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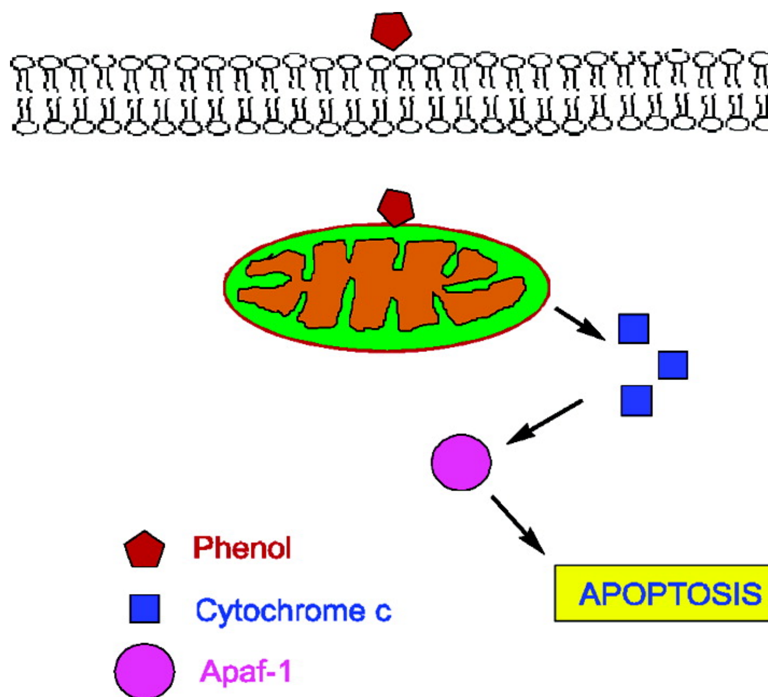

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Cellular Apoptosis and Cytotoxicity of Phenolic Compounds: A Quantitative Structure−Activity Relationship Study

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In this comprehensive study on the caspase-mediated apoptosis-inducing effect of 51 substituted phenols in a murine leukemia cell line (L1210), we determined the concentrations needed to induce caspase activity by 50% (I_{50}) and utilized these data to develop the following quantitative structure−activity relationship (QSAR) model: $\log 1/I_{50} = 1.06\ \mathrm{B5}_2 + 0.33\ \mathrm{B5}_3 - 0.18\pi_{2,4} - 0.92$. $\mathrm{B5}_3$ and $\mathrm{B5}_2$ represent steric terms, while $\pi_{2,4}$ represents the hydrophobic character of the substituents on the ring. The strong dependence of caspase-mediated apoptosis on mostly steric parameters suggests that the process is a receptor-mediated interaction with caspases or mitochondrial proteins being the likely targets. Conversely, cytotoxicity studies of 65 electron-releasing phenols in the L1210 cell line led to the development of the following equation: $\log 1/\mathrm{ID}_{50} = -1.39\sigma^+ - 0.28\ \mathrm{B5}_{2,6} + 0.16 \log P - 0.58I_2 - 1.04I_1 + 3.90$. The low coefficient with $\log P$ may pertain to cellular transport that may be enhanced by a modest increase in overall hydrophobicity, while the presence of σ^+ is consistent with the suggestion that radical stabilization is of prime importance in the case of electron-releasing substituents. On the other hand, the QSAR for the interactions of 27 electron-attracting phenols in L1210 cells, $\log 1/\mathrm{ID}_{50} = 0.56 \log P - 0.30\ \mathrm{B5}_2 + 2.79$, suggests that hydrophobicity, as represented by $\log P$ is of critical importance. Similar cytotoxicity patterns are observed in other mammalian cell lines such as HL-60, MCF-7, CCRF-CEM, and CEM/VLB. The significant differences between the cytotoxicity and apoptosis QSAR for electron-releasing phenols suggest that cytotoxicity involves minimal apoptosis in most of these substituted monophenols.

Introduction

In recent years, rapid advances have been made in delineating the molecular mechanisms underlying apoptosis or programmed cell death.[1,2] Apoptosis is mostly mediated through the activation of caspases that are aspartate-specific cysteine proteases that target a wide spectrum of specific proteins for limited proteolysis.[3,4] Two major pathways of caspase activation have been identified: a transmembrane receptor-mediated extrinsic pathway initiated by the binding of death activator proteins and an intrinsic pathway targeting mitochondrial membranes that results in the release of factors such as cytochrome *c* that promote caspase-9 activation.[5] It is well established that perturbations in cell cycle or metabolism, oxidative stress, growth factor availability, and genotoxic agents that diminish mitochondrial integrity, can also initiate cell death.[6] Many chemotherapeutic agents activate apoptosis, an activity often mediated by the p53 pathway.[7−9]

The ability of various phenolic compounds to play conflicting and complex roles as radical scavengers, antioxidants, and prooxidants provides a rationale for an examination of their apoptotic activities. Phenolic compounds are ubiquitous in nature. They are universally distributed throughout the plant kingdom as mostly hydroxylated aromatic rings that may also be conjugated with other natural products such as fatty acids and flavanoids or polymerized into larger entities such as lignins. The use of phenolic-based phytochemicals as chemopreventive agents coupled with the extensive usage and prevalence of various phenols as industrial chemicals or environmental pollutants warrants a thorough and systematic study of the variables that define their chemical reactivities and subsequent biological activities.[10]

In recent years, work in our laboratory has focused on the myriad biological activities of X-phenols and their ability to form radical species.[11] Brown's variant (σ^+) of Hammett's electronic σ constant has been particularly pertinent in delineating quantitative structure−activity relationships (QSARs) pertaining to the cytotoxicity of various substituted, albeit homogeneous, phenols in murine leukemia cells. These early QSAR models are described in eqs 1 and 2.[12] Bond dissociation energies as represented by BDE speak directly of the homolytic cleavage of the O−H bond and subsequent formation of the phenoxyl radical, a reactive oxygen species. In both cases, the low coefficient with the hydrophobic term ($\log P$) suggests that the radical may be interacting with a receptor such as DNA or it may represent the slightly enhanced transport of the X-phenoxy radical in the cellular environs. Phenols with substituents of an electron-withdrawing nature do not subscribe to this type of toxicity. Their mostly nonspecific cytotoxicities are modeled by hydrophobicity, as reflected in eq 3.[13]

In these QSAR equations, ID_{50} represents the molar concentration of X-phenol that induces 50% growth inhibition versus murine leukemia L1210 cells, and thus, $\log 1/\mathrm{ID}_{50}$ is the dependent variable that defines the biological activity in QSAR studies. σ^+ represents

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Cytotoxicity of electron-releasing X-phenols in L1210 cells:

$$\log 1/\mathrm{ID}_{50} = (-1.35\sigma^{+} \pm 0.15) + (0.18 \pm 0.04)\log P + (3.31 \pm 0.11) \quad (1)$$

$$n = 51, \quad r^2 = 0.895, \quad s = 0.227, \quad q^2 = 0.882$$

$$\log 1/\mathrm{ID}_{50} = (-0.19 \pm 0.02)\mathrm{BDE} + (0.21 \pm 0.03)\log P + (3.11 \pm 0.10) \quad (2)$$

$$n = 52, \quad r^2 = 0.920, \quad s = 0.202, \quad q^2 = 0.909$$

Cytotoxicity of electron-attracting X-phenols in L1210 cells:

$$\log 1/\mathrm{ID}_{50} = (0.62 \pm 0.16)\log P + (2.35 \pm 0.31) \quad (3)$$

$$n = 15, \quad r^2 = 0.845, \quad s = 0.232, \quad q^2 = 0.800$$

Brown's refinement of the Hammett σ constant, while $\log P$ is a calculated partition coefficient (octanol−water system).

