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The antineoplastic and cytotoxicity of benzohydroxamic acids and related derivatives in murine and human tumor cells

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Benzohydroxamic acids proved to be potent cytotoxic agents suppressing the growth of a number of murine and human cell lines grown in tissue culture, e.g. leukemia, colon, uterine and glioma. Selected compounds demonstrated activity against the growth KB nasopharynx, bronchogenic lung, osteosarcoma and skin cancer. *In vivo* activity against Ehrlich ascites carcinoma growth was shown with certain compounds. In L1210 cells compound 2 inhibited DNA synthesis significantly within 60 min. The site of action of the agent appears to involve the purine *de novo* synthesis pathway at PRPP amido transferase and IMP dehydrogenase. Dihydrofolate reductase and nucleoside kinase activities were inhibited by the agent. The levels of d(NTP)s in L1210 cells were reduced after drug treatment. The drug did not appear to affect the DNA template directly causing any damage which might alter transcription and replication nor was there any inhibition of HeLa topoisomerase activity by the drug. Thus the drug appears to be a metabolic inhibitor of nucleoside metabolism.

Key words: Antineoplastic agents, benzohydroxamic acids, DNA synthesis inhibitors, leukemia.

Introduction

Niacin and 6-aminonicotinamides were active against adenocarcinoma 753 growth, a mammary gland tumor in C_5-B1/6 mice.[1] 2-Amino-1,3,4-thiadiazole derivatives proved to have antileukemic activity.[2] Nicotinohydroxamic acids were shown to have hypolipidemic activity.[3] We have previously shown that benzohydroxamic acids were potent hypolipidemic agents.[4,5] A strong correlation exists in several instances between hypolipidemic activity and antineoplastic activity, e.g. di- and tripeptide boron derivatives,[6] boron cyano- and carboxyboron amines,[7,8] helenalin,[9] and 2,3-dihydrophthalazine-1,4-diones[10] have all demonstrated dual pharmacological activities. The current study involves the investigation of benzohydroxamic acids as cytotoxic agents.

Materials and methods

The benzohydroxamic acids and dibenzohydroxamic acids (**1–10**) were synthesized as previously described and their chemical and physical characteristics were identical.[4] 2-Methoxybenzohydroxamic acid (**11**) was prepared by the method of Hauser and Renfrow[11] and was isolated as a white solid: m.p. 126–128°C; IR (Nujol) 3255 (m), 1610 (s), 1585 (s) cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) S 3.83 (s, 3H), 7.06 (t and d, 4H), 9.14 (s, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) S 55.69, 111.80, 122.48, 130.09, 131.89, 142.31, 156.67, 163.27. 2,6-Dimethoxybenzo-hydroxamic acid was prepared by the method of Konaje and Hosangadi[12] and was isolated as a white solid: m.p. 195–196°C; IR (Nujol) 3323 (m), 1624 (s), 1574 (s) cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) S 3.82 (s, 6H), 6.68 (d, 2H), 7.35 (t, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) S 55.46, 103.94, 130.44, 142.11, 142.13, 157.19.

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).

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Pharmacology methods

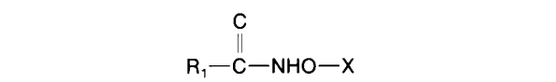
Cytotoxic activity. Compounds **1**–**10** (Table 1) were tested for cytotoxic activity by preparing a 1 mM solution of the drugs in 0.05% Tween 80/H_2O by homogenization. The drug solutions were sterilized by passing them through an acrodisc (45 μM). The following cell lines were maintained by the literature techniques:[8] murine L1210 lymphoid leukemia,[13] P388 lymphocytic leukemia,[13] human $Tmolt_3$ acute lymphoblastic T cell leukemia, colorectal adenocarcinoma SW480, lung bronochogenic MB-9812, osteosarcoma TE418, KB epidermoid nasopharynx, HeLa-S^3 suspended cervical carcinoma, epidermoid skin carcinoma A431 and glioma EH 118 MG. The protocol used to assess cytotoxicity was that of Geran *et al.*[13] Standards were determined in each cell line. Values are expressed for the drug's cytotoxicity as ED_{50} in μg/ml, i.e. the concentration which inhibits 50% of the cell growth, was 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 the method of Liebovitz *et al.*[14]

