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The cytotoxicity and mode of action of 2,3,4-trisubstituted pyrroles and related derivatives in human $Tmolt_4$ leukemia cells

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4-Carbethoxy-1-methyl-2-phenacyl-3-phenylpyrrole (**9**), 4-carbethoxy-2-(4-methoxybenzoyl)-3-(4-methoxyphenyl)pyrrole (**10**) and 2-(4-methoxybenzoyl)-3,4-bis-(4-methoxyphenyl)pyrrole (**11**) proved to be potent cytotoxic agents against the growth of murine and human leukemias and lymphomas. Selective toxicity was demonstrated against the growth of solid tumors, e.g. human adenocarcinoma of the colon SW480 and ileum HCT-8, glioma U-87-MG, and rat UMR-106 osteosarcoma. A mode of action study in $Tmolt_4$ leukemia cells demonstrated that the agents inhibited *de novo* purine synthesis at the regulatory sites PRPP-amido transferase, IMP dehydrogenase as well as dihydrofolate reductase resulting in significant inhibition of DNA synthesis in 60 min. Other biochemical sites which were affected significantly were thymidylate synthetase, DNA polymerase α, RNA polymerases, nucleoside kinase and ribonucleoside reductase.

1. Introduction

Recently we have been interested in vinylogous iminium salt based synthesis [1–4] of highly functionalized pyrroles and their applications to the synthetic modifications of marine natural products [5, 6] that contain such functionality. The compounds included in this study have been synthesized in this context and are structurally related to or are potential precursors or metabolites of these pyrrole containing marine natural products. Previously, two alkyl-3,4-bis(4-methoxyphenyl)pyrrole-2-carboxylates proved to be potent cytotoxic agents in the murine L1210 lymphoid leukemia screen [7]. 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 marginally by the drugs were DNA polymerase α, RNA polymerases, ribonucleoside reductase and dihydrofolate reductase. 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. The present study involves a series of 2-carboalkoxy-pyrroles or 2-phenacyl pyrroles with diaryl substitutions in positions 3 and 4 which are precursors or analogs of the marine natural products lukianol A and polycitone A. Also included is a series of 4-carbethoxy-2-phenacyl-3-aryl pyrroles which are precursors of the marine natural product rigidin.

2. Investigations and results

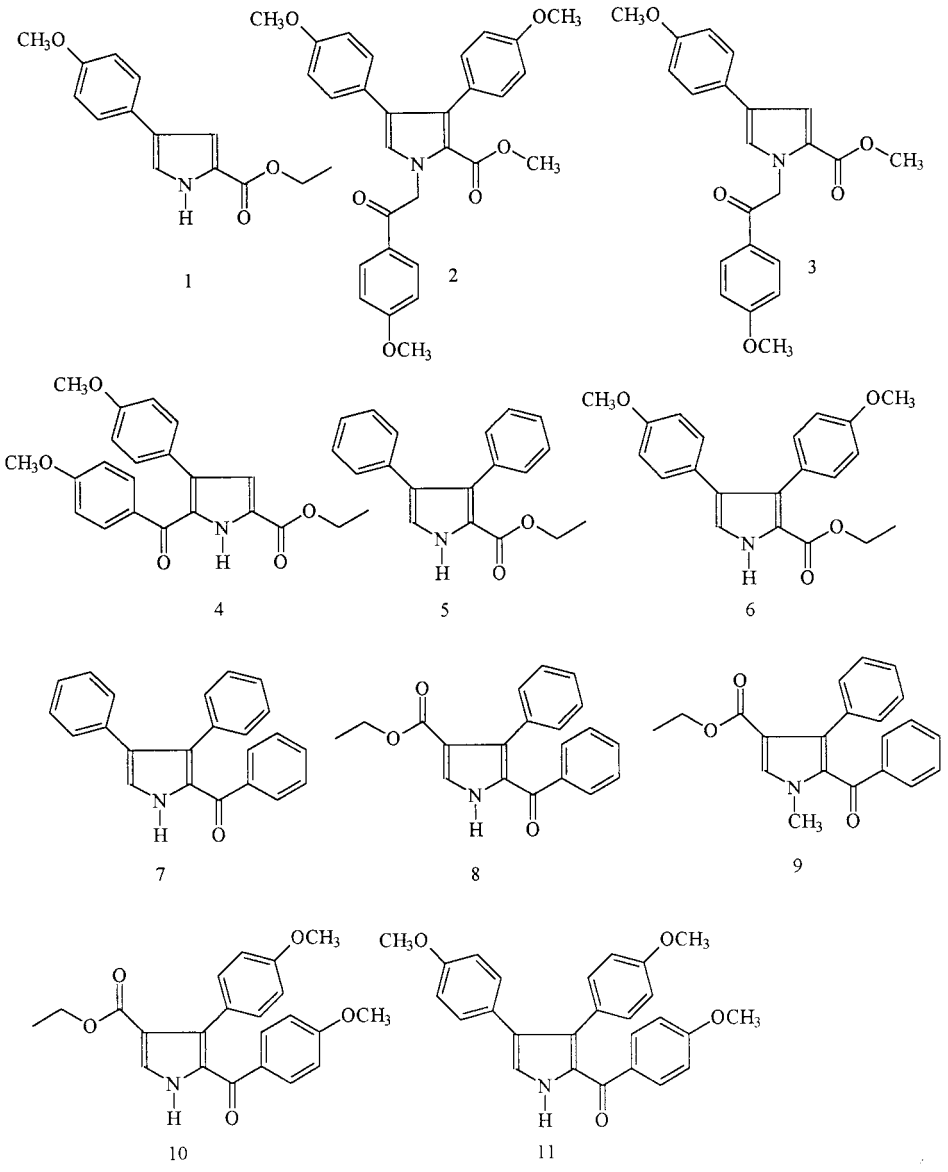
The pyrrole derivatives demonstrated potent cytotoxicity against the growth of murine and human tumor cells (Table 1). An ED_{50} value of less than 4 μg/ml is required for significant activity. In the mouse L1210 lymphoid leukemia screen all of the compounds were active with compounds **2**, **3**, **5**, **7**, **9** and **11** producing the most potent ED_{50} values of <2.00 μg/ml. In the P388 lymphocytic leukemia screen all of the compounds were active with compound **7** producing a value of 1.18 μg/ml. In the human leukemic screens, i.e. $Tmolt_3$, $Tmolt_4$ and HI-60, all of the compounds were active with compounds **2**, **3**, **5**, **6**, **9** and **11** having an ED_{50} value less than 2.00 μg/ml in the $Tmolt_3$ screen. Compounds **2** and **6** afforded ED_{50} values <2.00 μg/ml in the $Tmolt_4$ screen and compounds **2**, **3**, **6**, **7** and **10** were the most active in the Hl-60 screen. In the HuT-78 lymphoma screen all of the compounds were active with compound **10** affording an ED_{50} value of 0.93 μg/ml. In the THP-1 monocytic leukemia screen all of the compounds were active with compound **4**, **5**, and **6** producing a value of less than 1 μg/ml. In the HeLa-S^3 uterine suspended carcinoma compounds **3**, **4**, **6**, **9** and **10** afforded values <3.00 μg/ml. In the KB nasopharynx screen only compound **8** was active. Growth of adenocarcinoma of the SW480 colon was reduced significantly by compounds **4**, **5**, **6**, **8**, and **9**, and of adenocarcinoma of the ileum HCT-8 was suppressed by compounds **7**, **8**, **9**, **10** and **11**. Lung A549 growth was reduced only by compounds **7**, **8**, and **10**. Glioma U-87-MG growth was reduced by compounds **3** and **11**. None of the compounds were active against the growth of lung MB9812, solid HeLa uterine carcinoma, and melanoma. In the rat UMR-106 osteosarcoma screen compounds **3**, **7**, **8**, **9**, and **11** demonstrated potent activity with ED_{50} values of <2.00 μg/ml.

