Anti-Cancer Drugs, **4**, pp. 389 393

The cytotoxicity of *N*-substituted indazolones in murine and human tumor cells

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***N*-Substituted indazolones are effective cytotoxic agents, causing cell death in a number of tissue culture lines, e.g. L1210, $Tmolt_3$, colon adenocarcinoma and HeLa-S^3. Selected agents were also active against the growth of KB, bronchogenic lung, osteosarcoma and glioma. The mode of action of the derivatives involves inhibition of *de novo* purine synthesis of L1210 cells, which reduces DNA and RNA syntheses. Agents lowered d(NTP) pools, further reducing DNA synthesis. DNA strand scission was evident after incubation with *N*-substituted indazolones for 24 h at 100 μM, lowering DNA synthesis and causing cell death.**

Key words: Cytotoxicity, IMP dehydrogenase, indazolones, nucleic acid inhibitors, purine inhibitors.

Introduction

A series of cyclic imides and related derivatives have proved to possess cytotoxicity, e.g. indan-1,3-dione,[1] diphenamides 6,7-dihydro-5*H*-dibenz(*c,e*) azepine,[2] 2,3-dihydrophthalazine-1,4-dione[3] and 1,2,4-triazolidine-3,5-dione.[4] Cross-over between hypolipidemic and antineoplastic activities has been noted for a number of chemicals, e.g. the HMG-CoA reductase inhibitor Compactin, also inhibits DNA synthesis.[2] Heterocyclic amine boranes,[6] sesquiterpene lactones,[7] 2,3-dihydrophthalazine-1,4-dione,[3] and di- and tri-peptides of trimethylamine boranes[8] demonstrated similar hypolipidemic and antineoplastic cross-over activities. For this reason, *N*-substituted indazolones, known hypolipidemic agents,[9,10] were tested for cytotoxicity.

Materials and methods

Source of compounds

The indazolone *N*-substituted derivatives were previously synthesized and characterized (Figure 1).[9,10] All radioisotopes were purchased from New England Nuclear (Boston, MA) unless otherwise indicated. Radioactivity was determined in Fisher Scintiverse scintillation fluid with correction for quenching. Substrates and cofactors were obtained from Sigma (St Louis, MO).

Pharmacological methods

Compounds **1–26** (Table 1) were tested for cytotoxic activity by homogenizing drugs in a 1 mM solution in 0.05% Tween 80/H_2O. These solutions were sterilized by passing them through an acrodisc (45 μM). The following cell lines were maintained by literature techniques:[8] murine L1210 lymphoid leukemia,[11] human $Tmolt_3$ acute lymphoblastic T cell leukemia, colorectal acenocarcinoma SW480, lung bronchogenic MB-9812, osteosarcoma TE418, KB epidermoid nasopharynx, HeLa-S^3 suspended cervical carcinoma and glioma EH 118 MG. Geran *et al.*'s protocol[13] was used to assess the cytotoxicity of the 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. A value of less than 4 μg/ml was required for significant activity of growth inhibition. Solid tumor cytotoxicity was determined by Liebovitz *et al.*'s method[12] utilizing crystal violet/MeOH and read at 580 nm (Molecular Devices).

Incorporation of labeled precursors into [^{3}H]DNA, [^{3}H]RNA and [^{3}H]protein for 10^6 L1210

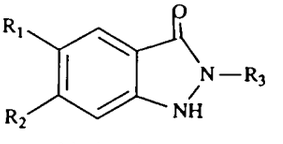

Figure 1. Structure of indazolones.

