100 ± 6	109 ± 4	89 ± 4	76 ± 5*
d[ATP]	100 ± 5			62 ± 4*
d[GTP]	100 ± 6			76 ± 4*
d[CTP]	100 ± 5			98 ± 6
d[TTP]	100 ± 4			68 ± 5*

chromic shift indicating no SN^1 alkylation reactions, and there was no change in the Tm value during the ct-DNA denaturation studies suggesting intercalation of the agents between the base pairs of the DNA molecule. On the other hand, the ct-DNA viscosity studies indicated less viscosity in the treated samples compared to the control. Follow up studies in the P388 cells showed that the compounds caused DNA strand scission after 24 h incubation at 100 μM (Fig.).

3. Discussion

The 2-ethenyl-2,3-dihydrophthalazine-1,4-diones appear to have significant cytotoxicity in murine and human cultured tumor cells. The substitution of 2-(1-phenylethenyl) groups afforded no reduction in cytotoxicity activity of the compounds in the suspended cell tumors. In fact, the new compounds in the P388 lymphocytic screen afforded better activity compared to the previous derivatives [1]. This study also showed that the compounds were active against human tumors previously not tested, e.g. HuT-78 lymphoma, THP-lacute monocytic leukemia, and HL-60 leuke-

Table 7: Effects of compound 5 in P388 lymphocytic leukemia metabolism after 60 min incubation

(N = 6) Assay	Percent of control (X ± S.D.)			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 5	92 ± 6	81 ± 3*	63 ± 4*
RNA synthesis	100 ± 6	92 ± 5	52 ± 4*	43 ± 2*
Protein synthesis	100 + 5	108 ± 6	97 ± 5	87 ± 5
DNA polymerase α	100 ± 6	113 ± 6	113 ± 5	97 ± 5
mRNA polymerase	100 ± 7	74 ± 4*	72 ± 5*	54 ± 4*
rRNA polymerase	100 ± 4	70 ± 6*	66 ± 5*	55 ± 5*
tRNA polymerase	100 ± 7	91 ± 6	87 ± 5	53 ± 4*
Ribonucleoside reductase	100 ± 5	57 ± 4*	47 ± 3*	37 ± 2*
Dihydrofolate reductase	100 ± 5	142 ± 6*	125 ± 5	88 ± 6
Purine de novo synthesis	100 ± 5	130 ± 7*	48 ± 4*	28 ± 3*
PRPP amido transferase	100 ± 6	53 ± 4*	28 ± 3*	15 ± 2*
IMP dehydrogenase	100 ± 5	74 ± 6*	71 ± 5*	58 ± 5*
Pyrimidine de novo synthesis	100 ± 5	98 ± 6	87 ± 5	85 ± 5
Carbamyl phosphate synthetase	100 ± 8	96 ± 5	93 ± 6	93 ± 5
Aspartate transcarbamylase	100 ± 6	102 ± 6	102 ± 5	102 ± 5
Thymidylate synthetase	100 ± 5	117 ± 6	109 ± 5	96 ± 5
Thymidine kinase	100 ± 6	90 ± 5	88 ± 5	52 ± 4*
Thymidine monophosphate kinase	100 ± 7	116 ± 6*	77 ± 5*	58 ± 4*
Thymidine diphosphate kinase	100 ± 6	89 ± 6	54 ± 5*	45 ± 4*
d[ATP]	100 ± 5			59 ± 5*
d[GTP]	100 ± 6			75 ± 5*
d[CTP]	100 ± 5			94 ± 6
d[TTP]	100 ± 4			49 ± 4*

mia. In the solid tumors the same pattern of selectivity was demonstrated by the new derivatives, and in addition activity was demonstrated against breast MCK-7 growth. The original derivatives showed better activity in the KB nasopharynx screen than the current derivatives [1]. In the mode of action the substitution of 2-(1-phenylethenyl) groups in P388 lymphocytic cells demonstrated similar results as did the previous 2,3-dihydrophthalazine-1,4-diones in L1210 lymphoid leukemia cells [1]. These agents appear to be anti-metabolities as opposed to interacting with the DNA molecule itself via alkylation, intercalation between base pairs or cross-linking of the DNA strands. Due to the similarity of their structure to the purine ring, it is not surprising that they interfere with the *de novo* purine synthesis specifically at the IMP dehydrogenase enzyme site. This action of the derivatives would account for the observed reduction of the deoxyribopurines d[ATP] and d[GTP] after 60 min as well as the reduction of DNA and RNA syntheses. The fact that the agents inhibit dihydrofolate reductase and the nucleotide kinases would add to the over all suppression of DNA synthesis rather than RNA synthesis. The reduction of the RNA polymerase activities by the compounds would account for the observed reduction of RNA synthesis.