In the present study, we examine the activation of caspases and subsequent apoptosis of a library of phenols against a mouse leukemia cell line. These results are then compared with their corresponding cytotoxicities in the same cell line to determine if apoptosis plays a major role in the overall cytotoxicity of monophenolic compounds as delineated by the form of the corresponding QSAR and the type and magnitude of the important descriptors.

Results

The heterogeneous set of phenols in Table 1 includes mono-, di-, and trisubstituted phenols with a large number of sterically hindered derivatives. The range in apoptotic activity is around 3 log units, and the highly sterically hindered phenols generally have the highest activities. With the data in Table 1, the following QSAR equation was formulated, with one compound, 2-iodophenol, omitted from the analysis.

Induction of caspase-mediated apoptosis in L1210 cells by X-phenols:

$$\log 1/I_{50} = (1.06 \pm 0.12)\mathrm{B5}_2 + (0.33 \pm 0.20)\mathrm{B5}_3 - (0.18 \pm 0.09)\pi_{2,4} - (0.92 \pm 0.46) \quad (4)$$

$$n = 51, \quad r^2 = 0.886, \quad s = 0.349, \quad q^2 = 0.866, \quad F_{3,47} = 121.72$$

In eq 4, I_{50} represents the concentration of X-phenol that induces caspase-mediated apoptosis by 50%. $\mathrm{B5}_2$ is Verloop's sterimol descriptor and is a measure of the width of the larger substituent in the ortho position, while $\mathrm{B5}_3$ represents the width of the larger substituent in the meta position. In most cases, ambiguity in position assignment is minimized because of the symmetrical nature of the substitution patterns. However, in the case of ortho monosubstituted analogues, e.g., 2-methoxyphenol, the methoxy substituent is placed in the 2-position. The hydrophobic parameter $\pi_{2,4}$ represents the sum of the hydrophobicity of substituents in the para position and the bulkier ortho position. In this model, 81% of the variance in the data is explained by the steric descriptor $\mathrm{B5}_2$ while the hydrophobicity of the ortho and para substituents as well as $\mathrm{B5}_3$ accounts for 5% and 2%, respectively, of the variance in the data. This strong dependence on mostly steric terms suggests that the phenols may be interacting with critical apoptogenic proteins.

Table 1. Caspase-Mediated Apoptosis of Phenols Against L1210 Cells

no.	X	log 1/ID$_{50}$ obsd	log 1/ID$_{50}$ pred[a]	B5$_2$	$\pi_{2,4}$	B5$_3$
1	4-OCH$_3$	0.02	0.48	1.00	−0.02	1.00
2	4-CN	0.10	0.58	1.00	−0.57	1.00
3	4-NO$_2$	1.00	0.53	1.00	−0.28	1.00
4	4-OC$_4$H$_9$	0.14	0.20	1.00	1.52	1.00
5	4-OC$_6$H$_5$	0.19	0.10	1.00	2.08	1.00
6	4-OC$_3$H$_7$	0.09	0.29	1.00	1.05	1.00
7	4-C(CH$_3$)$_3$	0.11	0.12	1.00	1.98	1.00
8	4-COCH$_3$	0.03	0.58	1.00	−0.55	1.00
9	H	−0.20	0.48	1.00	0	1.00
10	3-NO$_2$	0.79	0.96	1.00	0	2.44
11	3-NHCOCH$_3$	1.10	1.35	1.00	0	3.61
12	3-Cl	0.74	0.74	1.00	0	1.80
13	3-Br	1.00	0.79	1.00	0	1.95
14	3-F	0.82	0.59	1.00	0	1.35
15	3-NH$_2$	1.25	0.80	1.00	0	1.97
16	3-CN	1.11	0.68	1.00	0	1.60
17	3-OCH$_3$	1.31	1.17	1.00	0	3.07
18	3-CH$_3$	0.45	0.82	1.00	0	2.04
19	3-OH	0.79	0.79	1.00	0	1.93
20	2,6-(C(CH$_3$)$_3$)$_2$,4-OCOCH$_3$	2.80	2.53	3.17	1.34	1.00
21	2,6-(C(CH$_3$)$_3$)$_2$,4-C$_2$H$_5$	2.43	2.23	3.17	3.00	1.00
22	2,6-(C(CH$_3$)$_3$)$_2$,4-CHO	2.49	2.54	3.17	1.33	1.00
23	2,6-(C(CH$_3$)$_3$)$_2$	1.90	2.42	3.17	1.98	1.00
24	2,6-(C(CH$_3$)$_3$)$_2$,4-CH$_2$OCH$_3$	2.90	2.46	3.17	1.77	1.00
25	2,6-(C(CH$_3$)$_3$)$_2$,4-OH	2.50	2.54	3.17	1.31	1.00
26	2,4,6-(C(CH$_3$)$_3$)$_3$	2.10	2.05	3.17	3.96	1.00
27	2,6-(C(CH$_3$)$_3$)$_2$,4-CN	2.88	2.52	3.17	1.41	1.00
28	2,6-(C(CH$_3$)$_3$)$_2$,4-CH$_2$OH	2.39	2.61	3.17	0.95	1.00
29	2,6-(C(CH$_3$)$_3$)$_2$,4-COCH$_3$	2.41	2.52	3.17	1.43	1.00
30	2,6-(C(CH$_3$)$_3$)$_2$,4-Br	2.58	2.26	3.17	2.84	1.00
31	2,6-(C(CH$_3$)$_3$)$_2$,4-CH$_3$ (BHT)	2.09	2.31	3.17	2.54	1.00
32	2,6-(C(CH$_3$)$_3$)$_2$,4-NO$_2$	2.49	2.47	3.17	1.70	1.00
33	2,6-(C(CH$_3$)$_3$)$_2$,4-OCH$_3$	2.87	2.42	3.17	1.96	1.00
34	2,6-(OCH$_3$)$_2$	2.70	2.68	3.07	−0.02	1.00
35	2,4,6-(OCH$_3$)$_3$	2.20	2.68	3.07	−0.04	1.00
36	2,6-(OCH$_3$)$_2$,4-CH=CHCHO	2.60	2.72	3.07	−0.25	1.00
37	2,6-(OCH$_3$)$_2$,4-NH$_2$	3.10	2.90	3.07	−1.25	1.00
38	2,6-(OCH$_3$)$_2$,4-COCH$_3$	2.80	2.78	3.07	−0.57	1.00
39	2,6-(OCH$_3$)$_2$,4-NHCOCH$_3$	2.50	2.85	3.07	−0.99	1.00
40	2,6-(OCH$_3$)$_2$,4-CH$_3$	2.40	2.57	3.07	0.54	1.00
41	2,6-(OCH$_3$)$_2$,4-CHO	2.90	2.80	3.07	−0.67	1.00
42	2-NH$_2$	2.29	1.73	1.97	−1.23	1.00
43	2-C(CH$_3$)$_3$	2.42	2.42	3.17	1.98	1.00
44	2-CH(CH$_3$)$_2$	2.38	2.50	3.17	1.53	1.00
45	2-CH$_3$	1.87	1.48	2.04	0.56	1.00
46	2-I[b]	2.38	1.49	2.15	1.12	1.00
47	2-C$_2$H$_5$	2.11	2.59	3.17	1.02	1.00
48	2-C$_3$H$_7$	2.16	2.83	3.49	1.55	1.00
49	2-NH$_2$,4-NO$_2$	2.46	1.78	1.97	−1.51	1.00
50	2-NH$_2$,4-CH$_3$	2.09	1.63	1.97	−0.67	1.00
51	2-OCH$_3$	2.41	2.68	3.07	−0.02	1.00
52	2-C(CH$_3$)$_3$, 4-OCH$_3$ (BHA)	2.71	2.42	3.17	1.96	1.00