Incorporation of labeled precursors into [^{3}H]DNA, [^{3}H]RNA and [^{3}H]protein for 10^6 L1210 cells was determined by the method of Liao *et al.*[15] The concentration (10, 25, 50 and 100 μM) response for inhibition of DNA, RNA and protein synthesis was determined at 60 min. [^{14}C]Glycine (53.0 mCi/nmol) incorporation into purines was determined by the method of Cadman *et al.*[16] [^{14}C]Formate (53.0 mCi/nmol) incorporation into pyrimidines was determined by the method of Christopherson *et al.*[17]

Enzyme assays

Inhibition of various enzyme activities was carried out by first preparing the appropriate L1210 cell homogenate or subcellular fraction, then adding the drug to be tested during the enzyme assay. For the concentration response studies, the inhibition of enzyme activity was determined at 10, 25, 50 and 100 μM of compound **2** for 60 min incubations. DNA polymerase α activity was determined in a cytoplasmic extract isolated by the method of Eichler *et al.*[18] Nuclear DNA polymerase (β) was determined by isolating nuclei.[19] The polymerase assay for both α and β was that of Sawada *et al.*[20] with [^{3}H]TTP. Messenger, ribosomal and transfer RNA polymerase enzymes were isolated with different concentrations of ammonium sulfate and the individual RNA polymerase activities were determined using [^{3}H]UTP.[21,22] Ribonculeotide reductase activity was measured with [^{14}C]CDP with and without dithioerythritol.[23] 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/nmol) and the medium of Maley and Ochoa.[24] Carbamyl phosphate synthetase activity was determined by the method of Kalman *et al.*[25] and citrulline was determined colori-

Table 1. The *in vivo* antineoplastic activity of benzohydroxamic acids in the Ehrlich ascites carcinoma screen in CF_1 mice at 8 mg/kg/day (i.p.)



	R_1	X	Inhibition of Ehrlich ascites carcinoma growth (%)
1	Ph	H	62
2	4-MePh	H	96
3	4-MeOPh	H	86
4	4-Cl-Ph	H	34
5	4-NO_2-Ph	H	84
6	3,4-$(MeO)_2$-Ph	H	77
7	3,4,5-$(MeO)_3$-Ph	H	83
8	3,4,5-$(MeO)_3$-Ph	3,4,5$(MeO)_3$-Ph-CO	82
9	4-NO_2-Ph	4-NO_2Ph-CO	58
10	Ph	Ph-CO	71
11	6-MeoPh	H	23
12	1,6-$(MeO)_2$-Ph	H	64
	6-Mercaptopurine		99

$N = 6$.

metrically.[26] Asparate transcarbamylase activity was determined by the method of Kalman *et al.*[25] and carbamyl asparate was determined colorimetrically.[27] Orotidine-5-monophosphate (OMP) decarboxylase activity was determined using [carboxyl-^{14}C]OMP (34.9 μCi/mmol) by the method of Appel.[28] Thymidylate synthetase activity was analyzed by the method of Kampf *et al.*[29] 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.*[30] Phosphoribose pyrophosphate (PRPP) amido transferase activity was determined by the method of Spassova *et al.*[31] and inosine monophosphate (IMP) dehydrogenase activity was determined with [8-^{14}C]IMP (54 mCi/mmol) (Amersham, Arlington Heights, IL) where XMP was separated on PEI plates (Fisher Scientific by TLC).[32] Protein was determined for all of the enzymatic assays by the Lowry technique.[33]

