Polyhydroxylated Phenylacrylic Acid Derivatives as New Anti-tumor Agents

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Abstract □ Preliminary evidence indicates that antitumor agents containing the *o*-dihydroxybenzene moiety exhibit enhanced antitumor activity toward malignant cells of high oxidative potential, such as melanoma cells. Based on this consideration, 11 hydroxybenzene acrylic acid derivatives of differing redox potential were prepared as potential substrates for the melanoma specific enzyme tyrosinase, that might exhibit general antitumor activity and enhanced cytotoxicity toward melanoma cells. Five of these compounds [α-cyano-β-(4-hydroxyphenyl)-, α-cyano-β-(3,4-dihydroxyphenyl)-, and α-cyano-β-(3,4,5-trihydroxyphenyl)acrylic acid (THPPA), and 3,4-dihydroxy- and 3,4,5-trihydroxybenzalcyanoacetamide] were found to be substrates for tyrosinase with k_m values from 0.08 to 4.13 mM and V_{max} values from 0.18 to 3.02. These data indicate that as the number of hydroxy groups increases, the rate of oxidation increases, and that cyanoamides were faster reacting than corresponding cyanoacids, with dicyanides the least reactive. In contrast, cyanoamides were less effective as substrates than cyanoacids. In vitro studies showed all but two compounds were active against L1210 (IC_{50} range 21–980 μM), SK-MEL-28 (IC_{50} range 54–950 μM), and SK-MEL-30-3 (IC_{50} range 54–190 μM). Only THPPA was active in vivo against L1210 and B-16 melanoma.

The identification of *o*-dihydroxybenzene derivatives that are structurally related to levodopa and dopamine, as potential antitumor agents was based on the observation that levodopa, a metabolic precursor of the pigment melanin, was highly toxic to pigmented melanoma cells in vitro.[1,2] These findings have recently been confirmed by others.[3] We postulated that the cytotoxicity was mediated through oxidation products formed by the action of the melanoma specific enzyme tyrosinase on excess exogenous levodopa. Our most recent data have demonstrated that these compounds also act as potent inhibitors of DNA synthesis in other types of rapidly proliferating eucaryotic cells (e.g., L1210 leukemia cells). There are several possible mechanisms including direct, reversible inhibition of ribonucleotide reductase by the reduced form of the catechol,[4] inhibition of DNA polymerase by the oxidized quinone (semiquinone),[5] and indirect inhibition of thymidylate synthase which only occurs in situ.[6,7] Preliminary evidence suggested that a precise reducing potential was necessary for antitumor activity caused by the selective inactivation of certain enzymes central to DNA synthesis and not the entire cytosol. It is possible that there are important differences between normal and malignant cells in their ability to interact with redox reagents, since several other types of antitumor agents, such as anthracyclines, possess redox active features.

In order to define the structural parameters necessary for activity and to provide reagents for mechanistic studies, we prepared a series of compounds that would assist in examining the effect of appending unsaturated side chains of different oxidation states, including mono- and dicyanoacrylic acids and cyanoacetamides. Nitrile, carboxy, and amide groups can each activate the β-carbon atom, generating a potent electrophilic site. As a result, it might be possible to synthesize antitumor agents containing the catechol moiety that could be selectively converted by oxidation to parent electrophiles within certain tumor cells having a high oxidative state (e.g., tyrosinase positive melanoma cells).

Experimental Section

Radiolabeled thymidine ([^{3}H]thymidine; specific activity, 80 Ci/mmol) was obtained from New England Nuclear Corporation, Boston, MA. The isomeric hydroxybenzaldehydes were purchased from Aldrich Chemical Company, Milwaukee, WI. 3,5-Dihydroxybenzaldehyde was obtained from Spectrum Chemical Manufacturing Corporation, Gardena, CA. Mushroom tyrosinase (2000 units per mg of solid) was obtained from Sigma Chemical Company, St. Louis, MO. Melting points were measured in open Pyrex capillary tubes in an electrothermal melting point apparatus (Electrothermal Engineering Limited, Neville Road, London E.7., U.K.) and are uncorrected.

Chemical Synthesis—Compounds **1**–**3** were prepared by condensation reaction between ethyl cyanoacetate and the corresponding hydroxybenzaldehydes.

