Cytotoxicity of Substituted Alkyl-3,4-bis(4-methoxyphenyl)pyrrole-2-carboxylates in L1210 Lymphoid Leukemia Cells

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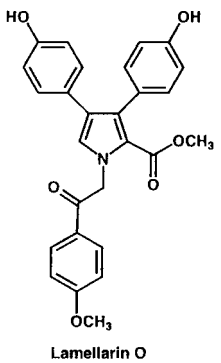
***Key Words**: Alkyl-3,4-bis(4-methoxyphenyl)pyrrole-2-carboxylates; antineoplastic activity; purine synthesis inhibitors*

Summary

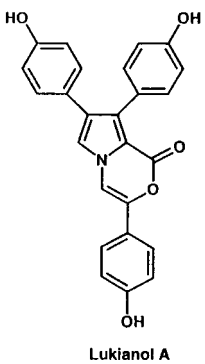
Two alkyl-3,4-bis(4-methoxyphenyl)pyrrole-2-carboxylates proved to be potent cytotoxic agents in the murine L1210 lymphoid leukemia screen. DNA synthesis was preferentially inhibited with the major target of the agents being *de novo* purine biosynthesis at the regulatory enzyme sites of PRPP-amido transferase and IMP dehydrogenase. Other enzymatic activities which were suppressed by the drugs were DNA polymerase α, RNA polymerases, ribonucleoside reductase and dihydrofolate reductase. The d[NTP] pools, nucleoside kinase and the pyrimidine pathway were not affected by the presence of drugs. The DNA molecule itself was not the target of the agents, i.e. no alkylation of nucleotide bases, intercalation between bases or cross-linking of DNA strands occurred. The agents did cause L1210 DNA fragmentation after 24 h incubation at 100 μM.

Introduction

Antineoplastic activity has been demonstrated by a number of heterocyclic five membered ring structures. These include 1-acyl- and 1,2-diacyl-4,4-diethyl-3,5-pyrazolidinediones[1], 1-acyl- and 1,2-diacyl-1,2,4-triazolidinedione-3,5-diones[2], 3,5-isoxazolidinediones and 2-isoxazoline-5-ones[3] , 2-alkoxycarbonyl-5-aryl-1,3,5-triazine-4,6(1*H*,5*H*)diones[4], 3-imino-1-oxaisoindolines [5], 5,6-substituted 1(2)*H*-indazole-4,7-diones[6], *N*-pyridinyl and *N*-quinolinyl substituted phthalimides and succinimides[7], diazomethyl ketone and chloromethyl ketone analogues of *N*-tosyl amino acids[8], furan derivatives[9], and vinyl, cyanomethyl and pyranyl activated esters of 2-furoic acid and 2-furylacrylic acids [10], [(*N*-alkyl-1,3-dioxo-1*H*,3*H*-isoindolin-5-yl)oxy]-alkanoic acids[11], [(*N*-alkyl-1*H*,3*H*-1-oxoisoindolin-5-yl-oxyalkanoates[12], and 5,6-disubstituted 1(2)*H*-indazole- 4,7-diones [13]. Recently, the marine natural product rigidin, which is a pyrrolopyrimidine, has demonstrated several types of biological activity [14]. Lamellarin O and Lukianol A also represent new and related marine natural products which possess a highly functionalized pyrrole nucleus. Further modification has yielded 2,3- and 2,5-disubstituted pyrroles. The purpose of this investigation is to explore two substituted alkyl-3,4-bis(4-methoxyphenyl)pyrrole-2-carboxylates for their antineoplastic activities since some similarity in structural features exist with the above compounds in that these low molecular weight derivatives have been shown to be potent antimetabolites in cancer cells.