In the mode of action study in human $Tmolt_4$ leukemia cells both compounds **9** and **10** demonstrated preferential inhibition of DNA synthesis in a concentration dependent manner over 60 min resulting in 93% and 66% reduction, respectively at 100 μM (Tables 2–4).

Compound **11** had less effects with 46% reduction after 60 min. RNA synthesis was inhibited, i.e. 21% by compound **10**, 54% by compound **9** and 75% by compound **11** at 100 μM and protein synthesis was not significantly affected by compounds **9** and **10** but **11** caused 40% reduction over 60 min. DNA polymerase α activity was suppressed 32% by compound **11**, 44% by compound **9** and 55% by compound **10**. mRNA polymerase activity was inhibited 44% by compound **10**, 36% by compound **11** and 32% by compound **9**. r-RNA polymerase activity was reduced 33% by compound **9** and 58% by compound **10** while t-RNA polymerase activity was inhibited 61% by compound **10** and 19% by compound **11**. Ribonucleoside reductase activity was suppressed 28% and 32% by compounds **9** and **10** and dihydrofolate reductase activity was reduced 71% and 78% by the compounds **9** and **10** but compound **11** caused only 32% reduction at 100 μM over 60 min. *De novo* purine synthesis was suppressed 24% by

Table 1: Cytotoxicity of 2,3,4-trisubstituted pyrroles and related derivatives (ED_{50} = µg/ml, N = 6)

Compd.	L1210 Leukemia	P388 Leukemia	$Tmolt_3$ Leukemia	$Tmolt_4$ Leukemia	Hl-60 Leukemia	HuT-78 Lymphoma	THP-1 Acute monocytic	HeLa-S^3 uterine	KB Naso-pharynx	Colon SW 480	Lung A549
1	2.73	3.37	4.31	3.61	2.25	2.96	2.25	5.12	5.23	7.26	7.35
2	1.51	2.87	1.47	1.58	1.46	2.32	1.12	4.18	6.92	5.26	4.46
3	1.41	3.04	1.59	2.14	1.01	1.68	1.56	2.94	7.05	7.67	4.41
4	3.39	1.95	2.24	3.88	3.04	1.42	0.81	2.69	6.16	3.28	4.81
5	1.79	2.81	1.07	2.61	2.03	2.71	0.75	3.58	6.69	3.52	5.24
6	3.76	2.99	1.20	1.43	1.01	1.41	0.62	2.56	7.95	3.56	5.13
7	1.41	1.18	4.22	4.59	1.91	2.06	1.19	4.99	4.60	5.31	3.53
8	2.39	1.89	3.7	3.41	2.25	2.96	1.75	4.99	2.87	3.42	3.67
9	1.6	3.4	1.98	4.97	2.25	2.06	1.69	2.42	4.36	2.09	5.92
10	2.10	3.61	2.18	2.04	1.58	0.93	1.25	2.43	6.68	5.25	2.88
11	1.84	2.40	1.19	2.06	2.25	1.42	2.63	3.46	4.69	7.09	4.16
std											
6-MP	2.43	2.04	0.43	2.67	6.36	1.63	3.34	2.12	5.74	3.61	4.71
Ara-C	2.07	0.79	1.29	2.36	3.90	2.50	2.54	2.13	2.84	3.42	5.62
HU	2.67	1.30	4.47	6.68	5.22	3.87		1.96	5.27	7.33	8.89
5-FU	1.41	0.99	2.14	2.75	5.28	1.50	0.49	2.21	1.25	2.47	3.58
VP-16	1.83	0.99	1.00	1.92	3.49	1.13	3.27	1.89	3.32	0.93	4.74