[a] Calculated using QSAR 4 [b] Not included in the derivation of QSAR 4

Equation 4 accounts for 89% of the variance in the data. The cross-validated r^2 (q^2), which is high (0.87), was obtained by using the leave-one-out (LOO) procedure.[14] Further validation of eq 4 was also carried out by using Y-randomization.[15] The biological data were randomly shuffled five times and the following statistical values were obtained for the five runs: $r^2 = 0.518$, $q^2 = 0.409$; $r^2 = 0.181$, $q^2 = 0.071$; $r^2 = 0.138$, $q^2 = -0.003$; $r^2 = 0.553$, $q^2 = 0.485$; $r^2 = 0.382$, $q^2 = 0.296$. These poor r^2 and q^2 values in the randomization test ensures the robustness of eq 4. Analysis of the data in

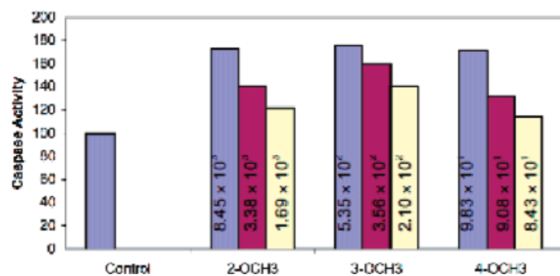

Figure 1. Induction of apoptosis by 2-, 3-, and 4-methoxyphenols.

Table 1 reveals that multisubstituted phenols are more adept at inducing apoptosis than the monosubstituted phenols. In the latter case, the following trend is observed: ortho phenols ≫ meta phenols ≫ para phenols. This disparity in apoptosis induction by positional isomers is well illustrated in Figure 1.

The presence of steric terms ($B5_2$, $B5_3$) and a hydrophobic term ($\pi_{2,4}$) suggests that large substituents, e.g., *tert*-butyl groups help to anchor the phenol on the surface of a hydrophilic receptor. The small and negative coefficient with the hydrophobic descriptor speaks to this type of interaction. Despite the heterogeneity of the substitutents' electronic attributes, a bifurcation in mechanism was not observed in the ability to induce apoptosis. Thus electron-attracting and electron-releasing phenols behave in a similar manner.

The monophenols that are most adept at inducing apoptosis are clearly the multisubstituted ones with bulky groups in the ortho positions. Thus, the most potent phenolics are the 2,6-di-*tert*-butyl, 4-methoxymethyl and 2,6-dimethoxy, and 4-amino analogues whose I_{50} values fall within the 1 mM range. At 0.1 mM, however, all of these monophenolic compounds exhibit minimal apoptosis (<15%).

To examine the relationship between caspase-mediated apoptosis and cytotoxicity, we extended our examination of cell growth inhibition by substituted monophenolics in L1210 cells. We expanded our study to include six new 4-X-2,6-dimethoxy phenols. Once again, a bifurcation in mechanism was observed depending on the electron density in the aromatic ring. This phenomenon is illustrated in QSAR eqs 5 and 6, pertaining to electron-releasing phenols (Table 2) and electron-attracting phenols (Table 3), respectively. The outliers (Δ

Cytotoxicity of electron-releasing phenols in L1210 cells:

$$\log {}^{1}/_{ID_{50}} = (-1.39 \pm 0.19)\sigma^+ - (0.28 \pm 0.05)B5_{2,6} + (0.16 \pm 0.05)\log P - (0.58 \pm 0.24)I_2 - (1.04 \pm 0.25)I_1 + (3.90 \pm 0.19) \quad (5)$$

$$n = 65, \quad r^2 = 0.840, \quad s = 0.271, \quad q^2 = 0.808, \quad F_{5,59} = 62.07$$