Lamellarin O



Lukianol A

Results

Synthesis

Gupton and co-workers [14] in recent years have been actively involved in developing methods for the regiochemically controlled synthesis of polysubstituted pyrroles from appropriately substituted derivatives of vinylogous iminium salts. Pyrroles, compounds **1** and **2**, were prepared as depicted in Scheme 1 from the commercially available 2-(4-methoxyphenyl)-4′-methoxyacetophenone (**3**). This ketone is efficiently converted to a vinylogous amide (**4**) by reaction with *N*,*N*-dimethylformamide dimethyl acetal in *N*,*N*-dimethylformamide. The vinyloglous amide (**4**) appeared to be a single stereoisomer as determined by tlc and NMR and was used in the next step without further purification. Subsequent treatment of the crude vinylogous amide (**4**) with phosphorus oxychloride in methylene chloride followed by hydrolysis of the crude product in water/tetrahydrofuran produces the respective β-chloroenal (**5**, a mixture of *E* and *Z* isomers with the *E* isomer predominating) in good yield. The mixture β-chloroenal is then reacted with either glycine methyl ester or *N*-methylglycine ethyl ester in refluxing *N*,*N*-dimethylformamide to produce the respective 2-carboalkoxy-3,4-diarylpyrrole (**1** or **2**) in high overall yield. A complete account of this new synthetic procedure is in preparation and will be published in the future. All compounds gave ^{3}H NMR and high resolution MS data consistent with their proposed structures and were found to be greater than 95% pure as

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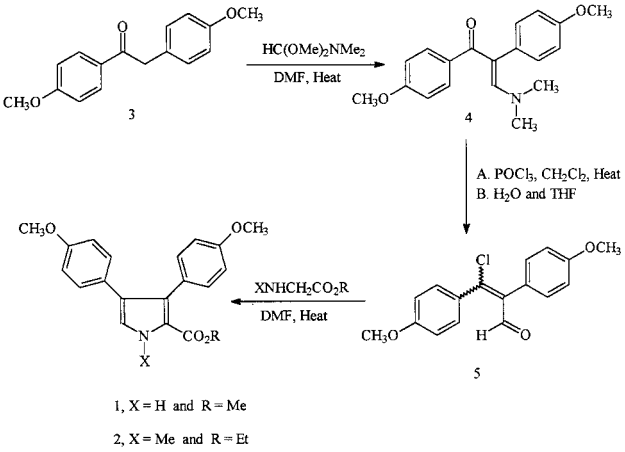

Scheme 1. General synthetic route for the preparation of 2-carboalkoxy-3,4-diarylpyrroles.

determined by tlc analysis. It should also be pointed out that compound **1** has previously been prepared by Furstner et al.[15] as part of the total synthesis of the marine natural product Lukianol A; our material **(1)** had physical properties (mp, NMR) indentical to those previously reported [15].

Compounds **1** and **2** afforded excellent cytotoxicity in the murine L1210 lymphoid leukemic, P388 lymphocytic leukemia, and human $Tmolt_3$ and $Tmolt_4$ T cell leukemia, HL-60 leukemia, HuT-8 lymphoma, THP-1 monocytic leukemia, EH118 MG glioma and HeLa-S^3 uterine carcinoma screens with ED_{50} values less than 20 μM which compared well with the clinical anti-neoplastic standards [Table 1].

The mode of action study in L1210 cells demonstrated that DNA synthesis was suppressed by 64% and 43% at 100 μM with less effect on RNA and protein syntheses after 60 min incubation. Compound **1** caused a 24% elevation of RNA syntheses with a 43% reduction of protein synthesis. Compound **2** caused 28% reduction of RNA synthesis and 25% reduction of protein synthesis. DNA polymerase α activity was suppressed 62% and 56% , m-RNA polymerase was reduced 56% and 42%, r-RNA polymerase was inhibited 41% and 31% and t-RNA polymerase activity was lowered 44% and 31% by compounds **1** and **2**, respectively at 100 μM after 60 min. Ribonucleoside reductase activity was reduced 36% to 39%, but dihydrofolate reductase activity was reduced more effectively by 78% and 53%. *De novo* purine synthesis was reduced significantly at 31% and 41% with the activities

Table 2. Effects of compound **1** on L1210 lymphocytic leukemia cell metabolism over 60 min.