Lung MB 9812	HeLa solid uterine	Melanoma Sk-2	HCT-8 Ileum	UMR-106 bone	Glioma U-87-MG
6.13	6.56	8.47	4.32	2.05	5.92
7.32	9.01	6.94	8.81	10.14	4.16
5.95	6.40	8.65	4.67	0.39	3.40
8.85	10.56	10.01	5.72	7.53	4.83
7.51	7.62	7.43	8.31	9.55	4.31
6.83	7.73	10.01	9.88	8.95	4.82
6.60	4.64	8.12	2.51	1.57	7.46
4.81	6.34	8.12	2.34	0.86	7.51
5.09	6.72	7.6	2.51	1.54	4.90
8.75	8.91	8.61	3.7	3.36	7.03
7.67	7.36	8.00	3.85	0.63	3.40
4.29	5.61	6.86	1.15	9.13	4.46
6.16	4.74	10.53	2.54	0.86	1.88
7.18	8.12		1.77	2.87	2.27
5.07	4.11	5.93	1.12	3.52	1.28
3.50	3.05	3.53	3.78	3.57	2.44

compound **10**, 42% by compound **9** and 48% by compound **11** at 100 μM while the activity of the regulatory enzymes of this pathway were also inhibited, i.e. PRPP-amido transferase activity 48% by compound **10** and 63% by compound **9** while IMP dehydrogenase activity 35% by compound **10** and 72% to 73% by compounds **9** and **11** over 60 min. *De novo* pyrimidine synthesis after 60 min incubation at 100 μM by compound **9** was inhibited 37%. The activity of the early regulatory enzymes of the pathway were not affected by the agents except aspartate transcarbamylase activity was reduced 24% at 100 μM by compound **11**. Thymidylate synthetase activity over 60 min at 100 μM was suppressed 62% by compound **9** and 81% by compound **10**. Thymidine kinase activity was reduced 25% by compound **9** and 39% by compound **10** but elevated 110% by compound **11**. TMP and TDP kinase activities were inhibited 24% and 60%, respectively by compound **10** at 100 μM over 60 min. Compound **11** elevated TMP kinase activity 99% after 60 min. Compound **10** lowered the pool levels of d[GTP] by 77% and d[TTP] by 82% and compound **9** lowered d[TTP] pool levels by 71% after 60 min incubation at 100 μM. Compound **11** lowered d[ATP] pools 27%, d[GTP] pools 54% and d[TTP] pools 24% at 100 μM.

Calf-thymus DNA studies demonstrated that the UV absorption from 220 to 340 nm was not affected by compounds **9** and **10** whereas compound **11** causes a hyperchromic shift to a higher wavelength. ct-DNA denaturation studies showed that the control Tm value was 85 °C while compound **11** was 77.5 °C, compound **10** was 72.5 °C and compound **9** was 45 °C. ct-DNA viscosity for the control was 607 s whereas compound **10** was 345 s, compound **9** was 601 s and compound **11** was 608 s. $Tmolt_4$ DNA fragmentation studies after 24 h incubation at 100 μM showed that compounds **10** and **11** caused DNA strand scission (Fig., low molecular weight) but compound **9** appears to cause cross-linking of DNA since a higher percentage of radioactivity appeared at fraction number 17 (the highest molecular weight).

3. Discussion

2,3,4-Trisubstituted pyrroles proved to be potent cytotoxicity agents suppressing the growth of suspended cells, e.g. murine L1210 and P388 leukemia cells, human $Tmolt_3$, $Tmolt_4$ and Hl-60 leukemia, Hut-78 lymphomas, THP-1 monocytic leukemia, and suspended HeLa uterine carcinoma. More selectivity was demonstrated by the compounds against the growth of tissue cultured cells derived from solid tumors. Selected compounds were active against human KB nasopharynx, SW480 colon, lung A549, HCT-8 ileum, glioma U-87-MG and rat UMR-106 osteosarcoma

Table 2: Effects of compound 9 on $Tmolt_4$ leukemia cell metabolism over 60 min

[N = 4]	Control	Percent of control (X ± S.D.)		
		25 μM	50 μM	100 μM
Assay				
DNA synthesis	100 ± 5[a]	17 ± 3*	8 ± 2*	7 ± 2*
RNA synthesis	100 ± 6[b]	51 ± 4*	46 ± 4*	46 ± 5*
Protein synthesis	100 ± 5[c]	99 ± 6	86 ± 5	86 ± 6
DNA polymerase alpha	100 ± 4[d]	126 ± 7	112 ± 5	56 ± 4*
mRNA polymerase	100 ± 7[e]	156 ± 6	84 ± 5	68 ± 4*
rRNA polymerase	100 ± 4[f]	79 ± 6	68 ± 4*	67 ± 4*
tRNA polymerase	100 ± 7[g]	103 ± 8	87 ± 5	85 ± 6
Ribonucleoside reductase	100 ± 5[h]	86 ± 6	78 ± 5*	72 ± 4*
Dihydrofolate reductase	100 ± 5[i]	52 ± 4*	49 ± 3*	22 ± 3*
Purine *de novo* synthesis	100 ± 5[j]	70 ± 4*	62 ± 5*	58 ± 4*
PRPP amido transferase	100 ± 6[k]	96 ± 6	63 ± 5*	37 ± 4*
IMP dehydrogenase	100 ± 5[l]	71 ± 4*	52 ± 4*	27 ± 2*
Pyrimidine *de novo* synthesis	100 ± 6[m]	90 ± 5	82 ± 4	63 ± 4*
Carbamyl phosphate synthetase	100 ± 7[n]	120 ± 6	116 ± 5	115 ± 6
Aspartate transcarbamylase	100 ± 6[o]	100 ± 5	99 ± 5	99 ± 6
Thymidylate synthetase	100 ± 5[p]	85 ± 5	69 ± 4*	38 ± 4*
Thymidine kinase	100 ± 6[q]	139 ± 7*	109 ± 6	76 ± 4*
Thymidine monophosphate kinase	100 ± 7[r]	248 ± 8*	238 ± 7*	113 ± 6
Thymidine diphosphate kinase	100 ± 6[s]	128 ± 6	143 ± 7*	96 ± 5
d[ATP]	100 ± 5[t]			109 ± 5
d[GTP]	100 ± 6[u]			91 ± 4
d[CTP]	100 ± 5[v]			106 ± 4
d[TTP]	100 ± 4[w]			29 ± 4*