Table 2. Cytotoxicity of Electron-Releasing Phenols in L1210 Cells

		log $^1/_{ID_{50}}$									log $^1/_{ID_{50}}$						
no.	X	obsd	pred[a]	σ^+	$B5_{2,6}$	log P [b]	I_1	I_2	no.	X	obsd	pred[a]	σ^+	$B5_{2,6}$	log P [b]	I_1	I_2
1	H	3.27	3.56	0.00	2.0	1.48	0	0	38	2-OC_2H_5[c]	3.25	4.08	−0.81	4.36	1.85	0	0
2	4-OCH_3	4.48	4.08	−0.78	2.0	1.57	0	1	39	2-$NHCONH_2$	3.50	3.45	−0.60	4.61	0.18	0	0
3	4-OC_2H_5	4.64	4.79	−0.81	2.0	2.10	0	0	40	2-OH,4-CH_3	5.03	4.99	−1.23	2.93	1.38	0	0
4	4-OC_3H_7	4.85	4.90	−0.83	2.0	2.63	0	0	41	2,6-$(CH_3)_2$	3.02	2.93	−0.62	4.08	2.37	1	0
5	4-OC_4H_9	5.20	4.95	−0.81	2.0	3.16	0	0	42	2,6-$(OCH_3)_2$	3.86	3.91	−1.56	6.14	1.11	0	1
6	4-OC_6H_{13}	5.50	5.12	−0.81	2.0	4.22	0	0	43	2,4,6-$(CH_3)_3$	3.20	3.44	−0.93	4.08	2.87	1	0
7	4-OC_6H_5	4.97	4.59	−0.50	2.0	3.57	0	0	44	2,6-$(C(CH_3)_3)_2$	3.85	3.60	−0.52	6.34	4.93	0	0
8	4-CH_3	3.85	4.07	−0.31	2.0	1.97	0	0	45	2,6-$(C(CH_3)_3)_2$-4-CH_3(BHT)	4.04	4.11	−0.83	6.34	5.43	0	0
9	4-C_2H_5	3.86	4.14	−0.30	2.0	2.50	0	0	46	2,6-$(C_2H_5)_2$	3.26	3.47	−0.60	6.34	3.43	0	0
10	4-C_3H_7	4.04	4.21	−0.29	2.0	3.03	0	0	47	2,6-$(CH(CH_3)_2)_2$	3.25	3.50	−0.56	6.34	3.93	0	0
11	4-C_4H_9	4.33	4.29	−0.29	2.0	3.56	0	0	48	2,4,6-$(C(CH_3)_3)_3$	3.90	4.25	−0.78	6.34	6.75	0	0
12	4-C_5H_{11}	4.47	4.38	−0.29	2.0	4.09	0	0	49	2-$C(CH_3)_3$−6-CH_3[c]	3.73	2.74	−0.57	5.21	3.65	1	0
13	4-C_7H_{15}	4.49	4.54	−0.29	2.0	5.15	0	0	50	2,6-$(C(CH_3)_3)_2$-4-C_2H_5	3.91	4.18	−0.82	6.34	5.96	0	0
14	4-C_8H_{17}	4.62	4.63	−0.29	2.0	5.68	0	0	51	2,6-$(C(CH_3)_3)_2$-4-Br	4.11	3.57	−0.37	6.34	6.09	0	0
15	4-C_9H_{19}	4.75	4.71	−0.29	2.0	6.21	0	0	52	2,4-$(C(CH_3)_3)_2$	4.24	4.23	−0.52	4.17	5.03	0	0
16	4-$C(CH_3)_3$	4.09	4.21	−0.26	2.0	3.30	0	0	53	2-$C(CH_3)_3$-4-CH_3 (BMP)	3.80	4.09	−0.57	4.17	3.70	0	0
17	4-F	3.83	3.73	−0.07	2.0	1.91	0	0	54	2,4-$(CH_3)_2$	3.04	3.23	−0.62	3.04	2.42	1	0
18	4-NH2	5.09	5.17	−1.30	2.0	0.25	0	0	55	2-CH_3-4-F	3.09	2.89	−0.38	3.04	2.36	1	0
19	4-OH	4.59	4.73	−0.92	2.0	0.81	0	0	56	2-CH_3-4-Br[c]	3.46	2.70	−0.16	3.04	3.08	1	0
20	4-$NHCOCH_3$	3.73	4.24	−0.60	2.0	0.49	0	0	57	2-CH_3-4-OCH_3	3.39	3.24	−1.09	3.04	2.02	1	1
21	3-$C(CH_3)_3$	3.88	3.99	−0.10	2.0	3.30	0	0	58	2-$C(CH_3)_3$-4-C_2H_5	3.80	4.16	−0.56	4.17	4.23	0	0
22	3-CH_3	3.54	3.74	−0.07	2.0	1.97	0	0	59	2,4,6-$(OCH_3)_3$	5.05	4.99	−2.34	6.14	1.07	0	1
23	3-$N(CH_3)_2$	4.11	3.81	−0.16	2.0	1.64	0	0	60	2,6-$(OCH_3)_2$-4-CHO	3.10	3.25	−1.09	6.14	1.01	0	1
24	3-C_2H_5	3.71	3.82	−0.07	2.0	2.50	0	0	61	2,6-$(OCH_3)_2$-4-$COCH_3$	3.06	3.24	−1.09	6.14	1.00	0	1
25	3-NH_2	4.11	3.59	−0.16	2.0	0.25	0	0	62	2,6-$(OCH_3)_2$-4-CHCHCHO	4.25	4.12	−1.72	6.14	1.01	0	1
26	2-CH_3[c]	3.52	2.73	−0.31	3.04	1.92	1	0	63	2,6-$(OCH_3)_2$-4-CH_3[c]	2.89	4.42	−1.87	6.14	1.61	0	1
27	2-F	3.20	3.60	−0.07	2.35	1.71	0	0	64	2,6-$(OCH_3)_2$-4-NH_2	5.52	5.53	−2.86	6.14	−0.09	0	1
28	2-OCH_3	3.78	3.45	−0.78	4.07	1.32	0	1	65	2,6-$(OCH_3)_2$-4-$NHCOCH_3$[c]	3.99	4.60	−2.16	6.14	0.15	0	1
29	2-C_2H_5	3.75	3.52	−0.30	4.17	2.45	0	0	66	2-$C(CH_3)_3$,4-OCH_3 (BHA)	3.82	4.10	−1.04	4.17	3.30	0	1
30	2-OH	4.92	4.48	−0.92	2.93	0.88	0	0	67	2,6-$(C(CH_2)_3)_2$,4-$OCOCH_3$	4.35	3.79	−0.71	6.34	4.46	0	0
31	2-NH_2	5.16	4.96	−1.30	2.97	0.62	0	0	68	2,6-$(C(CH_2)_3)_2$,4-CH_2OCH_3	3.93	3.64	−0.57	6.34	4.72	0	0
32	2-$C(CH_3)_3$	4.00	3.58	−0.26	4.17	3.20	0	0	69	2,6-$(C(CH_2)_3)_2$,4-OH[c]	4.16	4.77	−1.44	6.34	4.26	0	0
33	2-SCH_3	3.70	3.84	−0.60	4.26	2.03	0	0	70	2,6-$(C(CH_2)_3)_2$,4-CH_2OH	3.88	3.49	−0.56	6.34	3.89	0	0
34	2-$CH(CH_3)_2$	3.50	3.53	−0.28	4.17	2.70	0	0	71	2,6-$(C(CH_2)_3)_2$,4-OCH_3	3.71	4.11	−1.30	6.34	5.03	0	1
35	2-$CH_2CH\ (CH_3)_2$[c]	3.90	3.30	−0.30	5.45	3.38	0	0	72	2,6-$(C(CH_2)_3)_2$,4-CHO[d]	4.06	4.25	−0.99	6.34	4.89	0	0
36	2-CH_2OH	2.70	2.97	−0.04	3.70	0.39	0	0	73	2,6-$(C(CH_2)_3)_2$,4-$COCH_3$[d]	3.94	4.12	−0.90	6.34	4.91	0	0
37	2-C_3H_7	3.49	3.50	−0.29	4.49	2.98	0	0									

[a] Predicted using eq 5. [b] Calculated using ClogP version 4.23. [c] Not included in derivation of QSAR 5. [d] Approximately values of σ^+ calculated according to ref 34.

Table 3. Cytotoxicity of Electron-Attracting Phenols versus L1210 Cells

no.	X	log $^1/_{ID_{50}}$ obsd	log $^1/_{ID_{50}}$ pred[a]	log P [b]	$B5_2$
1	H	3.27	3.32	1.48	1.00
2	4-$CONH_2$	2.48	2.67	0.33	1.00
3	4-NO_2	3.45	3.53	1.85	1.00
4	4-I	3.86	4.12	2.89	1.00
5	4-SO_2NH_2	2.50	2.49	0.01	1.00
6	4-CHO	3.08	3.30	1.44	1.00
7	4-Cl	4.29	3.89	2.48	1.00
8	4-Br	4.20	3.97	2.63	1.00
9	4-CN	3.44	3.39	1.60	1.00
10	3-NO_2	3.48	3.53	1.85	1.00
11	3-$NHCOCH_3$	2.65	2.77	0.49	1.00
12	3-Cl	3.87	3.89	2.48	1.00
13	3-OCH_3	3.71	3.37	1.57	1.00
14	3-Br	3.82	3.97	2.63	1.00
15	3-CN	3.11	3.39	1.60	1.00
16	3-F	3.46	3.57	1.91	1.00
17	3-OH	3.46	2.94	0.81	1.00
18	2-Cl	3.22	3.46	2.15	1.80
19	2-CN	3.30	3.21	1.60	1.60
20	2-NO_2	3.34	3.10	1.85	2.44
21	2-Br	3.44	3.53	2.35	1.95
22	2-I	3.95	3.58	2.54	2.15
23	2-CF_3	3.22	3.58	2.80	2.61
24	2,6-$(C(CH_3)_3)_2$-4-NO_2	4.90	4.83	5.31	3.17
25	2-CH_3-4-NO_2	3.49	3.47	2.30	2.04
26	2-CH_3-4-$COCH_3$	3.14	3.25	1.90	2.04
27	2,6-$((CH_3)_3)_2$, 4-CN	4.68	4.69	5.05	3.17

[a] Calculated using eq 6. [b] Calculated using ClogP, version 4.23.

> 2 SD) are 2-CH_3; 2-$CH_2CH(CH_3)_2$; 2-OC_2H_5; 2-$C(CH_3)_3$, 6-CH_3; 2-CH_3, 4-Br; 2,6-$(OCH_3)_2$, 4-CH_3; 2,6-$(OCH_3)_3$, 4-$NHCOCH_3$; and 2,6-$(C(CH_3)_3)_2$, 4-OH.