	Percent of Control [X ± S.D]			
Assay	Control	25μM	50μM	100μM
DNA synthesis	100±5[a]	65±4*	43±4*	34±3*
RNA synthesis	100±6[b]	110±5	119±5	124±6*
Protein synthesis	100±5[c]	74±4*	62±3*	57±4*
DNA polymerase α	100±6[d]	82±5	53±4*	38±3*
mRNA polymerase	100±7[e]	65±5*	58±4*	44±3*
rRNA polymerase	100±4[f]	82±5	76±4*	59±4*
tRNA polymerase	100±7[g]	84±6	73±4*	56±4*
Ribonucleoside reductase	100±5[h]	69±4*	66±3*	61±4*
Dihydrofolate reductase	100±5[i]	63±3*	47±3*	22±2*
Purine *de novo* synthesis	100±5[j]	87±4	76±4*	79±5*
PRPP amido transferase	100±6[k]	61±4*	53±4*	49±4*
IMP dehydrogenase	100±5[l]	56±4*	39±4*	35±3*
Pyrimidine *de novo* synthesis	100±5[m]	104±4	107±5	115±6
Carbamyl phosphate synthetase	100±7[n]	98±6	92±5	87±5
Aspartate transcarbamylase	100±6[o]	98±5	99±6	108±7
Thymidylate synthetase	100±5[p]	127±6*	149±6*	92±5
Thymine kinase	100±6[q]	104±5	107±6	113±7
Thymidine monophosphate kinase	100±7[r]	100±6	78±4*	72±4*
Thymidine diphosphate kinase	100±6[s]	92±6	117±6	147±5
d[ATP]	100±5[t]			105±5
d[GTP]	100±6[u]			99±6
d[CTP]	100±5[v]			59±4*
d[TTP]	100±4[w]			102±6

Control values for 10^6 cells over 60 min

* $p \leq 0.001$

a 26152 dpm
b 4851 dpm
c 7461 dpm
d 47804 dpm
e 1502 dpm
f 4239 dpm
g 6400 dpm
h 2744 dpm
i 0.868 net OD units
j 92551 dpm
k 0.121 net OD units
l 76058 dpm
m 19758 dpm
n 0.392 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

Table 1. Cytotoxicity of Substituted Alkyl-3,4-bis(4-methyoxyphenyl)pyrrole-2-carboxylates.

	ED_{50} values =μM																
N=6	Mouse		Human														
Cp'd #	L-1210 Leukemia	P388 Leukemia	$Tmolt_3$ Leukemia	$Tmolt_4$ Leukemia	HL-60 Lymphoma	HuT-78	THP-1 Monocytic	HeLa-S^3 Uterine	Solid	Adenocarcinoma Colon SW-480	Ileum HCT-8	KB Naso pharynx	Lung A549	M9812	Osteo-Sarcoma Bone	Glioma Brain	Melanoma Skin
1	8.87	4.29	5.12	4.47	5.00	3.81	2.96	10.97	22.71	15.61	14.61	19.49	16.92	15.85	27.89	7.39	21.88
2	7.78	3.30	3.63	6.50	4.61	4.59	2.89	8.38	20.40	10.62	14.23	17.37	16.52	18.27	25.75	10.30	22.15
Ara-C	9.99	9.797	10.98	2.36	3.90	11.76	2.54	8.76	19.49	14.06	10.44	11.68	25.82	25.33	3.54	7.73	26.89
Hydroxy-urea	35.10	17.09	58.77	6.68	5.22	50.88	–	25.77	106.76	96.37	23.27	69.29	116.88	94.40	37.73	29.84	120.3
6MP	15.96	14.19	10.64	2.67	6.36	35.94	3.34	13.93	36.86	23.72	7.55	72.54	30.95	28.19	59.99	29.30	79.30
5FU	10.84	10.84	16.45	2.75	5.28	44.66	0.49	18.99	31.59	23.75	8.61	9.61	27.52	43.36	27.06	9.84	18.83

Standard deviation were all within 4% of the stated values. Ara-C = cytosine arabinoside; 6MP = 6-Mercaptopurine; 5FU = Fluorouracil

Table 3. Effects of compound **2** on L1210 leukemia cell metabolism over 60 min.

