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The cytotoxicity of *N*-substituted diphenimides and 6,7-dihydro-5*H*-dibenz[*c,e*]azepines

IH Hall, OT Wong and LK Chi

Divison of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27559-7360, USA. Tel: (+1) 919 966 1121; Fax: (+1) 919 966 6919.

***N*-substituted diphenimides and 6,7-dihydro-5*H*-dibenz-[*c,e*]azepines demonstrated significant cytotoxic activity against the growth of murine and human cells. These derivatives were active against leukemias, carcinomas and sarcomas. Different derivatives with *N*-substitutions showed specific activity against the growth of several tumor types. These agents inhibited L_{1210} leukemia IMP dehydrogenase and PRPP amido transferase activities; this was reflected in the inhibition of purine and DNA synthesis. Other sites inhibited to a minor degree by these agents included DNA polymerase α, r- and tRNA polymerases, ribonucleoside reductase, dihydrofolate reductase, pyrimidine synthesis, and nucleoside kinase. d(NTP) pool levels were reduced after 24 h incubation with these derivatives. L_{1210} DNA strand scission was evident after drug treatment.**

Key words: Anti-neoplastics, diphenimides, azepine purine inhibitors, DNA synthesis inhibitors.

Introduction

N-substituted diphenimides and reduced diphenimides have recently been found to be potent hypolipidemic[1,2] and anti-inflammatory agents.[3] Since there is a cross-over in agents demonstrating hypolipidemic and anti-neoplastic pharmacological action, we were interested in testing these agents for cytotoxic activity. This cross-over is exemplified by Compactin, a known HMG-CoA reductase inhibitor, which also acts to inhibit significantly DNA synthesis of L929 cells.[4] Other agents which show similar cross-over activity include trimethylamine carboxyboranes,[5] heterocyclic amine boranes,[6] sesquiterpene lactones,[7] 2,3-dihydrophthalazine-1,4-diones[8] and triazolidinediones.[9] Consequently, we decided to test the *N*-substituted diphenimides and reduced diphenimides for cytotoxic activity.

Correspondence to IH Hall

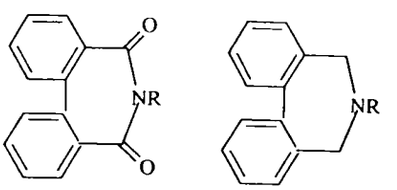
Materials and methods

Source of compounds

The diphenimide and 6,7-dihydro-5*H*-dibenz[*c,e*] azepine derivatives were synthesized previously. They possessed identical chemical and physical characteristics as reported (Figure 1).[1,2] All radioisotopes were purchased from New England Nuclear (Boston, MA) unless otherwise indicated. Radioactivity was determined in Fisher Scintiverse scintillation fluid with a correction for quenching. Substrates and cofactors were obtained from Sigma (St Louis, MO).

Pharmacological methods

Compounds **1–33** (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 L_{1210} lymphoid leukemia,[10] human $Tmolt_3$ acute lymphoblastic T cell leukemia, colorectal adenocarcinoma SW480, lung bronchogenic MB-9812, osteosarcoma TE418, KB epidermoid nasopharynx, HeLa-S^3 suspended cervical carcinoma, and glioma



1–26 **27–33**

Figure 1. *N*-substituted diphenimides

Table 1. Effects of *N*-substituted diphenimides and 6,7-dihydro-5*H*-dibenz[*c, e*]azepine on cell growth (ED_{50} = μg/ml)