Preparation of α-Cyano-β-(3,4-dihydroxyphenyl)acrylic Acid—Compound **2**, the cyanoacid, was prepared according to the procedure of Rosenmund and Boehm.[8] Sodium (400 mg) was slowly added to a solution of 13.8 g (100 mmol) of 3,4-dihydroxybenzaldehyde and 13.0 mL (122 mmol) of ethyl cyanoacetate in 100 mL of ethyl alcohol (95%) until a reddish brown solution formed. The solution was stirred for 24 h at room temperature and warmed for 10 min, cooled, and poured into 40 mL of 1% sulfuric acid. The yellow crystals of ethyl α-cyano-β-(3,4-dihydroxy)acrylate that formed were filtered and recrystallized from ethanol. The first crop weighed 5.60 g (25%), and leaving the solution for several days gave another 3.0 g (13%) of yellow crystal, mp 168–169 °C (lit.[8] mp 168 °C). This compound, without further characterization was subjected to hydrolysis. A solution of 9.32 g (40 mmol) of the ester in 80 mL of 2 M NaOH was stirred at room temperature for 10 min. The solution was cooled and made slightly acidic with 5% sulfuric acid, and the precipitate was filtered and washed with cold water. The yellow crystals after recrystallization from hot water gave 5.76 g (71%) of shiny bright, yellow crystals, mp 224–225 °C (lit.[8] mp 224–225 °C).

Compounds **1** and **3** were prepared in similar fashion and their properties are described in Table I.

Compounds **4**–**7** were prepared according to the procedure of Rosenmund and Boehm.[8]

Preparation of α,α-Dicyano-β-(3,4-dihydroxyphenly)acrylic Acid (5)—A mixture of 1.38 g (10 mmol) of 3,4-dihydroxybenzaldehyde, 94 mL (15 mmol) of malononitrile, and 10 mL of water was heated for 15 min. The bright yellow precipitate was filtered and washed with water. Recrystallization from a mixture of water and ethanol gave 1.38 g (78%) of bright yellow crystals, mp 226 °C with decomposition (lit.[8] mp 221 °C). Other acids (**4**–**7**) were prepared in an analogous fashion (Table I).

Compounds **8**, **9**, and **11** were prepared according to the procedure of Rosenmund and Boehm.[8]

Preparation of 3,4,5-Trihydroxybenzalcyanoacetamide (11)—Sodium (100 mg) was added to a solution of 1.72 g (10 mmol) of 3,4,5-trihydroxybenzaldehyde and 1.03 g (12.27 mmol) of cyanoacetamide in 50 mL of alcohol. The mixture was warmed, and then stirred at room temperature overnight. The solution was cooled in ice water and water was added. Recrystallization from water gave 1.76 g (80%) of bright yellow crystals, mp 270–271 °C (lit.[8] 268 °C).

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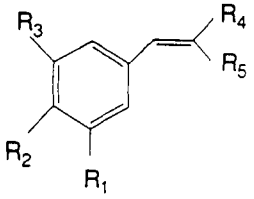
Table I—Synthesis of Polyhydroxylated Acrylic Acid Derivatives



Compound	R_1	R_2	R_3	R_4	R_5	Yield	mp Found (Lit.), °C
1	H	OH	H	CO_2H	CN	66	245 (244)
2	OH	OH	H	CO_2H	CN	90	224–5 (224–5)
3	OH	OH	H	CO_2H	CN	70	209–10 (210–2)
4	H	OH	H	CN	CN	90	195–200
5	OH	OH	H	CN	CN	92	226 (221)
6	OH	H	OH	CN	CN	92	190–1
7	OH	OH	OH	CN	CN	84	255 (250)
8	H	H	H	$CONH_2$	CN	98	124–5 (121–2)
9	H	OH	H	$CONH_2$	CN	88	195–200 (191)
10	OH	OH	H	$CONH_2$	CN	78	234–6 (231)
11	OH	OH	OH	$CONH_2$	CN	80	270–1 (268)

Other amides (**8**, **9**) were prepared similarly (Table I).

Preparation of 3,4-Dihydroxybenzalcyanoacetamide (10)—A mixture of 5.0 g of α,α-dicyano-β-(3,4-dihydroxyphenyl)acrylic acid and 40 mL of concentrated sulfuric acid was stirred at room temperature for 24 h and added in a dropwise manner to ice cold water. A yellow precipitate appeared, which was filtered, washed with water, and dried. Recrystallization from water gave 4.0 g (70%) of bright yellow crystals of cyanoacetamide, mp 234–236 °C (lit.[8] mp 231 °C).

Determination of Kinetic Constants—A reaction mixture consisted of a substrate at six different concentrations (0.1–1 mM) and 50 μL of mushroom tyrosinase (2 mg/mL) in 1.0 mL of 0.125 M sodium phosphate buffer at pH 7.3. The reaction was conducted at 25 °C, the disappearance of substrate and formation of pigment were monitored by UV spectrophotometry, and K_m and V_{max} were calculated from the Lineweaver–Burk analysis.