	Percent of Control [X ± SD]			
(*N*=6) Assay	Control	25 μM	50 μM	100μM
DNA synthesis	100±5	74±5*	69±4*	57±3*
RNA synthesis	100±6	116±6	96±7	72±4*
Protein synthesis	100±5	96±5	85±5	75±4*
DNA polymerase α	100±6	101±5	51±4*	44±3*
mRNA polymerase	100±7	136±6*	69±4*	58±4*
rRNA polymerase	100±4	90±4	76±4*	69±4*
tRNA polymerase	100±7	92±6	79±4*	78±3*
Ribonucleoside reductase	100±5	108±6	75±4*	64±5*
Dihydrofolate reductase	100±5	98±4	74±4*	47±3*
Purine *de novo* synthesis	100±5	94±4	86±5	49±4*
PRPP amido transferase	100±6	72±4*	61±3*	59±4*
IMP dehydrogenase	100±5	91±6	44±4*	37±3*
Pyrimidine *de novo* synthesis	100±5	109±6	106±5	103±3
Carbamyl phosphate synthetase	100±7	93±6	87±4	85±3
Aspartate transcarbamylase	100±6	95±5	92±6	91±4
Thymidylate synthetase	100±5	100±6	121±7	151±6*
Tymidine kinase	100±6	94±5	83±6	79±3*
Thymidine monophosphate kinase	100±7	98±5	82±5	73±4*
Thymidine diphosphate kinase	100±6	91±4	89±5	85±4
d(ATP)	100±5			112±5
d(GTP)	100±6			133±4
d(CTP)	100±5			99±5
d(TTP)	100±4			95±4

of regulatory enzymes PRPP amido transferase being reduced 51% and 41% and IMP dehydrogenase being suppressed 65% and 63% after 60 min at 100 μM. *De novo* pyrimidine synthesis as well as the initial two regulatory enzymes, carbamyl phosphate synthetase and aspartate transcarbamylase of the pathway as well as thymidylate synthetase were not significantly affected by the compounds after 60 min. Nucleoside kinase activities were generally not reduced by the agents except compounds **1** and **2** caused a 27% -28% reduction of TMP kinase activity but compound **1** resulted in elevated TDP kinase activity by 47%. d[NTP] pool levels were generally not affected by the agents after 60 min except compound **1** caused a 41% reduction of d[CTP] pool levels [Tables 2 and 3].

Calf thymus-DNA studies demonstrated that the agents did not directly interact with the DNA molecule based on DNA viscosity, Tm values for DNA thermal denaturation and U.V. absorption from 220 to 340 nm. L1210 DNA strand scission studies showed that compound **1** and **2** did cause DNA strand scission at 100 μM for 24 h [Fig 1].

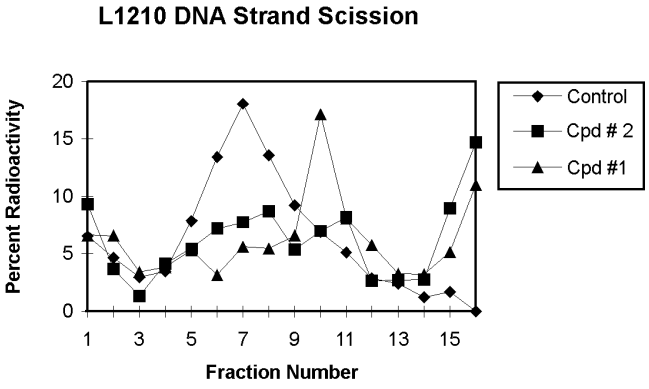

Figure 1. L1210 DNA strand scission after 24 h incubation at 100 μM of compounds **1** and **2**.