Compound	*R*	L_{1210}	$Tmolt_3$	SW40 adenocarcinoma (colon)	HeLa-S^3 uterine	KB nasopharynx	Lung bronchogenic	Osteosarcoma	Brain glioma
1	H	3.75	2.45	1.50	1.68	1.23	1.16	5.05	4.38
2	CH_3	2.45	3.17	1.73	2.41	1.18	1.45	7.84	1.96
3	CH_2CH_3	1.15	2.01	3.42	2.04	4.26	2.24	7.21	8.62
4	$CH_2CH_2CH_3$	1.61	2.59	3.22	2.77	4.64	3.94	7.61	6.52
5	$CH_2CH_2CH_2CH_3$	1.46	2.77	2.14	1.50	2.12	1.23	5.54	2.97
6	$CH_2CH_2C(O)CH_3$	2.84	2.77	1.39	1.96	1.31	1.41	2.24	4.38
7	$CH_2CH_2C(OH)CH_3$	1.15	3.71	3.84	2.09	4.34	1.85	4.85	5.76
8	CH_2CH_2COOH	1.83	3.31	1.44	1.18	1.75	0.94	6.25	7.56
9	Semicarbazone	2.52	2.46	1.39	1.91	1.91	1.41	7.85	5.22
10	C_6H_5	3.21	2.77	1.62	2.41	2.18	1.16	8.51	3.71
11	*o*-ClC_6H_4	3.29	2.01	1.73	1.54	3.18	3.94	4.13	7.46
12	*m*-ClC_6H_4	2.37	2.59	2.32	2.27	3.14	1.26	7.89	3.59
13	*p*-ClC_6H_4	1.76	1.38	2.55	2.00	1.56	0.81	8.33	6.04
14	*o*-$CH_3C_6H_4$	1.37	1.93	1.85	1.82	1.40	0.98	7.16	3.02
15	*m*-$CH_3C_6H_4$	2.29	2.82	2.19	1.77	3.89	1.11	6.00	1.96
16	*p*-$CH_3C_6H_4$	2.44	3.71	1.67	1.73	1.43	1.82	6.66	1.66
17	*o*-$C(O)CH_3C_6H_4$	1.37	2.10	1.15	2.27	2.69	3.82	1.31	1.94
18	*m*-$C(O)CH_3C_6H_4$	2.07	3.84	1.99	1.14	1.28	1.57	6.53	5.74
19	*p*-$C(O)CH_3C_6H_4$	3.37	2.06	1.27	1.82	2.13	1.36	7.66	3.53
20	*o*-$OCH_3C_6H_4$	1.22	2.28	1.73	2.41	5.65	1.31	6.43	7.13
21	*m*-$OCH_3C_6H_4$	2.29	2.68	1.89	2.68	1.28	1.57	6.53	5.74
22	*p*-$OCH_3C_6H_4$	2.44	2.82	1.39	1.82	2.71	2.46	4.49	3.48
23	Benzoyl	1.99	4.47	2.22	2.09	1.30	0.96	4.57	4.05
24	*o*-CH_2CH_3	0.98	3.04	1.89	1.14	1.08	1.18	6.53	7.67
25	*m*-CH_2CH_3	1.84	1.55	2.11	2.09	1.12	2.51	3.74	2.05
26	*p*-CH_2CH_3	1.45	1.61	2.89	1.59	1.95	1.33	3.38	1.96
27	H	0.94	1.76	5.22	2.21	1.76	7.06	1.96	7.08
28	CH_3	4.67	1.49	5.91	2.92	4.46	7.20	3.62	3.81
29	CH_2CH_3	1.33	1.78	6.33	2.38	8.24	5.77	3.16	2.65
30	$CH_2CH_2CH_3$	2.21	1.94	6.66	2.29	2.32	6.16	3.57	7.30
31	$CH_2CH_2CH_2CH_3$	2.84	1.94	7.23	1.84				2.75
32	$CH_2CH_2CH_2CH_2CH_3$	2.11	2.00	7.29	1.87	1.08	4.78	3.23	2.95
33	$CH_2CH_2C(O)CH_3$	2.00	0.82	7.32	1.96	2.12	7.70	5.55	2.52
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
Hydroxyurea		2.67	3.18	4.74	1.96	5.29	7.37	7.57	2.57

EH 118 MG. Geran *et al.'s* protocol[10] was used to assess the cytotoxicity of the compounds and standards in each cell line. Values for cytotoxicity (ED_{50}s) were expressed in 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 at 580 nm (Molecular Devices, Mealo Park, CA, USA) by Liebovitz *et al.'s* method[11] utilizing 2% crystal violet/MeOH.