Control values for 10^6 cells/h
[a] 12719 dpm, [b] 11261 dpm, [c] 14382 dpm, [d] 6090 dpm, [e] 2341 dpm, [f] 673 dpm, [g] 874 dpm, [h] 56734 dpm, [i] 0.198 O.D. units, [j] 27621 dpm, [k] 0.1236 O.D. units, [l] 15578 dpm, units, [m] 17342 dpm, [n] 0.912 mol N-carbamyl spartate, [o] 0.456 mmol citrulline, [p] 657642 dpm, [q] 2345 dpm, [v] 346579 dpm, [s] 2674 dpm, [t] 17.23 pmol, [u] 16.38 omol, [r] 23.45 pmol, [w] 18.14 pmol, * P ≡ 0.001 Student's "t" test

Table 3: Effects of compound 10 on Tmolt$_4$ leukemia cell metabolism over 60 min

[N = 4]	Control	Percent of control (X ± S.D.)		
		25 μM	50 μM	100 μM
Assay				
DNA synthesis	100 ± 5	66 ± 4*	61 ± 3*	34 ± 3*
RNA synthesis	100 ± 6	145 ± 5*	83 ± 5	79 ± 4*
Protein synthesis	100 ± 5	119 ± 6	114 ± 5	90 ± 5
DNA polymerase alpha	100 ± 4	105 ± 5	76 ± 4*	45 ± 4*
mRNA polymerase	100 ± 7	101 ± 6	61 ± 5*	56 ± 4*
rRNA polymerase	100 ± 4	50 ± 5*	46 ± 4*	42 ± 4*
tRNA polymerase	100 ± 7	87 ± 6	59 ± 5*	39 ± 4*
Ribonucleoside reductase	100 ± 5	82 ± 5	75 ± 4*	68 ± 4*
Dihydrofolate reductase	100 ± 5	57 ± 3*	30 ± 2*	29 ± 3*
Purine *de novo* synthesis	100 ± 5	109 ± 5	84 ± 5	76 ± 4*
PRPP amido transferase	100 ± 6	84 ± 5	83 ± 4	52 ± 3*
IMP dehydrogenase	100 ± 5	80 ± 4	65 ± 4*	65 ± 3*
Pyrimidine *de novo* synthesis	100 ± 6	111 ± 7	103 ± 6	95 ± 5
Carbamyl phosphate synthetase	100 ± 7	90 ± 6	89 ± 5	83 ± 5
Aspartate transcarbamylase	100 ± 6	100 ± 5	100 ± 6	89 ± 6
Thymidylate synthetase	100 ± 5	91 ± 5	82 ± 4	19 ± 2*
Thymidine kinase	100 ± 6	131 ± 5	81 ± 4*	61 ± 3*
Thymidine monphosphate kinase	100 ± 7	94 ± 5	93 ± 5	75 ± 4*
Thymidine diphosphate kinase	100 ± 6	78 ± 4*	52 ± 4*	40 ± 3*
d[ATP]	100 ± 5			108 ± 6
d[GTP]	100 ± 6			23 ± 3*
d[CTP]	100 ± 5			112 ± 6
d[TTP]	100 ± 4			18 ± 4*

growth. None of the compounds were active against the growth of lung MB9812, solid HeLa, or melanoma. The pattern of cytotoxicity demonstrated by these compounds is similar to known clinical antineoplastic agents which are metabolic inhibitors. A mode of action study in human Tmolt$_4$ leukemia cells demonstrated that the agents preferentially inhibited DNA synthesis after 60 min incubation followed by the suppression of RNA synthesis. Protein synthesis was not affected by the agents except by compound **11** over 60 min at these concentrations. Examination of sites within the nucleic acid pathway where the agents had their major effects appeared to be DNA polymerase α, dihydrofolate reductase, purine *de novo* synthesis and thymidylate synthetase. Reduction by the agents of all of these enzyme reactions would add to the overall suppression of DNA synthesis. The inhibition by the agents of the activities of the two regulatory enzyme of purine synthesis, i.e. PRPP-amido transferase and IMP dehydrogenase in a concentration dependent manner would lead to lower nucleotides for RNA and DNA synthesis but the suppression would appear in DNA synthesis quicker than RNA since the ratio of RNA to DNA pools in mam-

Table 4: Effects of compound 11 on Tmolt$_4$ leukemia cell metabolism over 60 min