Discussion

Methyl 3,4 -bis(4-methoxyphenyl)pyrrole-2-carboxylate **1** and ethyl-*N*-methyl-3,4-bis(4-methoxyphenyl)pyrrole-2-carboxylate **2** proved to effective cytotoxic agents in the a number of leukemia and lymphoma screens. They were also active in the human HeLa-S[3] uterine and glioma screens. Compound **2** demonstrated selective activity against SW-480 colon adenocarcinoma growth but the compounds in general were less active against the growth of solid tumors. Their principal biochemical effect was inhibition of L1210 lymphocytic leukemia DNA synthesis with less effects on RNA and protein synthesis. The inhibition of DNA synthesis appeared to be the result of additive inhibitory effects of the agents on several enzyme activities including DNA polymerase α, dihydrofolate reductase, PRPP-amido transferase and IMP dehydrogenase over 60 min. The suppression of the later three enzyme activities would effectively reduce purine synthesis in the cancer cells. The reduction in purines should reduce DNA synthesis initially before RNA synthesis is affected because deoxyribonucleotide pools are approximately 10% of the ribonucleotide pools in mammalian cells. The marginal reduction of ribonucleoside reductase activity by the agents would further reduce deoxyribonucleotide pools for incorporation into DNA. The deoxyribonucleotide pools were not actually reduced after 60 min incubation with the compounds. This was probably a net effect since the agents inhibition DNA polymerase α activity significantly. Not only would this reduce DNA synthesis but the unincorporated d[NTP]s would accumulate in the cell because they were not incorporated into the new strand of DNA. The agents appeared to have minimum effect of pyrimidine *de novo* synthesis and on nucleoside kinase activities. ct-DNA studies suggested that the agents were not alkylating agents attacking the bases of DNA, were not intercalators between base pairs of DNA and did not cause DNA cross-linking. However, they did cause DNA fragmentation which should be followed by reduction in DNA synthesis and apoptosis. The biochemical effects and concentration of these derivatives are typical of agents which function as antimetabolites in cancer therapy.

Acknowledgments

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Experimental Part

Chemical Synthesis

Reagents: All chemicals were used as received from the manufacturer (Aldrich Chemicals and Fisher). Nuclear magnetic resonance spectra were obtained on a Varian Gemini 2000 NMR spectrometer in either $CDCl_3$ or d_6-DMSO solution. Infrared spectra were recorded on a Perkin Elmer 1420 IR spectrometer as either nujol mulls or KBr pellets. High resolution mass spectra were supplied by Dr. James A. Sikorski of Searle Corporation. Melting points and boiling points were uncorrected.

*(E)-3-Dimethylamino-1,2-bis(4-methoxyphenyl)propenone (**4**)*

To a solution of desoxyanisoin (5.00g, 19.5 mmol) in 100 ml DMF was added 10.4 ml of *N,N*-dimethylformamide dimethyl acetal (9.30 g, 78.0 mmol). The solution was stirred under inert atmosphere at reflux for 18 h. The solvent was removed by Kugelrohr distillation affording a brown solid (5.0g, 91%): mp = 114–117 °C ; tlc (1 spot) R_f = 0.56 (8:2 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 2.73 (s, 6H), 3.78 (s, 6H), 6.73–6.82 (m, 4H), 7.05 (d, 2H, *J* = 9 Hz), 7.34 (s, 1H) , 7.42 (d, 2H, *J* = 9 Hz); HR-MS ($C_{19}H_{21}NO_3$, M+1) calcd 312.1600, found 312.1599.

*(E)-3-Chloro-2,3-bis(4-methoxyphenyl)propenal (**5**)*

To a solution of (*E*)-3-dimethylamino-1,2-bis(4-methoxyphenyl)-propenone (0.11g, 0.35 mmol) in 10 ml of dichloromethane was added 3 drops of phosphorousoxychloride (~0.15g, 0.98 mmol). The solution was stirred under inert atmosphere at reflux for 4 h. The solvent was removed under reduced pressure. The crude residue was taken up in 20 ml of THF:water [1:1] and stirred at room temperature for 24 h. The THF was removed under reduced pressure. The aqueous mixture was washed 4 × 15 ml with chloroform. The chloroform layers were combined and dried over anhydrous magnesium sulfate, filtered and the solvent removed under reduced pressure affording a brown solid (0.100 g, 94%): mp 132–134 °C; tlc (2 spots, *E* and *Z* isomers) R_f = 0.40 and 0.46 (8:2 hexanes: ethyl acetate); IR (nujol) 1675 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz, major isomer, *E*) δ 3.84 (s, 3H), 3.88 (s, 3H), 6.97 (d, 4H, *J* = 9 Hz), 7.24 (d, 2H, *J* = 9 Hz),7.51 (d, 2H, *J* = 9 Hz), 9.65 (s, 1H); HR-MS ($C_{17}H_{15}ClO_3$, M+1) calcd 303.0788, found 303.0782.