Incorporation of labeled precursors into [^{3}H]-DNA, [^{3}H]-RNA and [^{3}H]-protein for 10^6 L_{1210} cells was obtained.[12] 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 Chae *et al.*[13] Incorporation of [^{14}C]-formate (53.0 mCi/mmol) into pyrimidines was determined by the method of Christopherson *et al.*[14]

Enzyme assays

Inhibition of various enzyme activities was performed by first preparing the appropriate L_{1210} 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 10, 25, 50 and 100 μM of compounds **14** and **27** after 60 min incubations. DNA polymerase α activity was determined in cytoplasmic extracts isolated by Eichler *et al.'s* method.[15] Nuclear DNA polymerase (β) was determined by isolating nuclei.[16] The polymerase assay for both α and β was described by Sawada *et al.*[17] 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.[8,19] Ribonucleoside reductase activity was measured using [^{14}C]-CDP with and without dithioerythritol.[20]

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.[21] Carbamyl phosphate synthetase activity was determined with the method of Kalman *et al.*[22] Citrulline was determined colorimetrically.[23] Aspartate transcarbamylase activity was measured by the method of Kalman *et al.*[22] Carbamyl aspartate was determined colorimetrically.[24] OMP decarboxylase activity was determined using [carboxyl-^{14}C] orotidine-5-monophosphate (34.9 μCi/mmol) by Appel's method.[25] Thymidylate synthetase activity was analyzed by Kampf *et al.*'s method.[26] 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.*[27] PRPP amidotransferase activity was determined by Spassova *et al*'s method,[28] 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.[29] Protein content was determined for the enzymatic assays by the Lowry technique.[30]

After deoxyribonucleotide triphosphates were extracted,[31] levels were determined by the method of Hunting and Henderson[32] with calf thymus DNA, *Escherichia coli* DNA polymerase I, nonlimiting amounts of the three deoxyribonucleotide triphosphates not being assayed, and either 0.4 μCi of [^{3}H-methyl]dTTP or [5-^{3}H]dCTP.

The effects of compounds **14** and **27** on DNA strand scission were determined by the methods of Suzuki *et al.*,[33] Pera *et al.*[34] and Woynarowski *et al.*[35] L_{1210} lymphoid leukemia cells were incubated with 10 μCi [methyl-^{3}H] thymidine, 84.0 Ci mmol for 24 h at 37°C. L_{1210} 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 12 000 r.p.m. at 20°C for 60 min (Beckman rotor SW60). Fractions (0.2 ml) were collected from the bottom of the gradient, neutralized with 0.2 ml of 0.3 N HCl and measured for radioactivity. Thermal calf thymus DNA denaturation studies and DNA viscosity studies were conducted after incubation of compounds **14** and **27** at 100 μM at 37°C for 24 h.[36]

Statistics

The mean and standard deviation are designated by 'X + 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

The *N*-substituted diphenimides and 6,7-dihydro-5*H*-dibenz[*c*,*e*]azepines were very potent inhibitors of growth of single-cell suspensions of tumor cells (Table 1). With the single exception of Compound **28**, mouse L_{1210} lymphoid leukemia growth was inhibited by the 33 derivatives with ED_{50} values well below 4 μg/ml, the required ED_{50} value for significant cytotoxic activity. Compounds **3**, **7**, **20** and **24** all possessed ED_{50} values greater than 1.25 μg/ml. Compounds **4**, **5**, **8**, **13**, **14**, **17**, **23**, **25–27** and **29** afforded ED_{50} values less than 2 μg/ml. Human $Tmolt_3$ T leukemia cell growth was inhibited significantly by all of the compounds except **23**. Compounds **13**, **14** and **25–27** and **33** afforded ED_{50} values of 2 μg/ml or less. Growth of SW40 adenocarcinoma cells from the colon was significantly inhibited by the diphenimide (**1–26**), but not by the reduced diphenimide (**27–33**), compounds. Incubation with compounds **1**, **2**, **6**, **8–11**, **14**, **16–22**, and **24** resulted in ED_{50} values less than 2 μg/ml. Compounds **1–33** were also potent against the growth of suspended HeLa cells, affording ED_{50} values between 1.14 and 2.92 μg ml. KB nasopharyngeal growth was inhibited by all the compounds except **4**, **7**, **20**, **28** and **29** with ED_{50} values between 1.09 and 3.89 μg ml. Lung bronchogenic tumor growth was inhibited significantly by the compounds with ED_{50} values less than 1 μg ml for compounds **8**, **13**, **23** and **24**. Only compounds **3**, **4**, **11**, **17** and **25** produced ED_{50} values above 2 μg ml. The reduced diphenimide derivatives **27–33** were inactive. The osteosarcoma