[N = 4]	Control	Percent of control (X ± S.D.)		
		25 μM	50 μM	100 μM
Assay				
DNA synthesis	100 ± 5	88 ± 5	66 ± 5	54 ± 4*
RNA synthesis	100 ± 6	53 ± 3*	30 ± 2*	25 ± 4*
Protein synthesis	100 ± 5	132 ± 6*	98 ± 5	60 ± 4*
DNA polymerase α	100 ± 6	72 ± 4*	70 ± 5*	68 ± 4*
mRNA polymerase	100 ± 7	85 ± 5	76 ± 4*	64 ± 4*
rRNA polymerase	100 ± 4	94 ± 5	94 ± 4	84 ± 5
tRNA polymerase	100 ± 7	103 ± 4	81 ± 5	81 ± 4*
Ribonucleoside reductase	100 ± 5	125 ± 6	93 ± 5	86 ± 4
Dihydrofolate reductase	100 ± 5	126 ± 6	112 ± 5	68 ± 4*
Purine *de novo* synthesis	100 ± 5	70 ± 3*	61 ± 4*	52 ± 3*
PRPP amido transferase	100 ± 6	105 ± 5	94 ± 4	88 ± 4
IMP dehydrogenase	100 ± 5	55 ± 5*	44 ± 3*	28 ± 3*
Pyrimidine *de novo* synthesis	100 ± 5	107 ± 4	97 ± 4	93 ± 5
Carbamyl phosphate synthetase	100 ± 8	116 ± 4	115 ± 5	85 ± 5
Aspartate transcarbamylase	100 ± 6	112 ± 5	98 ± 6	76 ± 4*
Thymidylate synthetase	100 ± 5	139 ± 7*	118 ± 4	94 ± 5
Thymidine kinase	100 ± 6	149 ± 6*	202 ± 7*	210 ± 6*
Thymidine monphosphate kinase	100 ± 7	120 ± 6	144 ± 5*	199 ± 6*
Thymidine diphosphate kinase	100 ± 6	153 ± 5*	113 ± 4	82 ± 5
d[ATP]	100 ± 5			73 ± 4*
d[GTP]	100 ± 6			46 ± 5*
d[CTP]	100 ± 5			96 ± 5
d[TTP]	100 ± 4			76 ± 4*

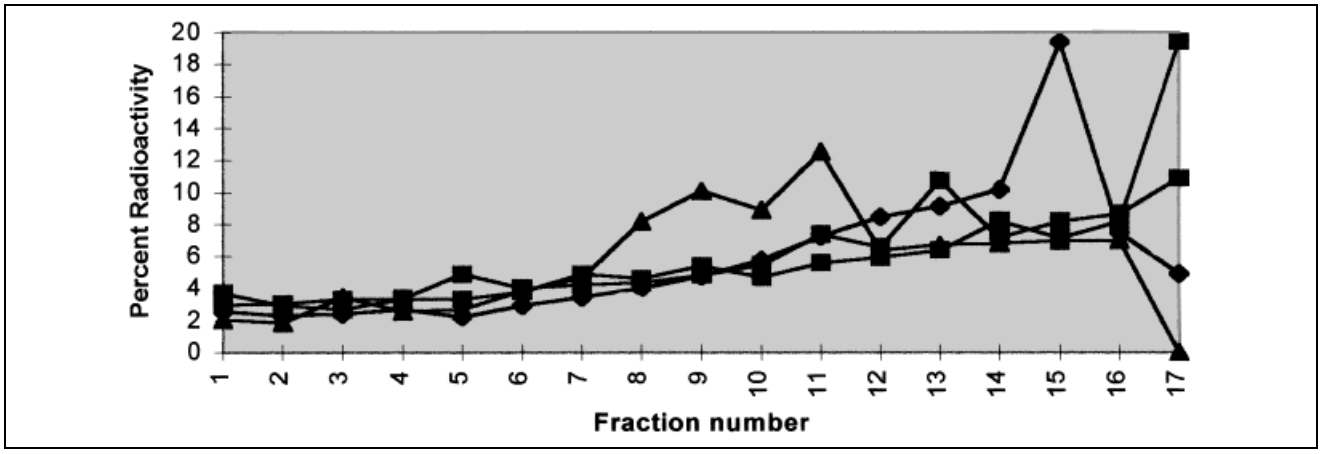


Fig.: $Tmolt_4$ leukemia DNA strand scission after 24 h incubation at 100 μM

malian cells is 90 : 10 [34]. The suppression of dihydrofolate reductase activity afforded by the compounds would cause suppression of one-carbon transfer required for purine synthesis. Reduction of thymidylate synthetase activity caused by the agents would lead ultimately to reduced TTP pool levels for incorporation into DNA. Interestingly these reduced pool levels were evident after 60 min. Other marginal effects of the agents were on ribonucleoside reductase activity which would lower the levels of deoxyribonucleotides for incorporation into the new strand of DNA. In addition, RNA synthesis was probably suppressed due to the inhibitory effects of the agents on RNA polymerase activities over 60 min.

4-Carbethoxy-2-(4′-methoxybenzoyl)-3-(4′-methoxyphenyl)pyrrole (**10**) appeared to have some additional effects on nucleic acid metabolism in that thymidine, TMP and TDP kinase activities were suppressed. 4-Carbethoxy-1-methyl-2-phenacyl-3-phenylpyrrole (**9**) suppressed only thymidine kinase activity but was more effective in blocking *de novo* pyrimidine synthesis. Thus, the substitution of different functional groups on the aromatic rings and the nitrogen of the pyrrole do make some difference in their effects on enzymes of nucleic acid metabolism.

ct-DNA studies suggested that the agents did not cause akylation of the bases of DNA since there was no hyperchromic shift of the absorption of DNA except with compound **11**. On the other hand, reduction in ct-DNA viscosity and $Tmolt_4$ DNA strand scission by compound **10** suggest this agent caused DNA fragmentation after 24 h. Conversely compound **9** increased ct-DNA viscosity and probably caused cross-linking of the two strands of DNA. The changes in the Tm value with compound **9** suggest a reaction with the DNA molecule also. At first these effects of the two agents on ct-DNA appear different from each other. One explanation may be that the compounds interact with DNA in a deleterious manner which is not stable and that compound **10** causes DNA fragmentation quicker than compound **9**. More detailed studies are required to address this effect of the agents.