Deoxyribonucleoside triphosphates were extracted by the method of Bagnara and Finch.[34] Deoxyribonucleoside triphosphates were determined by the method of Hunting and Henderson[35] 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 the agents on isolated DNA topoisomerase II activity were determined by the method of Miller *et al.*[36] Knotted DNA was prepared from bacteriophage P4 as outlined in the literature.[37] The enzyme DNA topoisomerase II was isolated from HeLa uterine carcinoma cells. The reaction medium contained 0.20 M Tris, pH 7.5, 0.4 M KCl, 0.04 M $MgCl_2$, 120 μg/ml bovine serum albumin, 2.0 M EDTA 4.0 mM DDT and 4 mM ATP. For the enzyme assay 2.5 μl of the reaction medium and 0.25 μl knotted DNA were added and diluted to 8 μl with distilled water. The agents were added with 1.0 μl of enzyme for a final volume of 10 μl which was incubated for 60 min at 37°C. Buffer (50% w/v sucrose, 0.5% w/v sodium dodecyl sulfate, 0.25% w/v bromophenyl blue and 0.25% xylene cyanol), 2.5 μl, was added to stop the reaction. Samples of the reaction, pure DNA and pure enzyme were placed on an agrose electrophoresis gel at 23 V. VP-16 (etoposide) was used as an internal standard. Inhibition of the activity is noted in the gel when topoisomerase II reduces the ability to unknot the knotted DNA. The inhibition of the activity appears as a smear of DNA in the gel as opposed to distinct separation of bands.

The effects of compounds **1** and **2** on DNA strand scission was determined by the method of Suzuki *et al.*,[38] Pera *et al.*[39] and Woynarowski *et al.*[40] L1210 lymphoid leukemia cells were incubated with 10 μCi [methyl-^{3}H]thymidine, 84.0 Ci/mmol for 24 h at 37°C. After harvesting the L1210 cells (10^7), the cells were centrifuged at 600 *g* for 10 min in phosphate buffered saline (PBS), 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 M KCl and 0.01 M EDTA) followed by 0.2 ml cell preparation. After incubating for 2.5 h at room temperature, the gradient 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 radioactivity measured. 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.[41]

Statistics

The mean and standard deviation are designated by the letter '*N*'. The probable level of significance (p) between test samples and control samples was determined by the Student's *t*-test with raw data.

Results

In vivo compounds **2**, **3**, **5**, **7** and **8** demonstrated greater than 80% inhibition of Ehrlich ascitic growth at 8 mg/kg/day (Table 1). Compound **2** compared favorably with 6-mercaptopurine. The benzohydroxamic and dibenzohydroxamic acids were shown to be effective cytotoxic agents against murine and human tumor growth. All of the compounds except **5** were active against L1210 and P388 murine tumor growth, e.g. compound **2** demonstrated significant activity (Table 2). In human tumors, all of the compounds except **11** were active in the $Tmolt_3$, colon adenocarcinoma and HeLa-S^3 growth. Compounds **1**, **4**, **5**, **7**, **9** and **10** were active in the KB nasopharynx screen. Compounds **4**, **7** and **9** were active in the bronchogenic screen. Compounds **3**, **4**, **5** and **9** were active against osteosarcoma growth. Glioma growth was inhibited by **2**, **4**, **5**, **7**, **9** and **10**. Skin epidermoid carcinoma was inhibited by **2**, **5** and **9**.

Table 2. Cytotoxicity of benzohydroxamic acids in murine and human tumor growth (ED_{50})