bone cancer growth was not inhibited by as many of the compounds. Only **6**, **17** and **25** produced significant activity against osteosarcoma growth. Brain glioma growth was significantly inhibited by all the compounds except **1**, **3**, **4**, **6–9**, **11**, **12**, **18**, **20**, **21**, **23**, **24** and **27**.

The mode of action of selected compounds was evaluated in the L_{1210} lymphoid leukemia cell model. Compound **14** was selected from the diphenimides and compound **27** was selected from the 6,7-dihydro-5*H*-dibenz[*c*,*e*]azepines as being typical of their chemical classes with regard to their cytotoxic profile. The L_{1210} lymphoid leukemia cell model was selected because of its well-known characteristics.

Both drugs caused a concentration dependent suppression of both DNA and RNA syntheses, but no protein synthesis inhibition, from 25 to 100 μM (Table 2). DNA polymerase α activity was inhibited 38% by **14** and 48% by **27** at 100 μM. r-RNA and t-RNA polymerase activities were inhibited by 31–44% at 100 μM; however, mRNA polymerase activity was not inhibited in a consistent manner. Ribonucleoside reductase activity was only inhibited 21% by **27** at 100 μM. Similarly, dihydrofolate reductase activity was inhibited 26% by **27** at 100 μM. Compound **14** had no effect on ribonucleoside reductase, and in contrast to **27** actually elevated dihydrofolate reductase activity. More importantly, compounds **14** and **27** inhibited *de novo* synthesis of purines greater than 50% in a concentration dependent manner. Compound **14** was more effective in blocking purine *de novo* synthesis. The activity of two regulatory enzymes in the purine pathway was inhibited by **14** and **27** in a concentration dependent manner. The drugs' inhibition of activity on either PRPP amido transferase or IMP dihydrogenase was of sufficient magnitude to account for the observed inhibition of DNA or RNA synthesis. The pyrimidine *de novo* synthetic pathway was only marginally (24–25%) reduced by compounds **14** or **27**. This marginal effect of the drugs was also observed in the regulatory enzymes, i.e. carbamyl phosphate synthetase, aspartate transcarbamylase and thymidylate synthetase. Thymidine, TDP and TMP kinases were inhibited markedly by **14**, e.g. thymidine and

Table 2. The effects of compounds **14** and **27** on L_{1210} metabolism and enzyme activities