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Table 1. Structures and cytotoxicity of *N*-substituted indazolones in murine and human tissue culture lines (ED_{50}, in μg/ml)

Compound	Structures				Murine			Human				
	R_1	R_2	R_3	R_4	T lymphoid leukemia L1210	T lymphoid leukemia $Tmolt_3$	Colon adeno-carcinoma	Uterine HeLa-S^3	Naso-pharyngeal KB	Broncho-genic lung	Bone osteo-sarcoma	Brain glioma
1	H	H	H	H	2.67	2.41	0.63	3.65	3.92	8.04	6.31	6.83
2	Cl	H	H	H	6.21	3.29	1.36	1.45	2.17	6.87	1.50	1.11
3	CH_3	H	H	H	5.89	4.00	7.16	2.72	0.81	3.16	1.83	0.85
4	H	H	$CO_2C_2H_3$	H	2.46	3.00	1.15	2.72	5.69	8.00	7.54	7.94
5	Cl	H	$CO_2C_2H_3$	H	2.52	4.64	2.62	1.36	3.83	7.75	6.91	7.16
6	H	Cl	$CO_2C_2H_3$	H	2.15	1.41	1.04	2.12	5.81	8.04	7.27	7.69
7	CH_3	H	$CO_2C_2H_3$	H	3.19	2.45	6.21	1.61	8.05	5.05	7.84	6.83
8	H	H	H	CH_3	2.29	—	—	—	—	—	—	—
9	H	H	H	C_2H_5	2.21	1.63	2.37	2.28	5.51	5.45	4.71	3.42
10	H	H	H	nC_3H_7	2.29	4.05	6.48	3.06	1.08	7.85	1.24	1.73
11	H	H	H	nC_4H_9	2.21	1.02	1.29	2.21	8.09	4.86	2.10	7.00
12	H	H	H	nC_5H_{11}	1.10	2.93	1.10	2.89	4.51	7.09	1.59	6.88
13	Cl	H	H	nC_4H_9	2.94	3.00	2.62	1.87	1.89	2.83	2.75	2.20
14	H	Cl	H	nC_4H_9	1.56	0.86	1.56	1.96	2.50	6.03	5.96	8.21
15	H	CH_3	H	nC_4H_9	1.79	4.00	1.91	2.89	1.22	6.71	2.75	6.48
16	CH_3	H	H	nC_4H_9	0.95	3.17	6.95	2.12	5.77	5.51	7.76	6.26
17	H	H	H	CH_2CH_2OH	3.17	2.94	1.15	3.40	7.67	8.04	7.46	8.21
18	H	H	H	$(CH)_2CH_2OH$	1.26	0.88	6.67	2.46	6.56	4.52	7.31	6.51
19	H	H	H	$C_6H_5CH_2$	2.21	1.22	1.66	1.79	3.48	3.97	5.77	—
20	H	H	H	$pCH_3COC_6H_5CH_2$	1.66	2.33	2.21	1.96	4.75	1.57	4.48	—
21	H	H	H	$mCH_3COC_6H_5CH_2$	2.48	0.94	2.48	2.46	3.88	7.25	2.81	5.14
22	H	H	H	$oCH_3COC_6H_5CH_2$	2.00	2.23	6.67	1.95	7.08	4.32	5.57	1.85
23	H	H	H	C_6H_5	2.67	2.41	0.63	3.66	3.93	8.04	6.31	6.83
24	H	H	H	$CH_2CH_2COCH_3$	2.84	1.53	2.15	2.67	1.42	7.82	1.78	6.61
25	H	H	$CH_2CH_2COCH_3$	$CH_2CH_2COCH_3$	2.63	4.35	1.67	2.46	2.89	7.74	1.83	3.31
26	H	H	$COOC_2H_5$	$CH_2CH_2COCH_3$	2.21	2.52	1.91	2.89	1.22	6.78	2.75	4.24
5 FU					1.41	2.14	3.09	2.47	1.25	5.69	—	1.28
Ara C					2.76	2.67	3.42	2.13	2.84	4.60	—	1.88
6 MP												
Hydroxyurea					2.67	3.18	4.74	1.96	5.29	7.37	7.57	2.57

cells was obtained.[13] The concentration response at 10, 25, 50 and 100 μM required for inhibition of DNA, RNA and protein syntheses was determined after 60 min incubations. The incorporation of [^{14}C]glycine (53.0 mCi/mmol) into purines was obtained by the method of Cadman *et al.*[14] Incorporation of [^{14}C]formate (53.0 mCi/mmol) into pyrimidines was determined by the method of Christopherson *et al.*[15]