Cytotoxicity Assays—The origin and maintenance of L1210 lymphocytic leukemia cells has been described.[9] The cells were maintained in a suspension of Eagle's minimum essential medium containing 10% fetal bovine serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin in a 5% CO_2 humidified air incubator at 37 °C. The medium was supplemented with 50 μM 2-mercaptoethanol.[10] The technique for radiolabeled precursor incorporation has been described previously.[9] The cells were checked for the presence of mycoplasma using the radiolabeled method described by Schneider et al.[11] The SK-MEL-30 and SK-MEL-28 cell lines were obtained from the American Type Culture Collection. The SK-MEL-30 cell line was maintained in RPMI-1640 medium that was supplemented with 0.1 mM nonessential amino acids. The SK-MEL-30-3 cell line is a heavily pigmented clone of the SK-MEL-30 cell line developed in our laboratory.

Cell Growth—Exponentially growing cultures were harvested and subcultures plated in Linbro multiwell tissue culture trays. The drug was added 24 h after plating, and the cells were continuously exposed to the drug for 48 h. Triplicate cultures were harvested and cells were counted in a model Z Coulter Counter.

Macromolecular Assay—The previously described method for radiolabeled precursor incorporation[9] was used. Cells were subcultured in Linbro multiwell tissue culture trays for 48 h to establish log phase. The growth medium was aspirated and the monolayer was washed with fresh medium. Serum-free medium (1.0 mL) containing [^{3}H]thymidine was added with the drug. After 1 h at 37 °C, the medium was removed, cells were washed once with saline, and 0.1 mL of 10% TCA was added. The precipitate containing DNA was digested for 4 h at 37 °C and then prepared for scintillation counting as previously described.[12]

In Vivo Antitumor Evaluation—The $B_6D_2F_1$ male mice (5–6 weeks old and average weight of 20 ± 2 g) were obtained from Taconic Inc., Germantown, NY. The assay procedures were identical to those of standard National Cancer Institute protocols.[13] Groups of seven (nine for B16) mice were inoculated ip with 1×10^5 L1210 leukemic cells (1×10^6 melanoma cells for B16) on day 0, and treatment was begun on day 1 for 4 consecutive days (tid), or on day 1 for 18 consecutive days for B16 (qd). All compounds were administered in sterile saline. All animals were weighed on days 1 and 7, and 7-day weight changes were calculated as a percentage to determine toxic dose levels.

Results and Discussion

Chemical Synthesis—Compounds **1–3** were prepared by the condensation between ethyl cyanoacetate and corresponding hydroxybenzaldehydes in alcohol in the presence of metallic sodium. The esters were hydrolized with aqueous NaOH. For **4–6**, malononitrile, in the absence of base, was used instead of cyanoacetate. For **8**, **9**, and **11**, cyanoacetamide in alcohol and metallic sodium was used. The yields and melting points are given in Table I.

Tyrosinase Substrate Activity—Although it has been shown that tyrosinase does not have a strict substrate structural requirement,[14] Table II shows that only five (**1**, **2**, **3**, **10**, and **11**) of the 11 compounds prepared were actually substrates for this enzyme. The results for tyramine, 3,4-DHBA, and 3,4,5-THBA, as well as the natural substrate L-dopa and tyrosine, are included for comparison. The results (Table II) indicate that the order of reactivity for the phenylacrylic acid derivatives is trihydroxy > dihydroxy > monohydroxy. This finding parallels their half-wave potential as reducing agents.[15] Furthermore, within these hydroxy groups (tri, di, mono), the compounds with the amine side-chain have greater activity than the cyanoamide which, in turn, has greater activity than the cyanoacid. The presence of two cyano side-chain groups completely abolished activity.

The following competition experiment was performed in order to determine if the dicyano groups were preventing these compounds from binding to the active site of tyrosinase or whether the presence of these two side-chains was preventing their oxidation (e.g., by increasing the activation energy). Tyrosinase and a dicyano compound (di- or monohydroxy) were added to the reaction mixture. After 15 min of preincubation, 3,4-DHBA was added and the absorption spectra were determined immediately and again after 15 min. The result revealed that the rate of oxidation of 3,4-DHBA was slower than in the absence of the dicyano compound. This result would suggest that the dicyano compounds were interfering with the oxidation by competing with 3,4-DHBA for the tyrosinase binding site. The inability of tyrosinase to catalyze the oxidation of the dicyano compounds appears to be due to factor(s) other than substrate binding, such as an altered electronic potential.

Table II—Properties of Phenylacrylic Acid Derivatives as Substrates for Melanoma Cell Tyrosinase

Compound	V_{max}	K_m, mM	λ_{max}
1	0.18	0.11	258
2	0.64	0.08	268
3	1.87	1.02	266
4	N.S.[a]	N.S.	426
5	N.S.	N.S.	292
6	N.S.	N.S.	280
7	N.S.	N.S.	310
8	N.S.	N.S.	285
9	N.S.	N.S.	260
10	0.68	4.13	288
11	3.02	1.21	289
L-Dopa	2.72	0.29	484
DHBA	4.41	1.42	405
Tyrosine	0.63	0.16	484
Tyramine	0.58	0.87	450
THBA	0.24	0.24	357

[a] Not substrate.