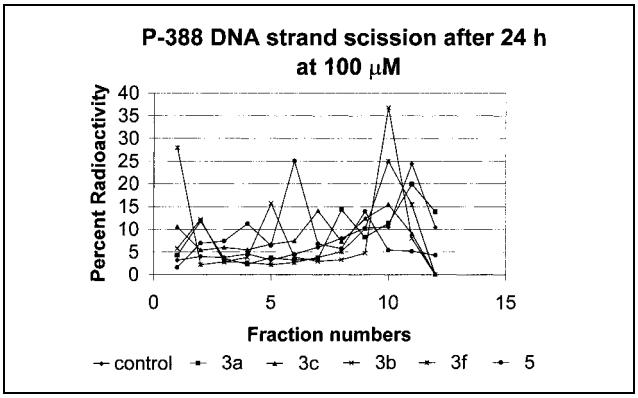


Fig.: P-388 DNA strand scission after 24 h at 100 μm

Nevertheless, since in mammalian cancer cells the ratio of deoxyribonucleotide to ribonucleotides is 9 : 1, affects by agents on their biosynthesis in 60 min should be reflected more in DNA synthesis rather than RNA synthesis. In addition the previously studied 2,3-dihydrophtalazine-1,4-diones were shown to cause DNA fragmentation in 24 h [1] as did the new derivatives. This action would not only reduce DNA synthesis but would cause apoptosis resulting in immediate death of the cancer cells.

4. Experimental

4.1. Synthesis

Melting points are uncorrected. IR spectra were recorded on a Perkin Elmer 1610 FT-IR Spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 300 NB NMR spectrometer. Tetramethylsilane (TMS) was the internal reference standard. Mass spectra (MS) were obtained with a Hewlett Packard 6890 GC-MS system. High resolution mass spectra (HRMS) were determined at the Center for Mass Spectrometry, Research Triangle Institute, Research Trinangle Park, North Carolina. Elemental analyses were performed by Desert Analytics, Tucson, Arizona. The C, H, N analysis for the compounds were all within ± 0.04% of the theoretical values. The acetophenone hydrazones were prepared according to the method of Newkome and Fishel [4].

4.1.1. 3-Methyl-2-(1-phenylethenyl)-2,3-dihydro-1,4-phthalazinedione (**3a**)*: general procedure for the preparation of the 3-methyl-2-ethenyl-1,4-phthalazine-2,4-diones* **3a–f**

A mixture of 6.0 g (50 mmol) of acetophenone and 2.76 g (60 mmol) of methylhydrazine in 25 ml of absolute ethanol was heated under reflux for 24 h. After cooling, the solvent was removed under reduced pressure. To the crude unstable acetophenone methylhydrazone was added 50 ml of dry 1,2-dimethoxyethane, 6.9 g (50 mmol) of anhydrous potassium carbonate, and, 0.05 g of tetrabutylammonium bromide. The mixture was cooled to 0 °C and 10.15 g (50 mmol) of phthaloyl dichloride was added over 20 min with stirring. The mixture was allowed to warm to room temperature and to stir for 2 days. The mixture was poured into ice-water, and the resulting mixture was extracted with CH_2Cl_2 (3 × 75 ml). The CH_2Cl_2 layer was dried ($MgSO_4$), and the solvent was removed under the reduced pressure to yield a solid residue. Recrystallization (MeOH) gave pure **3a** (27%) as a white solid: m.p.: 140–142 °C; FTIR (Nujol) 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.48 (s, 3 H), 5.72 (s, 1 H), 6.27 (s, 1 H), 7.37 (m, 5 H), 7.86 (m, 2 H), 8.40 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 33.9, 118.6, 125.3, 127.8, 128.1, 129.1, 128.2, 129.1, 129.5, 133.4, 133.8, 134.4, 141.5, 157.6, 157.7; HRMS, calc. for $C_{17}H_{14}N_2O_2$: 278.1055, found: 278.1057; MS, m/z 278.