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 compound **2** after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic extracts isolated by Eichler *et al.*'s method.[16] Nuclear DNA polymerase (β) was determined by isolating nuclei.[17] The polymerase assay for both α and β was described by Sawada *et al.*[18] with [^{3}H]TTP. 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.[19,20] Ribonucleoside reductase activity was measured using [^{14}C]CDP with and without dithioerythritol.[21] The deoxyribonucleotides [^{14}C]dCDP were separated from the ribonucleotides by thin layer chromatography (TLC) on PEI plates. Thymidine, TMP and TDP kinase activities were determined using [^{3}H]thymidine (58.3 mCi/mmol) in the medium of Maley and Ochoa.[22] Carbamyl phosphate synthetase activity was determined with the method of Kalman *et al.*;[23] citrulline was determined colorimetrically.[24] Aspartate transcarbamylase activity was measured by the method of Kalman *et al.*;[23] carbamyl asparate activity was determined colorimetrically.[25] OMP decarboxylase activity was

determined using orotidine-5-monophosphate[carboxyl-^{14}C] (34.9 μCi/mmol) by Appel's method.[26] Thymidylate synthetase activity was analyzed by Kampf *et al.*'s method.[27] 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.*[28] PRPP amidotransferase activity was determined by Spassova *et al.*'s method;[29] 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.[30] Protein content was determined for the enzymatic assays by the Lowry technique.[31]

After deoxyribonucleoside triphosphates were extracted,[32] levels were determined by the method of Hunting and Henderson[33] with calf thymus DNA, *Escherichia coli* DNA polymerase I, non-limiting amounts of the three deoxyribonucleoside triphosphates not being assayed and either 0.4 μCi of [^{3}H-methyl]dTTP or [5-^{3}H]dCTP.

The effects of compounds **1** and **2** on DNA strand scission were determined by the methods of Suzuki *et al.*,[34] Pera *et al.*[35] and Woynarowski *et al.*[36] L1210 lymphoid leukemia cells were incubated with 10 μCi [methy-^{3}H]thymidine, 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 phosphate buffered saline (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 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 0.3N HCl and measured for radioactivity. Thermal calf thymus DNA denaturation studies and DNA viscosity studies were conducted after incubation of compounds **1** and **2** at 100 μM at 37°C for 24 h.[37]

Statistics

The mean and standard deviation are designated by '$\bar{X} \pm$ SD'. The probable level of significance (p) between test and control samples was determined by the Student's t-test with the raw data.

Results

N-Substituted indazolones proved to be potent cytotoxic agents. In mouse L1210 lymphoid leukemia cells, compounds **12**, **14–16**, **18** and **19** afforded ED_{50} values less than 2 μg/ml (Table 1). Only Compounds **2** and **3** were inactive, i.e. they had ED_{50} values greater than 4 μg/ml. $Tmolt_3$ leukemia growth was inhibited by **6**, **9**, **11**, **14**, **18**, **19**, **21** and **24** with ED_{50} values less than 2 μg/ml. Colon adenocarcinoma growth was inhibited significantly by **1**, **2**, **4**, **6**, **11**, **12**, **14**, **15**, **17**, **19**, **23**, **25** and **26** with ED_{50} values less than 2 μg/ml. Compounds **3**, **7**, **10**, **16**, **18** and **22** were inactive against growth of colon carcinoma. HeLa-S^3 uterine growth was inhibited by **2**, **5**, **7**, **13**, **14**, **19**, **20** and **22** with ED_{50} values less than 2 μg/ml. All compounds were active against uterine carcinoma with ED_{50} values less than 4 μg/ml. KB nasopharynx carcinoma growth was inhibited significantly by **3**, **10**, **13**, **15**, **19**, **24** and **26** with ED_{50} values less than 2 μg/ml. Compounds **2**, **14**, **18**, **21**, **23**, **25** and **26** possessed ED_{50} values between 3 and 4 μg/ml. Lung bronchogenic growth was inhibited by **3**, **13**, **19** and **20**. Osteosarcoma growth was inhibited significantly by **2**, **3**, **10**, **12**, **24** and **25**; compounds **11**, **13**, **15**, **21** and **26** had ED_{50} values less than 4 μg/ml. Glioma growth was reduced by **2**, **3**, **10**, **13**, **22** and **25**.