Compound	Murine		$Tmolt_3$	Adenocarcinoma colon	HeLa-S^3	Nasopharynx KB	Bronchogenic lung	Osteosarcoma	Glioma	Skin
	L1210	P388								
1	2.63	3.02	0.59	0.42	2.84	2.34	—	—	—	—
2	0.64	1.82	2.45	0.11	2.02	7.08	5.44	7.15	3.02	2.89
3	1.23	3.18	2.75	2.54	1.33	5.03	6.22	2.34	5.32	2.69
4	1.63	3.35	2.43	2.19	2.26	1.18	1.09	2.66	1.07	2.07
5	1.93	4.36	1.74	2.07	1.28	3.18	5.25	1.79	1.24	3.64
6	1.16	2.77	1.42	3.11	1.69	4.89	7.68	8.30	5.74	—
7	2.63	2.62	3.00	1.77	2.75	3.16	3.13	5.60	1.77	6.68
8	2.12	3.76	2.95	2.87	2.82	5.04	7.79	4.03	3.97	—
9	1.62	4.29	2.35	1.43	1.81	1.16	3.02	1.37	1.22	1.79
10	3.73	2.32	2.49	2.33	2.34	3.72	—	—	2.62	5.28
11	2.09	—	4.31	2.87	3.52	—	5.88	7.85	—	—
12	3.19	—	3.42	2.53	3.84	—	5.34	8.00	—	—
5-FU	1.41	3.72	2.14	3.09	2.47	1.25	5.69	—	1.28	—
ARAC	2.76	4.23	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	2.27	—

Table 3. The effects of compound **2** on L1210 lymphoid leukemia metabolism as percent of control ($x \pm$ SD)

	Control	10 μM	25 μM	50 μM	100 μM
DNA synthesis	100 ± 6[a]	60 ± 5*	24 ± 4*	24 ± 3*	8 ± 2*
RNA synthesis	100 ± 5[b]	90 ± 6	75 ± 6*	65 ± 5*	62 ± 6*
Protein synthesis	100 ± 6[c]	78 ± 5*	52 ± 5*	49 ± 4	46 ± 4*
DNA polymerase α	100 ± 6[d]	107 ± 7	77 ± 6*	74 ± 5*	71 ± 6*
mRNA polymerase	100 ± 5[e]	99 ± 6	91 ± 7	85 ± 6	78 ± 7*
rRNA polymerase	100 ± 4[f]	89 ± 6	81 ± 6	79 ± 5*	78 ± 4*
tRNA polymerase	100 ± 6[g]	98 ± 7	104 ± 5	112 ± 6	136 ± 7
Ribonucleoside reductase	100 ± 7[h]	99 ± 6	102 ± 6	97 ± 5	96 ± 6*
[^{14}C]Glycine–purine	100 ± 8[i]	138 ± 7	106 ± 6	36 ± 4*	27 ± 3*
PRPP amido transferase	100 ± 6[j]	90 ± 5	58 ± 4*	44 ± 3*	26 ± 3*
IMP dehydrogenase	100 ± 7[k]	76 ± 5*	61 ± 5*	51 ± 4*	45 ± 4*
[^{14}C]formate—pyrimidine	100 ± 7[l]	105 ± 6	130 ± 7	117 ± 6	94 ± 5
Carbamyl phosphate synthetase	100 ± 7[m]	106 ± 5	105 ± 5	102 ± 6	91 ± 5
Aspartate transcarbamylase	100 ± 5[n]	123 ± 6	110 ± 6	89 ± 5	72 ± 5
OMP decarboxylase	100 ± 6[o]	111 ± 6	63 ± 5*	54 ± 4*	52 ± 5*
Thymidylate synthetase	100 ± 6[p]	77 ± 6*	72 ± 6*	69 ± 5*	60 ± 4*
Dihydrofolate reductase	100 ± 6[q]	99 ± 7	65 ± 6*	59 ± 5*	30 ± 3*
Thymidine kinase	100 ± 7[r]	143 ± 6	98 ± 5	92 ± 6	67 ± 5*
TMP kinase	100 ± 6[s]	87 ± 6	68 ± 6*	62 ± 5*	39 ± 4*
TDP kinase	100 ± 4[t]	56 ± 5*	27 ± 4*	4 ± 3*	3 ± 2*
d(ATP)	100 ± 4[u]	—	—	—	62 ± 6*
d(GTP)	100 ± 6[v]	—	—	—	73 ± 5*
d(CTP)	100 ± 5[w]	—	—	—	29 ± 4*
d(TTP)	100 ± 6[x]	—	—	—	57 ± 5*