In conclusion, these pyrroles offer a new chemical series of compounds which may be explored in the future as antineoplastic agents to be used in combination chemotherapy or in resistant cancer cells. They demonstrate a therapeutic profile which is very similar to current clinically used antineoplastic agents.

4. Experimental

4.1. Materials and apparatus

The following procedures are typical of the experimental conditions used for the reaction of vinylogous iminium salts and other three-carbon synthons with α-amino carbonyl compounds. The vinylogous iminium salts and β-chloroenals were prepared by standard methods [1–4]. All purified compounds gave a single spot upon TLC analysis on silica gel 7GF eluted with ethyl acetate/hexane mixture (30 : 70), respectively. Distillations were carried out on an Aldridge Kugelrohr apparatus at reduced pressure. Chromatographic separations were carried out on a Harrison Chromatotron using silica gel plates of 2 mm thickness with a fluorescent backing or by standard column chromatography techniques. IR spectra were recorded on a Perkin-Elmer-1600 FTIR and NMR spectra were obtained with a Varian Gemini 200 spectrometer. All samples gave ^{13}C NMR spectra consistent with a sample purity in excess of 98%. All melting and boiling points are uncorrected. High resolution MS were obtained by Midwest Center for Mass Spectrometry at the University of Nebraska-Lincoln. The following compounds were synthesized by previously published methods: 2-carbethoxy-4-(4-methoxyphenyl)pyrrole (**1**) [1], lamellarin-O-dimethyl ether (**2**) [5], and 2-carbethoxy-3,4-diphenylpyrrole (**5**) [4].

4.2. Synthesis

4.2.1. N-(4′-Methoxyphenethyl-2-oxo)-4-(4′-methoxyphenyl)-2-carbethoxy pyrrole (**3**)

Previously reported methods [1,5] were used to prepare compound **3**. This compound exhibited the following properties: m.p. 154–155 °C; ^1H NMR ($CDCl_3$) δ 3.74 (s, 3 H), 3.81 (s, 3 H), 3.88 (s, 3 H), 5.72 (s, 2 H), 6.88 (d, J = 8.8 Hz, 2 H), 6.96 (d, J = 8.8 Hz, 2 H), 7.20 (d, J = 1.8 Hz, 1 H), 7.25 (d, J = 1.8 Hz, 1 H), 7.44 (d, J = 8.8 Hz, 2 H), 7.98 (d, J = 8.8 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 51.3, 54.9, 55.4, 55.7, 114.3, 115.3, 122.9, 124.7, 126.2, 126.6, 127.4, 128.1, 130.5, 158.4, 162.0, 164.3, and 191.9; IR (KBr) 1600 cm^{-1}; MS (FAB) calcd. 380.1497, found 380.1535 (M + H). $C_{22}H_{21}NO_5$

4.2.2. 2-Carbethoxy-4-(4′-methoxyphenyl)-5-(4′-methoxybenzoyl)pyrrole (**4**)

To a solution of 3.13 g (20.57 mmol) of 4-methoxybenzoic acid in 25 ml dry methylene chloride was added 4.32 g (20.57 mmol) of trifluoroacetic anhydride. The resulting solution was stirred for 15–20 min at room temperature followed by the addition of 5.48 g (48.02 mmol) of trifluoroacetic acid and then stirred for 5 min at room temperature. Subsequently, 1.68 g (6.86 mmol) of 2-carbethoxy-4-(4′-methoxyphenyl)-pyrrole was added upon which the solution darkened immediately. This solution was then stirred at room temperature for 3 d and the reaction was quenched by the addition of sodium hydroxide pellets followed by stirring overnight at room temperature. The mixture was transferred to a separatory funnel where it was partitioned between chloroform and water (3 × 150 ml). The combined chloroform layers were dried over anh. magnesium sulfate, filtered and concentrated. The crude residue was found to be pure enough to be subjected to radial chromatography using hexane 90 : 10 hexane/ethyl acetate, followed by 80 : 20 hexane/ethyl acetate as eluant. After removal of the solvent, 2.45 g (6.47 mmol, 94.3%) of brownish orange crystals (**4**) were obtained. Compound **4** had the following properties: m.p. 145–

147 °C; ^{1}H NMR ($CDCl_3$) δ 1.39 (t, J = 7.1 Hz, 3 H), 3.73 (s, 3 H), 3.75 (s, 3 H), 4.39 (q, J = 7.1 Hz, 2 H), 6.63 (d, J = 8.9 Hz, 2 H), 6.66 (d, J = 8.9 Hz, 2 H), 6.99 (d, J = 2.9 Hz, 1 H), 7.06 (d, J = 8.9 Hz, 2 H), 7.54 (d, J = 8.9 Hz, 2 H) and 9.86 (bs, 1 H); ^{1}H NMR (DMSO-d6) δ 1.39 (t, J = 7.1 Hz, 3 H), 3.7 (s, 3 H), 3.8 (s, 3 H), 4.3 (q, J = 7.1 Hz, 2 H), 6.76 (d, J = 8.9 Hz, 2 H), 6.89 (d, J = 8.9 Hz, 2 H), 6.99 (d, J = 2.6 Hz, 1 H), 7.16 (d, J = 8.9 Hz, 2 H), 7.62 (d, J = 8.9 Hz, 2 H), 12.5 (bs, 1 H); ^{13}C NMR ($CDCl_3$) δ 16.4, 31.7, 57.2, 57.3, 63.1, 115.1, 115.5, 118.1, 127.5, 129.1, 131.5, 131.6, 132.5, 133.7, 134.1, 160.6, 162.4, 164.9 and 188.1; IR (KBr) 3309, 2970, 1711, 1597, 1453 and 1175 cm^{-1}; HRMS (EI) calcd. 379.1420, found 379.1421.
$C_{22}H_{21}NO_5$