Compound **20** was selected for a mode of action study because its activity and bioavailability were typical of this chemical class. The L1210 lymphoid leukemia tumor model was selected because of its well-documented characteristics. Compound **20** inhibited DNA and RNA syntheses in a concentration dependent manner with minimum reduction of protein synthesis at 100 μM (Table 2). DNA polymerase α and mRNA polymerase activities were not inhibited by **20**, whereas rRNA polymerase and tRNA polymerase were inhibited by 30–38% at 100 μM. Ribonucleoside reductase activity was inhibited in a concentration dependent manner with a 38% reduction at 100 μM. On the other hand, only a 22% inhibition of dihydrofolate reductase activity was observed at the same concentration. Purine *de novo* synthesis was significantly reduced by **20** with 63–65% reduction at 1, 50 and 100 μM. Although PRPP amidotransferase activity was significantly inhibited by 39% at 100 μM, the major site of inhibition was IMP dehydrogenase, with a reduction in enzymatic activity of 65% at 100 μM. The pyrimidine *de novo* synthetic pathway was not inhibited but rather stimulated by **20**. Aspartate transcarbamylase activity was inhibited only 25% at

Table 2. Effects of compound **20** on L1210 lymphoid leukemia cell metabolism over 60 min

Assay ($N = 6$)	Percent of control ($\bar{X} \pm$ SD)			
	Control	25 μM	50 μM	100 μM
DNA synthesis	100 ± 6[a]	73 ± 6*	60 ± 6*	30 ± 4*
RNA synthesis	100 ± 5[b]	67 ± 5*	61 ± 5*	49 ± 5*
Protein synthesis	100 ± 7[c]	120 ± 7	119 ± 4	91 ± 6
DNA polymerase α	100 ± 6[d]	141 ± 7	112 ± 5	90 ± 5
mRNA polymerase	100 ± 6[e]	107 ± 7	127 ± 6	99 ± 6
rRNA polymerase	100 ± 6[f]	81 ± 6	64 ± 6*	62 ± 5*
tRNA polymerase	100 ± 5[g]	96 ± 5	74 ± 7*	70 ± 5*
Ribonucleoside reductase	100 ± 8[h]	80 ± 7	64 ± 6*	62 ± 4*
Dihydrofolate reductase	100 ± 7[i]	100 ± 6	86 ± 7	78 ± 6*
Purine *de novo* synthesis	100 ± 8[j]	39 ± 4*	37 ± 6*	37 ± 5*
PRPP amido transferase	100 ± 6[k]	83 ± 5	68 ± 6*	61 ± 6*
IMP dehydrogenase	100 ± 7[l]	38 ± 4*	37 ± 4*	35 ± 5*
Pyrimidines *de novo* synthesis	100 ± 6[m]	145 ± 7*	186 ± 8*	196 ± 7*
Carbamyl phosphate synthetase	100 ± 7[n]	102 ± 6	105 ± 5	106 ± 6
Aspartate transcarboxylase	100 ± 8[o]	100 ± 5	86 ± 6	75 ± 5*
Thymidylate synthetase	100 ± 5[p]	86 ± 6	86 ± 7	85 ± 6
Thymidine kinase	100 ± 6[q]	99 ± 7	9 ± 8	87 ± 6
Thymidine monophosphate kinase	100 ± 5[r]	133 ± 8	138 ± 9	141 ± 8*
Thymidine diphosphate kinase	100 ± 5[s]	132 ± 6	142 ± 7*	142 ± 7*
d(ATP)	100 ± 6[t]	—	—	60 ± 5*
d(GTP)	100 ± 5[u]	—	—	74 ± 6*
d(CTP)	100 ± 6[v]	—	—	79 ± 5*
d(TTP)	100 ± 7[w]	—	—	115 ± 7*