$B5_{2,6}$ represents the sum of the width of the substituents in the ortho position, the two substituents that flank the labile hydroxy group. Cytotoxicity decreases as the width of these substituents increase. The negative coefficient with σ^+ (−1.39) implies that highly electron-releasing substituents (e.g., NH_2, OCH_3) enhance stabilization of the phenoxy radical and increase subsequent cytotoxicity. I_1 and I_2 are indicator variables, which pinpoint the unusual activities of the ortho methyl and methoxy substituents, respectively. They both have a deleterious effect on cytotoxicity as evidenced by their negative coefficients (−1.04 and −0.58). The various descriptors σ^+, $B5_{2,6}$, I_1, log P, and I_2 make the following sequential contributions to the variance in the data: σ^+ (17%), $B5_{2,6}$ (24%), I_1 (19%), log P (18%), and I_2 (6%). The coefficient with the hydrophobic descriptor is small and suggests a surface interaction with a minimally hydrophobic receptor. The most cytotoxic phenols tend to be the unhindered ones with high electron-releasing capability such as 2-NH_2 (5.16); 4-NH_2 (5.09); 4-OC_6H_{13} (5.50); and 2,6-$(OCH_3)_2$, 4-NH_2, (5.52) and thus favored with enhanced radical-stabilizing ability. In the hexyloxy case, the hydrophobicity of the long chain also enhances cytotoxicity. BHT and 2,6-di-*tert*-butyl-4-methylphenol (BMP) are moderately toxic with IC_{50} values of 91.2 and 158.5 μM, respectively. BHA is similar to BMP in terms of its cytotoxic potential, with an IC_{50} value of 151 μM. As in QSAR eq 1, the strong dependence on σ^+ is consistent with the suggestion that radical stabilization effects are of significance in the case of electron-releasing substituents.[16]

Table 4. Cytotoxicity of Phenolic Compounds versus HL-60 and MCF-7 Cells

no.	X	HL-60 log $^1/_{ID_{50}}$ obsd	HL-60 log $^1/_{ID_{50}}$ pred[a]	MCF-7 log $^1/_{ID_{50}}$ obsd	MCF-7 log $^1/_{ID_{50}}$ pred[b]	log P	σ^+
1	H	3.05	3.06	2.56	2.78	1.48	0.00
2	4-CH_3	3.35	3.34			1.97	−0.31
3	4-OCH_3	3.60	3.53	3.52	3.55	1.57	−0.78
4	4-$(CH_2)_3CH_3$	3.52	3.67			3.56	−0.29
5	4-OC_3H_7	3.70	3.78			2.63	−0.83
6	4-NH_2[c]	4.50	3.54	4.10	4.06	0.25	−1.30
7	diethylstilbesterol	4.00	3.89			4.96	−0.16
8	equilenin	3.90	3.68			3.27	−0.42
9	β-estradiol	3.60	3.75			3.78	−0.35
10	4-C_2H_5			2.91	3.07		−0.30
11	4-OC_2H_5			3.70	3.58		−0.81
12	4-OC_6H_5[d]			3.99	3.27		−0.50
13	4-$C(CH_3)_3$			3.11	3.03		−0.26

[a] Calculated using eq 7. [b] Calculated using eq 8. [c] Not included in the derivation of eq 7. [d] Not included in the derivation of eq 8.

The negative coefficients of the I_1 (−1.04) and I_2 (−0.58) variables are particularly interesting. They highlight the diminished cytotoxicity of ortho methyl and methoxy substituted phenols. In both cases, their diminished cytotoxicities could be attributed to their unbridled reactivities and subsequent indiscriminate reaction with the surrounding aqueous medium. Their enhanced reactivities are a function of their high σ^+ values and increased intermolecular hydrogen bonding with water.

Eight of the phenols (nos. 26, 35, 38, 49, 56, 63, 65, and 69) in Table 2 were deemed to be outliers on the basis of their deviations (2 SD). Ortho substitution appears to be a common factor in this subset, particularly when bulky ortho substituents flank the phenolic group as is seen in four of the cases. The general approach utilizing the additivity of σ^+ may not provide an accurate representation of electron density in these instances. Reasons for the anomalous behavior of 2-ethoxyphenol were not apparent. Three phenols with methyl groups in the ortho positions (nos. 26, 49, 56) but not the para position (**63**) were much more active than predicted.

With the data in Table 3, the following QSAR equation was generated for the inhibition of growth of L1210 cells by electron-attracting phenols.

Cytotoxicity of electron-attracting phenols in L1210 cells:

$$\log {}^1/_{ID_{50}} = (0.56 \pm 0.11)\log P - (0.30 \pm 0.18)B5_2 + (2.79 \pm 0.22) \quad (6)$$

$$n = 27, \quad r^2 = 0.848, \quad s = 0.233, \quad q^2 = 0.812, \quad F_{2,24} = 66.91$$

The log P parameter is of critical importance in describing the cytotoxicity in this cell line. It alone accounts for 84% of the variance in the data. Note that the coefficient with the log P term is much greater than that seen in eq 5. The negative coefficient with Verloop's $B5_2$ parameter suggests that steric hindrance plays a role, albeit a minor one in facilitating membrane penetration.

Table 4 includes a set of miscellaneous phenols whose cytotoxicities were assessed versus HL-60 (human

Table 5. Cytotoxicity of Phenolic Compounds versus CCRF (Sensitive) and CEM/VLB (Resistant) Cells

		CCRF-CEM log $^1/_{ID_{50}}$		CEM/VLB log $^1/_{ID_{50}}$			
no.	X	obsd	pred	obsd	pred	log *P*	σ^+
1	4-I	4.29	4.00[a]	3.86	4.00[b]	2.89	
2	4-NO_2	3.28	3.33[a]	3.36	3.34[b]	1.85	
3	4-SO_2NH_2	2.31	2.14[a]	2.11	2.16[b]	0.01	
4	4-$CONH_2$	2.23	2.35[a]	2.38	2.36[b]	0.33	
5	4-Cl	3.45	3.74[a]	3.89	3.74[b]	2.48	
6	4-NH_2[e]	4.61	4.74[c]	1.94	5.25[d]	0.25	−1.30
7	4-OC_6H_{13}	5.34	5.10[c]	5.37	4.33[d]	4.22	−0.81
8	4-CH_3	3.82	3.71[c]	4.05	3.61[d]	1.97	−0.31
9	4-$C(CH_3)_3$	3.86	4.01[c]	3.79	3.88[d]	3.30	−0.26
10	4-H	3.27	3.10[c]	2.65	2.80[d]	1.48	0.00
11	4-OCH_3	4.41	4.32[c]	4.36	4.51[d]	1.57	−0.78
12	4-C_3H_7	3.72	3.98[c]	3.73	3.87[d]	3.03	−0.29
13	4-$C(CH_3)_2C_6H_4$-4′-OH	4.18	4.15[c]	3.96	4.05[d]	3.67	−0.29
14	4-C_2H_5	3.79	3.84[c]	3.84	3.74[d]	2.50	−0.30
15	4-C_8H_{17}	4.66	4.71[c]	4.67	4.63[d]	5.68	−0.29

[a] Calculated using eq 9. [b] Calculated using eq 10. [c] Calculated using eq 11. [d] Calculated using eq 12. [e] Not included in the derivation of eq 12.

promylocytic leukemia cell line) and MCF-7 (human breast cancer cell line). Equations 7 and 8 were developed for the inhibition of growth of HL-60 and MCF-7, respectively, by these phenols.