4.2.3. 2-Carbethoxy-3,4-bis(4′-methoxyphenyl)pyrrole (**6**)

This compound was prepared in an analogous manner to compound **5** [4]. This compound exhibited the following properties: m.p. 119–123 °C; ^{1}H NMR ($CDCl_3$) δ 1.18 (t, J = 7 Hz, 3 H), 3.76 (s, 3 H), 3.81 (s, 3 H), 4.20 (q, J = 7 Hz, 2 H), 6.75 (d, J = 8 Hz, 2 H), 6.84 (d, J = 8 Hz, 2 H), 7.00–7.06 (m, 3 H), 7.20 (d, J = 8 Hz, 2 H) and 9.30 (broad s, 1 H); ^{13}C NMR ($CDCl_3$) δ 14.29, 55.32, 60.29, 113.19, 113.86, 119.96, 120.05, 126.63, 126.79, 127.36, 129.12, 129.67, 132.19, 158.25, 158.77 and 161.44; IR (KBr) 3300 and 1675 cm^{-1}; HRMS calcd. 351.1471, found 351.1443.
$C_{21}H_{21}NO_4$

4.2.4. 3,4-Diphenyl-2-phenacylpyrrole (**7**)

This compound was prepared in a manner analogous to the previously reported procedure [4] with the exception that α-aminoacetophenone hydrochloride was used in the place of glycine ethyl ester in the reaction. The purified product exhibited the following physical properties: m.p. 178–179 °C; ^{1}H NMR ($CDCl_3$) δ 6.80–7.30 (m, 14 H), 7.38 (d, J = 8.0 Hz, 2 H) and 9.68 (br. s, 1 H); ^{13}C NMR ($CDCl_3$) δ 125.1, 128.2, 128.5, 128.7, 128.8, 129.4, 129.6, 130.2, 130.4, 130.6, 131.1, 131.9, 132.7, 132.8, 133.0, 133.2, 136.1, 136.2, 136.4, 136.5, 139.8, 139.9 and 189.9; IR (CCl_4) 3446, 3283, 1602, 1544 and 1385 cm^{-1}; HRMS (EI) calcd. 323.1310, found 323.1297.
$C_{23}H_{17}NO$

4.2.5. 4-Carbethoxy-2-phenacyl-3-phenylpyrrole (**8**)

This compound was prepared in a manner analogous to a previously reported procedure [4] with the exception the α-aminoacetophenone hydrochloride was used in place of glycine ethyl ester in the reaction. The purified product exhibited the following physical properties: m.p. 141–142 °C; ^{1}H NMR (DMSO-d6) δ 1.11 (t, J = 7.1 Hz, 3 H), 4.06 (q, J = 7.1 Hz, 2 H), 7.00–7.18 (m, 7 H), 7.2–7.40 (m, 3 H), 7.73 (d of d, J = 3.5 Hz and 1.3 Hz, 1 H) and 12.58 (br. s, 1 H); ^{13}C NMR ($CDCl_3$) δ 16.2, 62.0, 118.9, 129.0, 129.1, 129.4, 130.8, 130.9, 131.7, 133.1, 133.4, 135.0, 135.4, 139.1, 165.9 and 190.0; IR (CCl_4) 3246, 1718 and 1610 cm^{-1}; HRMS (EI) calcd. 319.1208, found 319.1194.
$C_{20}H_{17}NO_3$

4.2.6. 4-Carbethoxy-1-methyl-2-phenacyl-3-phenylpyrrole (**9**)

This compound was prepared by the reaction of 4-carbethoxy-2-phenacyl-3-phenylpyrrole with iodomethane, sodium hydride in DMF in a manner previously reported [4]. A 100% crude yield was obtained and a 98% purified yield of a light yellow solid resulted after purification by radial chromatography. The purified product exhibited the following properties: m.p. 146–147 °C; ^{1}H NMR (DMSO-d6) δ 1.13 (t, J = 7.1 Hz, 3 H), 3.85 (s, 3 H), 4.08 (q, J = 7.1 Hz, 2 H), 6.96–7.18 (m, 7 H), 7.30 (t, J = 7.2 Hz, 1 H), 7.42 (d of d, J = 7.0 Hz and 1.2 Hz, 2 H) and 7.91 (s, 1 H); ^{13}C NMR ($CDCl_3$) δ 16.2, 39.3, 61.8, 115.4, 128.9, 129.5, 131.6, 132.2, 133.1, 133.9, 135.3, 135.7, 140.1, 165.8 and 191.0; IR (CCl_4) 3065, 2958, 1706, 1632 and 1252 cm^{-1}; HRMS (EI) calcd. 333.1365, found 333.1353.
$C_{21}H_{19}NO_3$

4.2.7. 4-Carbethoxy-2-(4′-methoxybenzoyl)-3-(4′-methoxyphenyl)pyrrole (**10**)