Assay ($N = 6$)	Control	Percent of control (mean ± SD)					
		14			**27**		
		25 μM	50μM	100 μM	25 μM	50 μM	100 μM
DNA synthesis	100 ± 6[a]	85 ± 6	71 ± 6*	44 ± 5*	81 ± 7	58 ± 5*	56 ± 5*
RNA synthesis	100 ± 5[b]	40 ± 4*	37 ± 4*	36 ± 6*	50 ± 6*	42 ± 4*	37 ± 4*
Protein synthesis	100 ± 7[c]	126 ± 6	122 ± 7	121 ± 6	100 ± 5	111 ± 7	110 ± 6
DNA polymerase α	100 ± 6[d]	70 ± 5*	64 ± 6*	62 ± 6*	86 ± 6	61 ± 6*	52 ± 8*
m-RNA polymerase	100 ± 5[e]	76 ± 4*	101 ± 7	111 ± 6	98 ± 7	102 ± 6	117 ± 7
r-RNA polymerase	100 ± 6[f]	90 ± 6	63 ± 6*	61 ± 6*	95 ± 6	59 ± 6*	56 ± 4*
t-RNA polymerase	100 ± 8[g]	85 ± 5	79 ± 7	69 ± 5*	82 ± 7	77 ± 7	68 ± 6*
Ribonucleoside reductase	100 ± 6[h]	96 ± 7	94 ± 6	93 ± 7	89 ± 8	84 ± 7	79 ± 5*
Dihydrofolate reductase	100 ± 7[i]	99 ± 6	112 ± 7	58 ± 8	120 ± 6	84 ± 5	74 ± 6*
Purine *de novo* synthesis	100 ± 6[j]	27 ± 3*	21 ± 4*	20 ± 4*	66 ± 5*	48 ± 5*	46 ± 5*
PRPP amido transferase	100 ± 5[k]	55 ± 6*	44 ± 5*	42 ± 5*	62 ± 4*	49 ± 5*	41 ± 4*
IMP dehydrogenase	100 ± 7[l]	51 ± 5	50 ± 4*	49 ± 4*	55 ± 6*	42 ± 5*	38 ± 5*
Pyrimidine *de novo* synthesis	100 ± 7[m]	106 ± 6	102 ± 6	75 ± 5*	90 ± 7	82 ± 8	76 ± 6
Carbamyl phosphate synthetase	100 ± 6[n]	97 ± 7	63 ± 6*	88 ± 5	94 ± 6	93 ± 7	90 ± 5
Aspartate transcarbamylase	100 ± 7[o]	95 ± 6	94 ± 5	82 ± 4	108 ± 7	97 ± 6	78 ± 6
Thymidylate synthetase	100 ± 6[p]	72 ± 7*	72 ± 6*	57 ± 5*	88 ± 7	87 ± 6	83 ± 7
Thymidine kinase	100 ± 5[q]	141 ± 7	86 ± 6	16 ± 5*	75 ± 5*	74 ± 5	74 ± 5
Thymidine monophosphate (TMP) kinase	100 ± 6[r]	128 ± 7	112 ± 7	23 ± 4*	152 ± 8*	92 ± 6	79 ± 6
Thymidine diphosphate (TDP) kinase	100 ± 6[s]	87 ± 6	58 ± 5*	21 ± 3*	132 ± 6	131 ± 5	91 ± 5
d(ATP)	100 ± 6[t]			71 ± 4*			66 ± 5*
d(GTP)	100 ± 5[u]			81 ± 5			73 ± 6*
d(CTP)	100 ± 6[v]			64 ± 3*			52 ± 4*
d(TTP)	100 ± 7[w]			90 ± 7			102 ± 7

Control values for 10 cells/h: [a]7719 dpm, [b]1014 dpm, [c]17492 dpm, [d]5318 dpm, [e]1343 dpm, [f]325 dpm, [g]400 dpm, [h]48780 dpm, [i]0.133 O.D. units, [j]28614 dpm, [k]19375 dpm, [l]0.0878 O.D. units, [m]19758 dpm, [n]0.273 μmol citrulline, [o]57387 dpm, [p]44743 dpm, [q]4362 dpm, [r] 646 dpm, [s]275 dpm, [t]32.38 dpm, [u]23.79 pmol, [v]86.24 pmol, [w]22.04 pmol. *$p \leq 0.001$, Student's *t*-test.

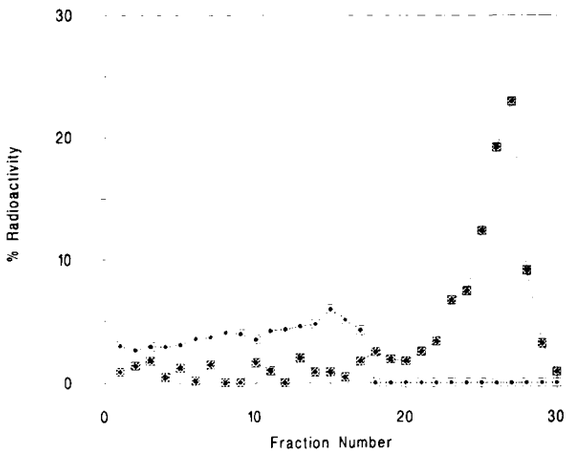


Figure 2. DNA strand scission L_{1210}: ⊙, **14**; ⊠, control.