Inhibition of growth of HL-60 by phenolic compounds:

$$\log {}^1/_{ID_{50}} = (0.21 \pm 0.13)\log P - (0.57 \pm 0.53)\sigma^+ + (2.75 \pm 0.48) \quad (7)$$

$$n = 8,\quad r^2 = 0.816,\quad s = 0.152,\quad q^2 = 0.626,\quad \text{outlier} = 4\text{-}NH_2$$

Inhibition of growth of MCF-7 by phenolic compounds:

$$\log {}^1/_{ID_{50}} = (-1.16 \pm 0.31)\sigma^+ + (2.65 \pm 0.23) \quad (8)$$

$$n = 6,\quad r^2 = 0.964,\quad s = 0.120,\quad q^2 = 0.916,\quad \text{outlier} = 4\text{-}OC_6H_5$$

In this size-limited set, growth inhibition is also minimally dependent on hydrophobicity and mostly affected by electron density as seen in eqs 1 and 5. The coefficient with the hydrophobic term is similar, but the coefficient with σ^+ is lower. However, the 95% confidence interval is also much larger. This is in keeping with the smaller data set and the subsequent limited range in σ^+ values. It also appears that HL-60 is less susceptible to electronic perturbations than the L1210 cell line. This data set does include three estrogenic phenols.

The small number of data points precluded the examination and inclusion of other descriptors. A strong dependence of growth inhibition on electron-releasing capabilities of the phenols is observed; a large negative coefficient with σ^+ is seen. Equations 1, 5, 7, and 8 reveal a recurring theme in these cytotoxicity studies, a strong dependence on σ^+ and hence radicalization of the phenol to its ensuing phenoxy radical. The use of BDE and their excellent correlations with inhibitory potencies (eq 2) validates the importance of hydrogen abstraction in this critical ligand−receptor interaction.

Table 5 includes a limited set of X-phenols whose cytotoxicities were assessed versus CCRF-CEM (parental human acute lymphoblastic cells) sensitive and resistant (CEM/VLB) to vinblastine. The phenols were sequestered according to their electron densities, and eqs 9−12 were formulated for their activities.

Inhibition of growth of CCRF-CEM cells by electron-attracting phenols:

$$\log {}^1/_{ID_{50}} = (0.64 \pm 0.32)\log P + (2.14 \pm 0.62) \quad (9)$$

$$n = 5,\quad r^2 = 0.929,\quad s = 0.265,\quad q^2 = 0.768$$

Inhibition of growth of CEM/VLB cells by electron-attracting phenols:

$$\log {}^1/_{ID_{50}} = (0.64 \pm 0.15)\log P + (2.15 \pm 0.29) \quad (10)$$

$$n = 5,\quad r^2 = 0.983,\quad s = 0.124,\quad q^2 = 0.949$$

Inhibition of growth of CCRF-CEM cells by electron-releasing phenols:

$$\log {}^1/_{ID_{50}} = (0.28 \pm 0.10)\log P - (1.52 \pm 0.39)\sigma^+ + (2.69 \pm 0.40) \quad (11)$$

$$n = 10,\quad r^2 = 0.933,\quad s = 0.176,\quad q^2 = 0.807$$

Inhibition of growth of CEM/VLB cells by electron-releasing phenols:

$$\log {}^1/_{ID_{50}} = (0.29 \pm 0.14)\log P - (2.16 \pm 0.74)\sigma^+ + (2.37 \pm 0.51) \quad (12)$$

$$n = 9,\quad r^2 = 0.935,\quad s = 0.219,\quad q^2 = 0.859,\quad \text{outlier} = 4\text{-}NH_2$$

Once again, a similar dependence of phenolic cytotoxicity on only hydrophobicity is seen in both sensitive and resistant cells in the case of electron-attracting phenols, which suggests that nonspecific toxicity at the membrane level is operating in both cell lines. A greater difference in response is seen in the case of electron-releasing phenols. Cytotoxicity is dependent on the lipophilic and electronic character of the phenols; the electron-releasing substituents play a greater role in stabilizing the reactive intermediate in resistant cells. CEM/VLB is a Pgp overexpressing resistant subline,[17] and the sensitivity of this line to electron-releasing phenols is enhanced by the electron-rich character of the substituents. The coefficient with the σ^+ term (−2.16) is a measure of the susceptibility of cytotoxicity to electronic effects. It has been determined that CEM/VLB has a more active mitochondrial electron transport chain than its parental CCRF-CEM cell line.[18] This could account for the close interaction between the easily available radical intermediate and the electron transport chain.

Discussion

The QSAR for apoptosis contrasts sharply to the QSAR for cytotoxicity (Table 6). A significant difference involves the lack of the electronic parameter σ^+. Thus, apoptosis must include a distinct step that focuses on the interactions of the phenolic moiety with a critical receptor such as a mitochondrial protein, procaspase, or cytochrome *c*. Mitochondrial membrane polarization (MMP) is a key step in the intrinsic pathway that

Table 6. Summary of Critical Variables in Eqs 4−12

QSAR equation no.	n	cell line	compd	descriptor coefficients: steric	hydrophobic	electronic	other	intercept
				Apoptosis				
4	51	L1210	all phenols	$0.33B5_2$ $1.06B5_2$	$-0.18\pi_{2,4}$			−0.92
				Cytotoxicity				
5	63	L1210	electron-releasing phenols	$-0.28B5_{2,6}$	$0.16 \log P$	$-1.40\sigma^+$	$-0.61I_2$ $-1.05I_1$	3.88
6	27	L1210	electron-attracting phenols	$-0.30B5_2$	$0.56 \log P$			2.79
7	8	HL-60	X-phenols		$0.21 \log P$	$-0.57\sigma^+$		2.75
8	6	MCF-7	X-phenols			$-1.16\sigma^+$		2.65
9	5	CCRF-CEM	electron-attracting phenols		$0.64 \log P$			2.14
10	5	CEM/VLB	electron-attracting phenols		$0.64 \log P$			2.15
11	10	CCRF-CEM	electron-releasing phenols		$0.28 \log P$	$-1.52\sigma^+$		2.69
12	9	CEM/VLB	electron-releasing phenols		$0.29 \log P$	$-2.16\sigma^+$		2.37

characterizes apoptosis.[19] Induction of MMP is sufficient to bring on apoptosis or necrosis. Reactive oxygen species can trigger MMP, which involves drastic alterations in cell metabolism and the release of apoptogenic proteins that initiate caspase-dependent as well as caspase-independent destructive pathways.[20,21]

Yu et al. in conclusive studies have shown that the cytotoxicity of BHA is due to the induction of apoptosis as a direct consequence of release of cytochrome *c* and subsequent activation of caspases.[22] Pretreatment of rat hepatocytes with antioxidants such as *N*-acetylcysteine and ascorbic acid had little effect on apoptosis, suggesting that induction of apoptosis by BHA appears to be independent of formation of reactive metabolites. Preliminary results in my laboratory with *N*-acetylcysteine and ascorbic acid support these findings in L1210 cells.[23] QSAR eq 4, pertaining to caspase-mediated apoptosis, is also supportive of this view because it lacks the ubiquitous σ^+ term that predominates in cytotoxicity QSAR. The mostly steric terms suggest that the phenols may be interacting with apoptotic proteins released by the direct action of phenols on mitochondria. The X-phenols in this study are moderately hydrophobic and will have the ability to partition into the hydrophobic phase of the membrane lipids, which may interfere with membrane integrity particularly in the case of the bulky analogues. Bulky substituents in the ortho and meta positions of relatively small hydrophilic phenols may enhance apoptosis by binding to a hydrophilic receptor like cytochrome *c* or maybe even the procaspases directly, resulting in caspase induction or activation.