This compound was prepared in a manner analogous to compound **8**. The purified product exhibited the following properties: m.p. 151–152 °C; ^{1}H NMR ($CDCl_3$) δ 1.21 (t, J = 7.1 Hz, 3 H), 3.70 (s, 3 H), 3.72 (s, 3 H), 4.17 (q, J = 7.1 Hz, 2 H), 6.52 (d, J = 9.0 Hz, 2 H), 6.60 (d, J = 9.0 Hz, 2 H), 7.02 (d, J = 9 Hz, 2 H), 7.37 (d, J = 9.0 Hz, 2 H), 7.72 (d, J = 3 Hz, 1 H) and 10.20 (broad s, 1 H); ^{13}C NMR ($CDCl_3$) δ 14.4, 55.3, 55.4, 60.0, 112.9, 113.0, 116.6, 125.8, 128.6, 129.8, 130.1, 131.7, 132.6, 159.0, 162.6, 164.3 and 187.1; IR (nujol) 3300 and 1680 cm^{-1}; HRMS calcd. 380.1498, found 380.1517.
$C_{22}H_{22}NO_5$

4.2.8. 2-(4-Methoxybenzoyl)-3,4-di-(4′-methoxyphenyl)pyrrole (**11**)

This compound was prepared in a manner analogous to compound **7**. The purified product exhibited the following properties: m.p. 77–79 °C; ^{1}H NMR ($CDCl_3$) δ 3.70 (s, 3 H), 3.72 (s, 3 H), 3.78 (s, 3 H), 6.52 (d, J = 8.8 Hz, 2 H), 6.54 (d, J = 8.8 Hz, 2 H), 6.75 (d, J = 8.8 Hz, 2 H), 6.80 (d, J = 8.8 Hz, 2 H), 7.00 (d, J = 8.8 Hz, 2 H), 7.15 (d, J = 2.9 Hz, 1 H), 7.40 (d, J = 8.8 Hz, 2 H) and 9.45 (broad s, 1 H); ^{13}C NMR ($CDCl_3$) δ 55.3, 55.4, 113.0, 113.5, 113.8, 113.9, 122.0, 127.0, 127.4, 129.3, 129.6, 129.9, 130.6, 131.7, 132.6, 158.4, 158.6, 162.3 and 186.7; IR (KBr) 3350 and 1600 cm^{-1}; HRMS (EI) calcd. 414.1705, found 414.1693.
$C_{26}H_{24}NO_4$

4.3. Pharmacological studies

4.3.1. Cytotoxicity

Compounds **1** and **11** (Table 1) were tested for cytotoxic activity by homogenizing the drugs as a 1 mM solution in 0.05% Tween® 80/water. These solutions were sterilized by passing them through an acrodisc (0.45 μm). The following cell lines were maintained by literature techniques [8]: murine L_{1210} lymphoid leukemia and P388 lymphocytic leukemia, human $Tmolt_4$ and $Tmolt_3$ 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 U-87-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} = μg/ml, 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 4 μg/ml was required for significant activity of growth inhibition. Solid tumor cytotoxicity was determined utilizing crystal violet/MeOH and read at 580 nm (Molecular Devices) [9].

4.3.2. Incorporation studies

Incorporation of labeled precursors into ^{3}H-DNA, ^{3}H-RNA and ^{3}H-protein for 10^6 $Tmolt_4$ leukemia cells was obtained [10] 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 [11] and the incorporation of ^{14}C-formate (53.0 mCi/mmol) into pyrimidines [12] was determined in a similar manner.

4.3.3. Enzyme assays

Inhibition of various enzyme activities was performed by first preparing the appropriate $Tmolt_4$ leukemia 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 **9**, **10** and **11**, after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic isolated extracts [13]. Nuclear DNA polymerase β was determined by isolating nuclei [14]. The polymerase assay for both α and β was determined with ^{3}H-TTP [15]. 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 [16, 17]. Ribonucleoside reductase activity was measured using ^{14}C-CDP with dithioerythritol [18]. 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) [19]. Carbamyl phosphate synthetase activity was determined [20] with citrulline quanititated colorimetrically [21]. Aspartate transcarbamylase activity was measured [20] and carbamyl aspartate was quanititated colorimetrically [22]. Thymidylate synthetase activity was analyzed by the ^3H_2O released which was proportional to the amount of TMP formed from ^{3}H-dUMP [23]. Dihydrofolate reductase activity was determined by a spectrophotometric method [24]. PRPP amidotransferase activity was determined by the method of Spassova et al. [25]. 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 [26]. Protein content was determined for the enzymatic assays by the Lowry et al. technique [27].

4.3.4. ct-DNA studies

After deoxyribonucleoside triphosphates were extracted [28], levels were determined by the method of Hunting and Henderson [29] 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 **9**, **10** and **11** on DNA strand scission was determined by the methods of Suzuki et al. [31], Pera et al. [30] and Woynarowski et al. [32]. $Tmolt_4$ leukemia cells were incubated with 10 μCi 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 for 10 min in PBS. They were later washed and suspended in 1 ml of PBS. Lysis buffer (0.5 ml; 0.5 M sodium hydroxide, 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 sodium hydroxide, 0.7M potassium

chloride 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 hydrochloric acid and measured for radioactivity. Thermal calf thymus DNA denaturation studies, DNA UV absorption studies and DNA viscosity studies were conducted after incubation of compounds **9**, **10** and **11** at 100 μM at 37 °C for 24 h [33].

4.3.5. Statistical analysis

Data is displayed in Tables and the Fig. as the means ± standard deviations of the mean. N is the number of samples or animals 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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