$N = 6$.
[a]7719 d.p.m., [b]1014 d.p.m., [c]17492 d.p.m., [d]5318 d.p.m., [e]1343 d.p.m., [f]325 d.p.m., [g]400 d.p.m., [h]48780 d.p.m., [i]28614 d.p.m., [j]19575 d.p.m., [k]0.0878 ΔOD units, [l]19758 d.p.m., [m]0.273 μ mol citrulline, [n]0.807 mol *N*-carbamyl aspartate, [o]57387 d.p.m., [p]77616 d.p.m., [q]0.114 ΔOD units, [r]1371 d.p.m., [s]1179 d.p.m., [t]1891 d.p.m., [u]17.07 pmol, [v]13.58 pmol, [w]33.60 pmol, [x]31.04 pmol. *$p \leq 0.001$.

Compound **2** was selected as a representative agent of the group. When it was examined for its mode of action in L1210 lymphoid leukemic cells (Table 3), DNA synthesis was inhibited in a concentration dependent manner achieving 92% inhibition at 100 μM. RNA and protein synthesis was inhibited significantly by the agent but their magnitude was less than the DNA synthesis inhibition. The suppression of DNA polymerase α, mRNA and rRNA polymerase activities was between 22 and 29% but they did not appear to be of magnitudes to explain the observed DNA synthesis inhibition. [^{14}C]Glycine incorporation into purines was significantly inhibited by **2** in a concentration manner with greater than 70% reduction at 100 μM. [^{14}C]Formate incorporation into pyrimidine was not significantly inhibited by **2**. The activities of the regulatory enzymes of the purine pathway, IMP dehydrogenase and PRPP amido transferase, were significantly inhibited in a concentration manner. In the pyrimidine pathway, the early steps in pathway are not significantly affected by compound **2**. The latter steps in the pyrimidine pathway appeared to be affected by the agent; OMP decarboxylase activity was suppressed greater than 45%. Thymidylate synthetase was suppressed 40% at 100 μM of **2**. Dihydrofolate reductase activity was suppressed by **2** in a concentration dependent manner with 70% reduction at 100 μM. The nucleoside kinase activities were also suppressed by **2**. The TDP kinase activity was reduced the most by **2** by 97%. TMP kinase was inhibited 61% and thymidine kinase activity 33%. d(NTP) levels were markedly reduced by **2**. Purine nucleotides dATP and dGTP reductions were probably due to suppression of the purine regulatory enzyme activities. Pyrimidine nucleotide levels, dCTP and dTTP were also reduced by **2** probably due to reduction of the nucleotide kinase by **2**.

Kinetic studies with compound **2** at 100 μM showed that 45 min was required to inhibit DNA synthesis greater than 50% (Table 4). The regulatory enzymes were inhibited in a similar manner. Only thymidine kinase activity was inhibited by 50% by 30 min.

Table 4. Kinetic effects of compound **2** at 100 μM on L1210 DNA synthesis and selected enzyme activities as percent of control ($x \pm$ SD)

		Minutes ($x \pm$ SD)			
	Control	15	30	45	60
DNA synthesis	100 ± 6[a]	76 ± 5	62 ± 5	24 ± 5	8 ± 2
PRPP amido transferase	100 ± 6[b]	98 ± 7	81 ± 6	59 ± 6	50 ± 4
IMP dehydrogenase	100 ± 7[c]	78 ± 6	75 ± 6	53 ± 5	40 ± 3
OMP decarboxylase	100 ± 6[d]	92 ± 5	84 ± 4	66 ± 4	52 ± 5
Thymidine kinase	100 ± 7[e]	90 ± 4	50 ± 5	26 ± 3	3 ± 2

$N = 6$.
[a]7719 d.p.m., [b]19575 d.p.m., [c]0.0878 ΔOD units, [d]57387 d.p.m., [e]1371 d.p.m.