Saito et al.[24] have shown that caspases 3, 8, and 9 can be activated to varying degrees by BHT, BHA, and BMP in HL-60 cells. The concentrations that induced DNA fragmentation were between 0.1 and 0.2 mM for both BHT and BHA. Exact I_{50} values were not available. In our assays, using HL-60 cells, the I_{50} values of BHT and BHA were assessed after 16 h and determined to be 3.18 and 1.38 mM, respectively. Thus, in both cell lines, L1210 and HL-60, BHA was found to be more apoptogenic than BHT. Recently, Okubo et al. assessed the cytotoxicity of BHA in human monocytic leukemia U937 cells. It caused nuclear condensation and fragmentation, structural damage in mitochondria, reduction in mitochondrial transmembrane potential, internucleosomal DNA cleavage, and induction of caspase activity that are hallmarks of apoptosis.[25] From their results, it was established that cytotoxicity induced by BHA was mostly attributable to apoptosis phenomena.

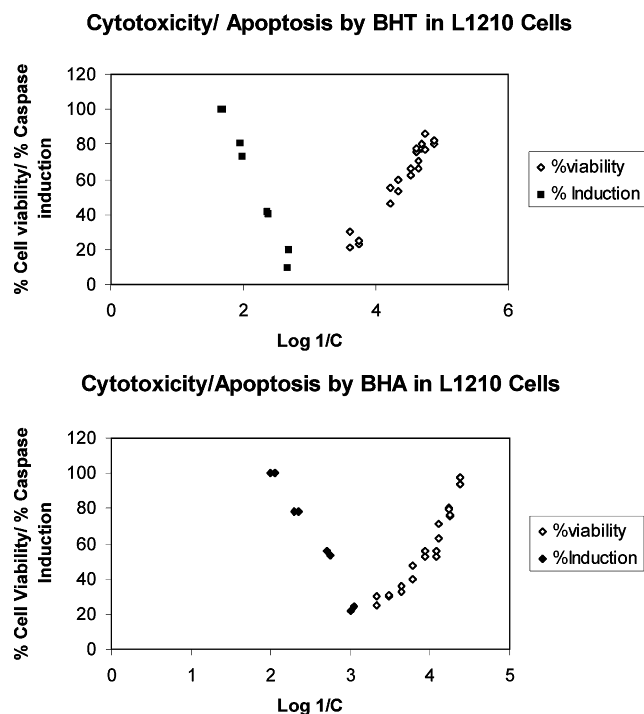

Figure 2. Contrasting behavior of BHA and BHT.

An analysis of the difference ($\Delta \log {}^1/_C$) between the cytotoxicity and apoptosis values for the phenols in this study revealed a number of compounds with values less than 1.70. They included phenols 11, 21, 30, 34, 36, 38, 39, 41, 43−48, 51, and 52. Phenols 43−48 and 51 are ortho mono-substituted phenols, which indicates that at cytotoxic concentrations, some apoptosis may ensue (~10%). Phenols that could induce considerable apoptosis at cytotoxic concentrations include 2,6-dimethoxyphenol (1.16), 2,6-dimethoxy-4-acetylphenol (0.26), 2,6-dimethoxy-4-formylphenol (0.20), 2-isopropylphenol (1.12), and BHA (1.11). However at its IC_{50}, the level of apoptosis induced by BHA is observed to be minimal, around 15−20%. BHT does not induce apoptosis at cytotoxic doses (as seen by its $\Delta \log {}^1/_C = 1.92$), which translates into a concentration differential of 80. Figure 2 illustrates the stark differences in behavior of BHA and BHT at a range of concentrations.

The significant presence of σ^+ in eqs 5, 7, and 8 underscores the importance of phenoxy radicals in mediating the ensuing inhibition of cell growth. It is well established that electron-donor groups enhance radical stabilization via an increased through-resonance ef-

fect.[26] Excellent correlations between BDE (O−H) and Brown's σ^+ have been obtained for para-substituted phenols.[16,27,28] Bordwell et al. obtained a linear plot of BDEs of the O−H bonds for 14 para-substituted phenols versus σ^+ with a ρ^+ value of 7.14.[16] Our similar analysis of a set of 28 phenols yielded a ρ^+ of 6.12 ± 0.47.

The negative sign and magnitude of the steric term ($B5_{2,6}$) indicate that extensive crowding of the O−H moiety by bulky ortho substituents leads to a small decrease in cytotoxicity. Perhaps the steric repulsion between the phenolic OH and the adjacent substituents with significant widths destabilizes the parent phenol as well as the ensuing radical.[29]

The indicator variable I_1 eludes to the observed diminished cytotoxicity of methyl-substituted phenols. This unusual behavior (considering its electron-donating capability) raises a question concerning the identity of the reactive site and the substituent. Should such compounds be considered as substituted phenols or substituted toluenes, both of which are subject to hydrogen abstraction?[30] There exists many examples of hydrogen abstraction from the benzylic carbon of toluene that are well correlated by σ^+.[31] The various coefficients with the σ^+ term (the ρ^+ values) range from −2.53 to −0.32, depending on the solvent, reagent, and temperature. It is also well established that benzylic carbon atoms are susceptible to oxidation, easily forming the corresponding alcohol metabolite.[32] These observations suggest that benzylic hydroxylation could well be occurring in the L1210 cell line, thus providing another avenue of metabolism for X-alkyl phenols.

The indicator variable I_2 pinpoints the slightly aberrant behavior of methoxy-substituted phenols, mostly those of the 2,6 variety. Using BDE data, various groups have stressed that hydrogen bonding is responsible for the stabilizing behavior of methoxy (hydrogen bond acceptors) phenols in what is deemed to be the "toward conformer" (where the hydrogen bond points toward the methoxy moiety).[26,29,33] Thus, intramolecular hydrogen bonding could contribute to reduced delocalization of the critical radical that mediates cytotoxicity. Hence, the utility of more lipophilic analogues of 2,4,6-trimethoxyphenol and 4-amino-2,6-dimethoxyphenol as potential effective antioxidants merits further attention. It must be noted that although quantum mechanical and empirical methods have been used extensively to assess the kinetic and thermodynamic stabilities of the phenolic bond, discrepancies in the published values for phenol itself have led to a recent study by Bosque and Sales, who advocated the use of molecular structural descriptors to calculate more accurate bond dissociation energies of phenols.[34]

Phenolic compounds with ortho or para alkyl substituents may be easily oxidized via an initial phenoxy radical to a more reactive quinone methide, which can subsequently alkylate cellular proteins and/or DNA to induce cytotoxicity.[35] The data set in Table 2 contains 27 of this class of phenols. Bolton et al.[36] have shown that the presence of bulky alkyl groups in the ortho positions provides little or no stabilizing influence on the oxo group of the quinone methide and also diminishes its interactions with the aqueous medium, unlike its charged resonance counterpart, in more accessible quinone methides. Thus, in the neutral form these reactive species react rapidly via nonenzymatic Michael additions to critical nucleophiles, to induce toxicity.