*Methyl 3,4 -bis(4-methoxyphenyl)pyrrole-2-carboxylate (**1**)*

To a solution of (*E*)-3-chloro-2,3-bis(4-methoxyphenyl)propenal (79 mg, 0.26 mmol) in 10 ml dry DMF was added methyl glycinate hydrochloride (54 mg, 0.43 mmol). The solution was stirred under inert atmosphere at reflux for 20 h. The solvent was removed by Kugelrohr distillation. A crude black residue was obtained which was purified by passing it through a short plug of silica gel affording a light brown solid (72 mg, 82%) : mp 168–171 °C [lit ref. 169–171 °C] ; tlc (1 spot) R_f = 0.24 (75:25 hexanes:ethyl acetate) . IR (KBr) 3300 and 1670 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 3.73 (s, 3H), 3.76 (s, 3H), 3.82 (s, 3H), 6.75 (d, 2H, *J* = 9 Hz), 6.84 (d, 2 H, *J* = 9 Hz), 6.98–7.07 (m, 3H), 7.19 (d, 2H, *J* = 9 Hz), 9.18 (br s, 1H); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 51.38, 55.23, 113.25, 113.80, 119.47, 120.41, 126.54, 126.63, 127.27, 129.24, 129.61, 132.04, 158.19, 158.71, 161.90; HR-MS ($C_{20}H_{19}NO_4$, M+1).

*Ethyl N-Methyl-3,4-bis(4-methoxyphenyl)pyrrole-2-carboxylate (**2**)*

To a solution of (*E*)-3-chloro-2,3-bis(4-methoxyphenyl)propenal (a mixture of isomers) (195 mg, 0.64 mmol) in 10 ml dry DMF was added ethyl sarcosinate hydrochloride (139 mg, 0.91 mmol). The solution was stirred under inert atmosphere at reflux for 20 h. The solvent was removed by Kugelrohr distillation. A crude black residue was obtained which was purified by passing it through a short plug of silica gel affording a yellow-green oil (200 mg; 92%); bp 110–120 °*C* at 2.0 torr; tlc (1 spot) R_f = 0.56 (75:25 hexanes:ethyl acetate). IR (KBr) 1690 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.02 (t, 3H, *J* = 7 Hz), 3.74 (s, 3H), 3.81 (s, 3H), 3.96 (s, 3H), 4.08 (q, 2H, *J* = 7 Hz), 6.73 (d, 2H, *J* = 9 Hz), 6. 83 (d, 2H, *J* = 9 Hz), 6.88 (s, 1H), 7.00 (d, 2H, *J* = 9 Hz), 7.13 (d, 2H, *J* = 9 Hz), ^{13}C NMR ($CDCl_3$, 200 MHz) δ 13.93, 37.66, 55.23, 59.75, 113.06, 113.74, 120.72, 123.96, 126.70, 127.33, 128.49, 129.37, 131.13, 131.98, 158.01, 158.53, 162.11; HR-MS ($C_{22}H_{23}NO_4$, M + 1).

Pharmacological Studies

Cytotoxicity

Compounds **1** and **2** (Table 1) were tested for cytotoxic activity by homogenizing the drugs as a 1 mM solution in 0.05% Tween 80/H_2O. These solutions were sterilized by passing them through an acrodisc (0.45 μm). The following cell lines were maintained by literature techniques [17]: murine L_{1210} lymphoid leukemia and P388 lymphocytic leukemia, human $Tmolt_3$ and $Tmolt_4$ acute lymphoblastic T cell leukemia, Hl-60 leukemia, Hut-78 cutaneous lymphoma, THP-1 monocytic leukemia, SW480 colorectal adenocarcinoma, HCT-8 ileocecal adenocarcinoma, MB-9812 lung bronchogenic, A-549 lung carcinoma, TE-418 osteosarcoma, KB epidermoid nasopharynx, HeLa-S^3 suspended and HeLa solid cervical carcinoma, Sk-MEL-2 malignant melanoma and EH 118 MG glioma. The NCI protocol was used to assess the cytotoxicity of the test compounds and standards in each cell line. Values for cytotoxicity were expressed as ED_{50} = μM, i.e. the concentration of the compound inhibiting 50% of cell growth. ED_{50} values were determined by the trypan blue exclusion technique[17] . A value of less than 20 μM was required for significant activity of growth inhibition. Solid tumor cytotoxicity was determined utilizing crystal violet/MeOH and read at 580 nm (Molecular Devices)[18].