4.1.2. 3-Methyl-2-(1-(3,4-dimethoxyphenyl)ethenyl)-2,3-dihydro-1,4-phthalazinedione (**3b**)

17%, m.p.: 150–152 °C (MeOH); FTIR (Nujol), 1639, 1624 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.50 (s, 3 H), 3.84 (s, 3 H), 3.87 (s, 3 H), 5.60 (s, 1 H), 6.15 (s, 1 H), 6.79 (d, 1 H, J = 8.41 Hz), 6.84 (d, 1 H, J = 8.41 Hz), 6.95 (s, 1 H), 7.85 (m, 2 H), 8.37 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 33.8, 55.9 (2 C's), 108.3, 111.3, 116.6, 118.2, 127.2, 127.7, 128.2, 128.9, 129.2, 133.4, 133.8, 141.3, 149.4, 150.3, 157.6, 157.7; MS, m/z 338.

4.1.3. 3-Methyl-2-(1-(3-methoxyphenyl)ethenyl)-2,3-dihydro-1,4-phtalazinedione (**3c**)

24%; m.p.: 123–125 °C (MeOH); FTIR (Nujol), 1644 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.49 (s, 3 H), 3.77 (s, 3 H), 5.70 (s, 1 H), 6.25 (s, 1 H), 6.25–6.95 (m, 3 H), 7.25–7.29 (m, 1 H), 7.84–7.87 (m, 2 H), 8.37–8.40 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 33.9, 55.3, 111.5, 114.6, 117.7, 118.8, 127.8, 128.1, 128.9, 129.3, 130.2, 133.4, 133.8, 135.9, 141.4, 157.6, 157.7, 160.1; MS, m/z 308.

4.1.4. 3-Methyl-2-(1-(4-methoxyphenyl)ethenyl-2,3-dihydro-1,4-phthalazinedione (**3d**)

23%, m.p.: 124–126 °C (MeOH); FTIR (Nujol), 1644 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.47 (s, 3 H), 3.79 (s, 3 H), 5.57 (s, 1 H), 6.12 (s, 1 H), 6.86 (d, 2 H), 7.29 (d, 2 H), 7.85 (m, 2 H), 8.38 (d, 2 H); ^{13}C NMR ($CDCl_3$) δ 33.8, 55.3, 114.5, 116.5, 126.7, 127.0, 127.7, 128.1, 128.9, 129.2, 133.4, 133.8, 141.1, 157.5, 157.6, 160.6; MS, m/z 308

4.1.5. 3,6-/3,7-Dimethyl-3-(1-(4-methoxyphenyl)ethenyl)-2,3-dihydro-1,4-phthalazinedione (**3e**)

4%, m.p.: 159–161 °C, purified by cc on silica gel (petroleum ether: EtOAc (90 : 10); FTIR (Nujol) 1651 cm^{-1}, ^1H NMR ($CDCl_3$) δ 3.48 (s, 3 H), 3.80 (s, 3 H), 4.75 (s, 3 H), 5.58 (s, 1 H, J = 1.0 Hz), 6.14 (s, 1 H, J = 1.0 Hz), 6.87 (d, 2 H), 7.28 (d, 2 H), 7.87 (d, 1 H), 8.38–8.40 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 33.9, 44.9, 55.4, 113.8, 114.6, 116.6, 126.7, 127.6, 128.7, 129.0, 129.6, 133.4, 141.0, 143.6, 157.1, 157.2, 160.7; MS, m/z, 322.

4.1.6. 3-Methyl-2-(1-(4-chlorophenyl)ethenyl)-2,3-dihydro-1,4-phthalazinedione (**3f**)

25%, m.p.: 146–148 °C (MeOH); FTIR (Nujol), 1666 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.47 (s, 3 H), 5.72 (s, 1 H, J = 1.1 Hz), 6.24 (s, 1 H, J = 1.1 Hz), 7.33 (s, 4 H), 7.86 (m, 2 H), 8.37 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 34.0, 118.9, 126.6, 127.8, 128.1, 128.7, 129.2, 129.3, 133.1, 133.5, 133.9, 135.5, 140.6, 157.6, 157.7; MS, m/z 312.