Finally, based on the large K_m values, it appears that the amide side-chain hinders the interaction of the substrate with tyrosinase, although it does not significantly affect the rate of the reaction. On the other hand, the presence of a basic group on the side-chain increases the binding of the substrate to tyrosinase.

In Vitro Cytotoxicity—The in vitro structure–activity analysis is shown in Table III. The results indicate that within each series of compounds, the trihydroxy derivatives are the most cytotoxic. The monohydroxy derivatives, on the other hand, have little if any cytotoxic activity with the notable exception of the monohydroxydicyano derivative. Our laboratory has prepared a number of hydroxybenzene derivatives and the monohydroxy or *m*-dihydroxy derivatives had never exhibited significant cytotoxicity. We believe that the cytotoxicity of the mono- and *m*-dicyano derivatives is due to the two cyano side-chains. Specifically, the presence of two cyano groups appears to confer a nonspecific cytotoxic effect.

The lack of activity of the dihydroxymonocyanoacrylic acid derivative is an interesting finding that might suggest chemical modifications to avoid when designing new hydroxybenzene derivatives. Specifically, the dihydroxyphenylacrylic acid, which has a negatively charged side group at physiological pH that hinders transport of these compounds into cells, has no in vitro activity; all other *o*-dihydroxy derivatives with neutral or basic side groups have significant cytotoxicity.

This restriction on the side-chain would not seem to apply to the trihydroxy derivatives. The results for the trihydroxymonocyanoacrylic acid suggest that the activity of this derivative (**3**) is most likely due to extracellular membrane effects. The trihydroxy compounds can readily undergo autooxidation. Since the acidic side group inhibits intracellular transport, the in vitro damage by the trihydroxycyanoacid would appear to be the result of oxidation of the cell membrane due to free radicals generated by the autooxidation of this derivative in the medium.

One of the initial objectives of this study was to design polyhydroxybenzene analogues that would act as improved substrates for tyrosinase and yield more of the reactive oxidation products. Instead, the derivatives synthesized in this report were, in general, less good substrates for tyrosinase. The results for the dihydroxybenzene derivatives suggest that despite the fact that the dihydroxycyanoamide, was sevenfold less active as a tyrosinase substrate than 3,4-DHBA, it retained the same level of cytotoxic activity against melanoma cells. This suggested that the cytotoxic activity of the dihydroxybenzene derivative might not be due to oxidation products, as had been proposed,[5,16] but instead due in large part to the reduced form of the dihydroxybenzene derivatives causing a direct inhibition of ribonucleotide reductase[4,5] and/or the indirect inhibition of other DNA synthetic enzymes such as thymidylate synthase.[8,9]

Table III—In Vitro Evaluation of Phenylacrylic Acid Derivatives

Compound	IC_{50}, μM[a]			
	SK-MEL-28	L1210		SK-MEL-30-3
	Growth	Growth	DNA Synthesis	Growth
1	none	none	none	none
2	none	908	none	none
3	66	21	84	70
4	54	260	80	95
5	82	27	27	77
6	224	24	37	192
7	71	25	76	54
8	none	980	896	none
9	957	452	300	none
10	85	230	161	38
11	107	52	24	17

[a] Concentration of drugs causing 50% inhibition of growth or DNA synthesis (determined as described in the *Experimental Section*).

Table IV—Antitumor Activity of α-Cyano-β-(3,4,5-trihydroxyphenyl)acrylic Acid (THPPA) in B16 Melanoma and L1210 Leukemia In Vivo at Optimal Doses

	Day of Death			
	L1210 Cells (200 mg/kg[2])		B16 Cells (100 mg/kg[a])	
	Control	Treated[b]	Control	Treated[b]
	9	14	20	22
	9	14	21	30
	9	14	15	28
	9	14	17	31
	9	15	22	22
	10	16	20	22
	10	18	20	23
	10		16	24
	11		18	23
Mean	9.56	15.00	18.8	25.0
Median	9	14	20	23

[a] Administered daily, days 1–9, for B16 melanoma and three times daily, days 1–4, for L1210 leukemia; the experiments were conducted according to standard NCI protocol (ref 13). [b] Values are significant at $p < 0.001$.

In Vivo Cytotoxicity—The in vivo antitumor activity of the trihydroxycyanoacid is shown in Table IV. All other compounds were either too toxic (**10** and **11**), had no other antitumor activity (**1** and **2**), or could not be tested in vivo due to a lack of solubility in the injection vehicle (**4**–**8**). The trihydroxyphenylacrylic acid demonstrates a significant and reproducible antitumor activity against the L1210 leukemia and B-16 melanoma models. Representative results are shown with $p < 0.001$.

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