Incorporation Studies

Incorporation of labeled precursors into ^{3}H-DNA, ^{3}H-RNA and ^{3}H-protein for 10^6 L1210 leukemia cells was obtained [19] using a concentration range of 25 50 and 100 μM of the test agents over a 60 min incubations. The incorporation of ^{14}C-glycine (53.0 mCi/mmol) into purines[20] and the incorporation of ^{14}C-formate (53.0 mCi/mmol) into pyrimidines[21] was determined in a similar manner.

Enzyme Assays

Inhibition of various enzyme activities was performed by first preparing the appropriate L1210 cell homogenates or subcellular fractions, then adding the drug to be tested during the enzyme assay. For the concentration response studies, inhibition of enzyme activity was determined at 25, 50 and 100 μM of compounds **1** and **2**, after 60 min incubations. These concentrations of the agents were selected for 1 hour incubation to establish the mode of action of the derivatives quickly and are consistent with literature models [1–13] . DNA polymerase α activity was determined in cytoplasmic isolated extracts[22] . Nuclear DNA polymerase β was determined by isolating nuclei[23] . The polymerase assay for both α and β was determined with ^{3}H-TTP[24] . Messenger-, ribosomal- and transfer-RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate; individual RNA polymerase activities were determined using ^{3}H-UTP[25,26]. Ribonucleoside reductase activity was measured using ^{14}C-CDP with dithioerythritol[27]. The deoxyribonucleotides ^{14}C-dCDP were separated from the ribonucleotides by TLC on PEI plates. Thymidine, TMP and TDP kinase activities were determined using ^{3}H-thymidine (58.3 mCi/mmol)[28]. Carbamyl phosphate synthetase activity was determined [29] with citrulline quanititated colorimetrically[30] . Aspartate transcarbamylase activity was measured [29] and carbamyl aspartate was quanititated colorimetrically [31]. Thymidylate synthetase activity was analyzed by the ^3H_2O released which was proportional to the amount of TMP formed from ^{3}H-dUMP [32]. Dihydrofolate reductase activity was determined by a spectrophotometric method [33] . PRPP amidotransferase activity was determined by the method of Spassova et al.[34] . IMP dehydrogenase activity was analyzed with 8-^{14}C-IMP (54 mCi/mmol) (Amersham, Arlington Heights, IL) after separating XMP on PEI plates (Fisher Scientific) by TLC[35] . Protein content was determined for the enzymatic assays by the Lowry et al. technique [36].

DNA studies

After deoxyribonucleoside triphosphates were extracted [37] , levels were determined by the method of Hunting and Henderson [38] with calf thymus DNA, *E. 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 **2** on DNA strand scission was determined by the methods of Suzuki et al. [39] , Pera et al. [40] and Woynarowski et al. [41] . L1210 leukemia cells were incubated with 10 Ci thymidine [methyl-^{3}H, 84.0 Ci/mmol] for 24 h at 37°C. L1210 cells (10^{7}) were harvested and then centrifuged at 600 g × 10 min in PBS. They were later washed and suspended in 1 ml of PBS. Lysis buffer (0.5 ml; 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 (5 ml; 0.3 M NaOH, 0.7 KCl and 0.01 M EDTA); this was followed by 0.2 ml of the cell preparation. After the gradient was incubated for 2.5 h at room temperature, it was centrifuged at 12,000 RPM 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 measured for radio-activity. Thermal calf thymus DNA denaturation studies, DNA u.v. absorption studies and DNA viscosity studies were conducted after incubation of compounds **1**, and **2** at 100 μM at 37°C for 24 h [42] .

References

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