4.1.7. 2-(1-(4-Methoxyphenyl)ethylideneamino)-1,3-isoindolinedione (**5**)

To a stirred mixture of 5.0 g (30 mmol) of 4′-methoxyacetophenone hydrazone, 6.06 g (60 mmol) of triethylamine and 0.05 g of tetrabutylammonium bromide in 60 ml of anhydrous ether was added at 0 °C degree 6.09 g (30 mmol) of phthaloyl chloride dropwise over 20 min. The reaction mixture was allowed to warm to room temperature and stir overnight. The resulting mixture was poured into ice-water and extracted with ether. The ether extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was recrystallized (CCl_4) to give 5.7 g (65%) of **5** as a yellow solid: m.p.: 129–130 °C; FTIR (Nujol), 1790, 1713 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.32 (s, 3 H), 3.85 (s, 3 H), 6.92–6.95 (m, 2 H), 7.72–7.98 (m, 6 H); ^{13}C NMR ($CDCl_3$) δ 17.7, 55.4, 113.8, 123.5, 128.8, 129.5, 131.4, 134.2, 162.5, 164.

4.2. Cytotoxicity

Compounds **3a**–**f** and **5** were tested for cytotoxic activity by homogenizing drugs as a 1 mg/ml solution in 0.05% Tween 80/H_2O. These solutions were sterilized by passing them through an acrodisc (45 μm) and tested serially from 0.2 to 15 μg/ml against the cell line. The following cell lines were maintained by literature techniques [5] and the growth mediums and growth conditions are according to American Type Culture Collection protocols: murine L1210 lymphoid leukemia and P388 lymphocytic leukemia, rat UMR-106 osteosarcoma, human $Tmolt_3$ and $Tmolt_4$ acute lymphoblastic T cell leukemia, HL-60 leukemia, HuT-78 lymphoma, THP-1 acute monocytic leukemia, HeLa-S^3 suspended cervical carcinoma, HeLa solid cervical carcinoma, KB epidermoid nasopharynx, Sk-Mel-2 malignant melanoma, colorectal adenocarcinoma SW480, HCT-8 ileocecal adenocarcinoma, lung bronchogenic MB-9812, A549 lung carcinoma, MCK-7 breast effusion, renal clear cell, Saos-2 osteosarcoma and glioma HS683. Geran et al.'s protocol [5] was used to assess the suspended cell cytotoxicity of the compounds and standards in each cell line. Cell numbers were determined by the trypan blue exclusion technique after three days incubation. Solid tumor cytotoxicity was determined by Leibovitz et al's method [6] utilizing crystal violet/MeOH and read at 562 nm (Molecular Devices) after 4–5 days incubation when the controls have converged. Values for cytotoxicity were expressed as ED_{50} [μg/ml], i.e. the concentration of the compound inhibiting 50% of cell growth. A value of less than 4 μg/ml was required for significant activity of growth inhibition [5].

4.3. Incorporation studies

The effects of drugs on the incorporation of ^3H-thymine, ^3H-uracil, or ^3H-leucine into ^3H-DNA, ^3H-RNA or ^3H-protein, respectively for 10^6 P388 lymphocytic leukemia cells at 25, 50 and 100 μM was determined for 60 min incubations [7]. The acid insoluble labeled DNA, RNA or protein was collected on discs which were counted in a Packard beta counter. The incorporation of ^{14}C-glycine (53.0 mCi/mmol) into purines was obtained by the method of Cadman et al. [8]. Incorporation of ^{14}C-formate (53.0 mCi/mmol) into pyrimidines was determined by the method of Christopherson et al. [9]. The final purines or pyrimidines were separated by TLC from starting components using the appropriate standard nucleoside bases and counted.