TMP kinase activities were inhibited 21–26% at 100 μM. d(NTP) pool levels were also modulated by the drugs. L_{1210} d(ATP), d(GTP) and d(CTP) pool levels were reduced significantly by **14** and **27**. cDNA–drug interaction studies demonstrated no evidence that the drug intercalated or bound to DNA bases; this evidence was based on UV absorption of cDNA, thermal denaturation, T_m values and DNA viscosity values. However, when **14** and **27** were incubated for 24 h at 100 μM with L_{1210} cells, there was evidence of DNA strand scission (Figures 2 and 3).

Discussion

The *N*-substituted diphenimides and azepines demonstrated potent cytotoxicity against murine

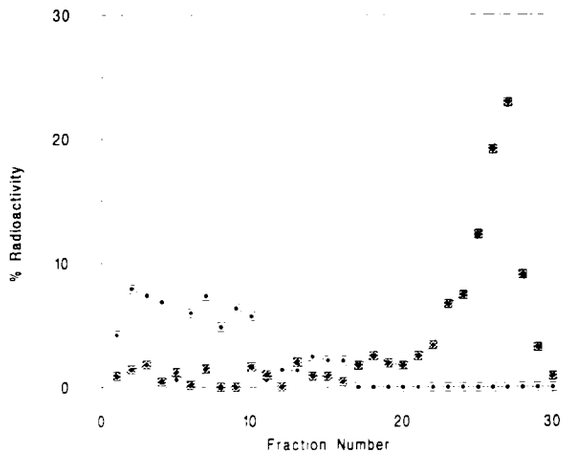


Figure 3. DNA strand scission L_{1210}: ⊙, **27**; ⊠, control.

L_{1210} growth. The compounds also showed potent activity against a number of human tumor cell lines. Activity was demonstrated against suspended tumor cells, e.g. L_{1210}, $Tmolt_3$, and HeLa-S[3]. Surprisingly, these derivatives also demonstrated good activity against the growth of certain solid tumors, e.g. lung, colon and KB nasopharynx. Approximately half of the agents were active against brain glioma growth and most of the compounds were inactive against the growth of osteosarcoma. Differences between the *N*-substituted diphenimides and 6,7-dihydro-5*H*-dibenz-[*c*,*e*]azepines in activity against certain cell lines emerged. *N*-substituted diphenimides were active against human lung growth and inactive against bone growth, whereas 6,7-dihydro-5*H*-dibenz[*c*,*e*]-azepines were generally less active against growths of lung and colon tissues. They were active against bone growth and generally more active against glioma growth. No clear-cut structure–activity relationship characteristic could be identified for the diphenimide *N*-substituted agents for the improvement of cytotoxicity. The different moieties substituted on the nitrogen group improved their activities against certain tumor cell growths and made them less active against other cell lines.

The mode of action of both of these chemical derivatives appears to be at regulatory sites in the purine synthetic pathway, i.e. PRPP amido transferase and IMP dehydrogenase. Inhibition at these sites should be reflected in both DNA and RNA syntheses, which was true for both agents. The large magnitude of reduction observed for RNA synthesis at 100 μM of drug may reflect additive effects due to the agents' inhibitions of r- and t-RNA polymerase activities. An additional site which appeared to be affected by **14** was nucleoside kinase; dihydrofolate reductase and ribonucleoside reductase both seemed to be affected by **27**. The mixed results from the drugs on d(NTP) pool levels may be due to reduced ribonucleoside reductase activity by **27** and reduced nucleoside kinase activity by **14**. However, inhibition of DNA polymerase α activity would lead to an elevation of d(NTP) levels due to the fact deoxyribonucleotides were not incorporated into new DNA strands. Thus, some d(NTP) levels may increase, as was observed for d(TTP) with both drugs and d(ATP) with **27**. There was no evidence of drug interactions directly with nucleic acid bases. However, the occurrence of DNA scission suggested that the drug affected DNA integrity. One possibility is that the drug might be incorporated into the DNA strand since it has

similar shape and size as a purine base. The new DNA strand may not be stable and may thus fragment

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