There was no evidence that the agent interacted with calf thymus DNA, e.g. there was no alteration DNA absorption, thermal denaturation or DNA viscosity after incubation for 24 h with the drug. When **2** was incubated with L1210 cells, there was no evidence of DNA strand scission after 24 h (Figure 1). HeLa-S^3 topoisomerase activity was not affected by **2** at 100 μM.

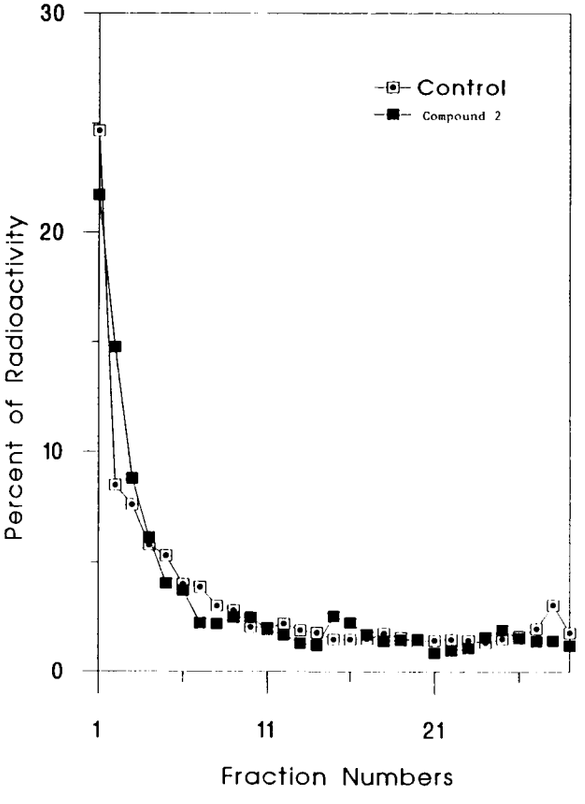


Figure 1. L1210 DNA strand scission.

Discussion

The benzohydroxamic acids were effective cytotoxic agents with a number of derivatives demonstrating activity comparable to the clinically used standards. In the Ehrlich ascites screen, the single substitution on the phenyl ring resulted in the best *in vivo* activity. Bulky substitutes led to less activity. These compounds were more active against leukemia single cell tumors, e.g. $Tmolt_3$, L1210 and P388. The growth of colon adenocarcinomas and HeLa-S^3 from uterine carcinoma derived from a solid human tumor was particularly susceptible to the benzohydroxamic acids. Selectivity of the compounds was demonstrated in some of the other solid tumors, e.g. lung bronchogenic, osteosarcoma and KB nasopharynx. Selected compounds were active against the growth of glioma. The drugs are of such a small molecular weight in all probability they cross the blood–brain barrier easily. Interestingly, a number of the compounds, but not all, were active against the growth of skin cancer.

The mode of action of compound **2**, the *para*-methyl substituted derivative, was demonstrated to involve DNA synthesis which was preferentially inhibited. The inhibition of L1210 DNA synthesis appeared to be the result of additive effects of the inhibition of regulatory enzymes in both the purine and pyrimidine pathways. The inhibition of the nucleotide kinase by compound **2** is sufficient alone to account for the observed reduction of deoxyribonucleotide levels and DNA synthesis of L1210 cells.

The effects on L1210 RNA and protein synthesis by **2** are probably secondary but additive. The lesser effects on RNA synthesis by **2** is probably the result of the ribonucleotides and ribonucleosides being in higher concentration in the cells than deoxyribonucleosides and deoxyribonucleotides. When sufficient d(NTP) and r(NTP) precursors were

added to the assay medium of DNA polymerase and mRNA and rRNA polymerase, the compound had only marginal effects indicating the polymerases were not a major site of the drug. There was no direct effect on the DNA template by compound **2** affecting its utilization for replication and transcription. Thus, the major effects of the benzohydroxamic acid appear to be as metabolic inhibitors for the precursor for DNA and RNA synthesis.

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