4.4. Enzyme assays

The effects of the 2,3-dihydrophthalazine-1,4-diones on nucleic acid metabolism were determined at 25, 50 and 100 μM of compounds **3a**, **3b**, **3c**, **3f** and **5** after 60 min incubations. P388 DNA polymerase α activity was determined in cytoplasmic extracts isolated by Eichler et al.'s method [10, 11]. The DNA polymerase α assay was described by Sawada et al. [12] with ^3H-2-deoxyribothymidine-5′-triphosphate [TTP]. Messenger-, ribosomal- and transfer-RNA polymerase nuclei enzymes were isolated with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using ^3H-uridine-5′-triphosphate [UTP] [13, 14]. The following enzyme activities were determined using P388 homogenates. Ribonucleotide reductase activity was measured using ^{14}C-cytidine-5′-diphosphate [CDP] with dithioerythritol [15]. ^{14}C-2′-Deoxyribocytidine-5′-diphosphate was separated from the ^{14}C-CDP by TLC on polyethyleneimine cellulose [PEI] plates. Thymidine, thymidine-5′-monophosphate [TMP] and thymidine-5′-diphosphate [TDP] kinase activities were determined using ^3H-thymidine (58.3 mCi/mmol) in the medium of Maley and Ochoa [16] and seperated by TLC. Carbamyl phosphate synthetase activity was determined by the method of Kalman et al. [17] and the product citrulline was determined colorimetrically [18]. Aspartate transcarbamylase activity was measured using the incubation medium of Kalman et al. [17]; the product carbamyl aspartate was determined colorimetrically by the method of Koritz et al. [19]. Thymidylate synthetase activty was analyzed by Kampf et al.'s method [20]. The ^3H_2O separated by charcoal was proportional to the amount of TMP formed from ^3H-2′-desoxyribouridine-5′-monophosphate [UMP]. Dihydrofolate reductase activity was determined by the NADH disappearance spectrophotometric method of Ho et al. [21] at 340 nm. Phosphoribosyl-pyrophosphate [PRPP]-amidotransferase activity was determined by Spassova et al.'s [22] method as the generation of NADH; inosine-5′-monophosphate [IMP] dehydrogenase activity was analyzed with 8-^{14}C-IMP (54 mCi/mmol) (Amersham, Arlington Heights, IL) after separating ^{14}C xanthosine-5′-monophosphate [XMP] on [PEI] plates (Fisher Scientific) by TLC [23] which was then counted. Protein content was determined for the enzymatic assays by the Lowry technique [24].

4.5. DNA studies

After deoxyribonucleoside triphosphates [d[NTP]] were extracted [25], d[NTP] levels were determined by the method of Hunting and Henderson [26] with calf thymus DNA, *E. coli* DNA polymerase I, non-limiting amounts of the three desoxyribonucleoside triphosphates not being assayed, and either 0.4 mCi of (^3H-methyl)-dTTP or (5-^3H)-dCTP. Thus, 2′-deoxyriboadenosine-5′-triphosphate [dATP], 2′-deoxyriboguanoside-5′-triphosphate [dGTP], 2′-deoxyribocytidine-5′-triphosphate [dCTP] and thymidine-5′-triphosphate [dTTP] levels were determined after incubation with the drugs for 60 min at 100 μM.

The effects of the compounds **3a**, **3b**, **3c**, **3f** and **5** on DNA strand scission were determined by the methods of Suzuki et al. [27], Pera et al. [28] and Woynarowski et al. [29]. P388 lymphoid leukemia cells were incubated with 10 μCi [methyl-^3H]-thymidine (84.0 Ci/mmol) for 24 h at 37 °C. P388 cells (10^7) were harvested and then centrifuged at 600 × g 10 min in phosphate buffered saline (PBS). They were later washed and suspended in 1 ml of PBS. Lysis buffer (0.5 ml; 0.5 MNaOH, 0.02 M EDTA, 0.01% Triton X-100 and 2.5% sucrose) was layered onto a 5–20% alkaline-sucrose gradient (5 ml; 0.3 M NaOH, 0.7 KCl and 0.01 M EDTA); this was followed by 0.2 ml of cell preparation. After the gradient was incubated for 2.5 h at room temperature, it was centrifuged at 12,000 × g 17 h at 8 °C. Fractions (0.2 ml) were collected from the bottom of the gradient, neutralized with 0.2 ml of 0.3 N HCl, and measured for radioactivity.

Thermal calf thymus DNA denaturation studies, changes in DNA UV absorption from 220–340 nm, and DNA viscosity studies were conducted after incubation of compounds **3a**, **3b**, **3c**, **3f** and **5** at 100 μM at 37 °C for 24 h [30].

4.6. Statistical analysis

Data is displayed in tables and figures as the means ± standard deviations expressed as percentage of control. N is the number of samples per group. The Student's "t"-test was used to determine the probable level of significance (p) between test samples and control samples.

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