Control values 10^6 cells/h: [a]7719 d.p.m., [b]1014 d.p.m., [c]17 492 d.p.m., [d]5318 d.p.m., [e]1343 d.p.m., [f]325 d.p.m., [g]400 d.p.m., [h]48 780 d.p.m., [i]0.133 ΔOD units, [j]28 614 d.p.m., [k]19 375 d.p.m., [l]0.0878 ΔOD units, [m]19 758 d.p.m., [n]0.273 μmol citrulline, [o]57 387 d.p.m., [p]44 743 d.p.m., [q]4362 d.p.m., [r]646 d.p.m., [s]275 d.p.m., [t]32.39 d.p.m., [u]23.79 pmol, [v]86.24 pmol, [w]22.04 pmol.

100 μM. Carbamyl phosphate synthetase, thymidylate synthetase, thymidine kinase, TMP and TDP kinase activities were not inhibited significantly at the concentrations of **20** used. The latter two enzyme activities were actually stimulated by **20**. d(ATP), d(GTP) and d(CTP) pool levels were reduced after incubation with **20** at 100 μM; however, d(TTP) levels were not affected.

Incubation of **20** at 100 μM for 24 h with cDNA showed that it did not alter UV absorption at 260 nm, T_m values for thermal denaturation or DNA viscosity. However, similar incubation of **20** with L1210 cells for 24 h resulted in DNA fragmentation (Figure 2).

Discussion

N-Substituted indazolones proved to be potent cytotoxic agents in suspended single cells, e.g. L1210, $Tmolt_3$ and HeLa-S^3 cells derived from solid tumors. There was no one compound that was active in all tissue culture cell types, indicating that different moieties for the *N*-substituted groups resulted in slightly different potencies against each tumor cell type. The major site for inhibition by compound **20** was at IMP dehydrogenase, which together with marginal inhibition of PRPP amidotransferase, accounted for the observed inhibition of *de novo* purine synthesis. Purine inhibition is of a magnitude to account for the observed inhibitions of both DNA and RNA syntheses and the resultant cell death.

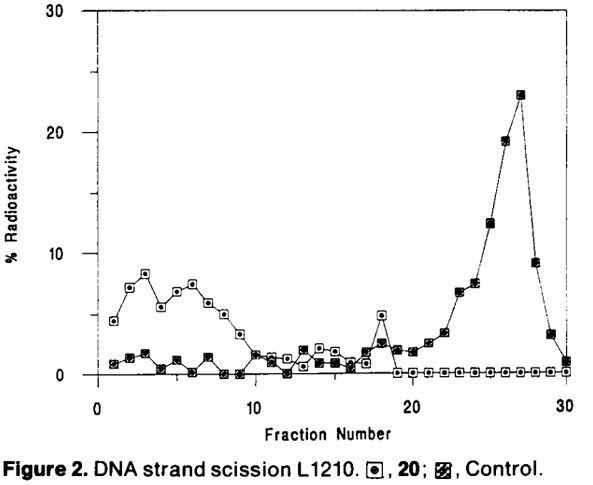

Figure 2. DNA strand scission L1210. ▣, **20**; ▨, Control.

Data from DNA strand scission studies suggested the possibility that the drugs are incorporated into DNA *in lieu* of a purine base. However, this may not be a stable situation and the DNA may fragment. There was no evidence that compound **20** binds to DNA bases or causes intercalation between strands of DNA.

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