Conclusion

In a comparison of the concentrations of phenols utilized to induce apoptosis after 12 h and cytotoxicity after 48 h, we see marked differences. Apart from the simple monosubstituted phenols, most of the multisubstituted phenols mediate apoptosis at the millimolar level (10^{-3} mol). This contrasts sharply with the cytotoxic concentrations that hover around the 0.1 millimolar range (10^{-4} mol). This difference indicates that at critical cytotoxic concentrations, apoptosis is mostly nonexistent or minimal at best (<5%). There are four phenols that do not fall under this umbrella, and they include 2,6-dimethoxy-4-acetylphenol and 2,6-dimethoxy-4-formylphenol, whose log $^1/_C$ values are observed to be similar in the cytotoxic and apoptosis assays. This behavior suggests that their cytotoxicity may be attributed to their apoptosis-inducing ability. The other two phenols, 2,6-dimethoxyphenol and 2-*tert*-butyl-4-methoxyphenol (BHA), show minimal (<15%) apoptosis at cytotoxic-inducing concentrations.

The distinct differences between the eqs 4 and 5 suggest that cytotoxicity may not be a direct result of apoptosis. The lack of a σ^+ term in the apoptosis QSAR downplays the importance of a reactive intermediate such as a phenoxy radical in its occurrence. The presence of hydrophobic and steric terms in eq 4 suggests that apoptosis is a receptor-mediated process with mitochondrial proteins or caspases being the likely and ultimate targets. Preliminary results with a few phenols indicate that at apoptosis-inducing concentrations, BHT, BHA, and 2-amino-*p*-cresol do disrupt mitochondrial transmembrane potential in L1210 cells.[37]

In summary, data on an extensive series of substituted monophenols in different murine and human cells indicate that apoptosis occurs at high concentrations in contrast to cytotoxicity, which in most cases is not mediated by apoptotic phenomena. Cytotoxicity is an end result of complex interactions of electronic, solvation, steric, and hydrophobic factors. The ability to form a reactive phenoxy species underscores the importance of cleavage of the O−H bond in their chemical reactivities and subsequent cytotoxic activities.

Experimental Procedures

Melting points were determined on an electrothermal melting point apparatus (MEL-TEMP II with digital thermometer). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX 400 MHz NMR spectrometer with TMS as the internal standard; chemical shifts are given in δ (ppm). Thin-layer chromatography was performed on silica gel plates (silica gel IB-G Baker). Chemical elemental analysis was carried out by Desert Analytics (Tucson, AZ).

Chemicals. Most of the phenols and estrogenic phenols are commercially available. The synthesis of some of the ortho-substituted phenols have been previously reported.[12,38] 2,6-Dimethoxyphenol, 2,6-dimethoxy-4-acetophenol, 2,6-dimethoxy-4-formylphenol, and 3,5-dimethoxy-4-hydroxycinnamaldehyde were purchased from Aldrich Chemical Co. (Milwaukee, WI), while 2,6-dimethoxy-4-methylphenol was purchased from Alfa Aesar Chemical Co. (Ward Hill, MA). 2,4,6-Trimethoxyphenol was obtained from Pfaltz & Bauer (Waterbury, CT). Two other phenols, 4-amino-2,6-dimethoxyphenol and 4-acetamido-2,6-dimethoxyphenol were synthesized as follows.

Syntheses. 4-Amino-2,6-dimethoxyphenol Hydrochloride. 4-Hydroxy-3,5-dimethoxyazobenzene (1.3 g, 0.005 mol), obtained from the reaction of 2,6-dimethoxyphenol with benzenediazonium chloride,[39] was dissolved in 25 mL of hot ethanol, and a solution of sodium hydrosulfite (3.5 g in 18 mL of water) was added slowly. The reaction mixture was heated at reflux for 2 h and then immediately filtered. The filtrate was concentrated to 10 mL and then cooled. The product so obtained was filtered, washed with water, and dried. It gave 800 mg of crude 4-amino-2,6-dimethoxyphenol, which was dissolved in 100 mL of ethanol, and dry hydrogen chloride gas was passed through it. The solvent was then evaporated, and the residue was washed with ether and dried. It was crystallized from ethanol to yield the hydrochloride of 4-amino-2,6-dimethoxyphenol (740 mg, 71.49%), mp 214−215 °C. ^{1}H NMR δ_H (400 MHz, DMSO-d_6) 3.82 (6 H, s, 2 × OCH_3), 6.62 (2 H, s, Ph), 8.70 (1 H, s, OH), 9.96 (3 H, s, NH_3^+). ^{13}C NMR δ_C (400 MHz, DMSO-d_6) 56.5 (2 × OCH_3), 101.0 (C3 and C5), 122.9 (C1), 135.3 (C4), 148.6 (C2 and C6). Anal. Found (%): C, 46.51; H, 5.99; N, 6.85; Cl, 16.97. Calcd for $C_8H_{12}NO_3Cl$: C, 46.7; H, 5.84; N, 6.81; Cl, 17.27.

4-Acetamido-2,6-dimethoxyphenol. 4-Amino-2,6-dimethoxyphenol (760 mg, 4.5 mmol), as prepared above, was dissolved in a mixture of glacial acetic acid (3 mL) and acetic anhydride (3 mL), and the mixture was stirred overnight at room temperature. The solvent was evaporated at reduced pressure, and the residue was dissolved in ethyl acetate. The ethyl acetate layer was washed with water, dried over magnesium sulfate, and finally filtered. The filtrate was evaporated to provide the crude product, which was crystallized from ethanol to give 4-acetamido-2,6-dimethoxyphenol (660 mg, 69.62%), mp 157−159 °C (lit. mp 141 °C).[40] ^{1}H NMR δ_H (400 MHz, DMSO-d_6) 2.01 (3 H, s, CH_3), 3.72 (6 H, s, 2 × OCH_3), 6.92 (2 H, s, Ph), 8.08 (1 H, s, OH), 9.68 (1 H, s, NH). ^{13}C NMR δ_C (400 NHz, DMSO-d_6) 24.3 (CH_3), 56.2 (2 × OCH_3), 97.8 (C3 & C5), 131.3 (C1), 131.7 (C4), 148.0 (C2 & C6), 168.0 (CO). Anal. Found (%): C, 56.69; H, 6.30; N, 6.62. Calcd (%) for $C_{10}H_{13}NO_4$: C, 56.87; H, 6.20; N, 6.63.

Evaluation of Cytotoxicity. L1210 (murine leukemia cells), HL-60 (human promylelocytic leukemia cells), CCRF (drug sensitive parental human acute lymphoblastic cells), and CEM-VLB (multidrug-resistant subline of CCRF-resistant to 100 ng mL^{-1} vinblastine) cells were maintained in asynchronous logarithmic growth at 37 °C in RPMI medium with L-glutamine supplemented with 10% (v/v) FBS. All stock solutions and dilutions were made in unsupplemented RPMI medium.

Cell cultures were seeded at (2−5) × 10^4 cells/mL in duplicate for each inhibitor concentration in a 96-well microtiter plate (180 μL/well). The test compounds (20 μL) were then added to the cell cultures in 1:10 dilution in order to achieve the desired concentration. Each inhibitor was tested at a minimum of eight concentrations. After 48 h of continuous drug exposure, the cells were counted by using the CyQUANT GR assay kit from Molecular Probes. For this purpose the media was removed from the plates, which were then frozen at −80 °C for a minimum of 1 h. The cells were thawed at 37 °C, and 200 μL of CyQUANT GR dye/cell lysis buffer was added to each well. The plates were incubated for 5 min at 37 °C, and their fluorescence was measured using a Cytofluor II multiwell fluorescence plate reader. The excitation maximum was 485 nm, and the emission maximum was 530 nm. From the data, a dose response curve was drawn and the IC_{50} was determined. The CyQUANT GR assay measures the ability of CyQUANT GR dye to bind to cellular nucleic acids. Cytotoxicity is expressed as the concentration of the phenol (IC_{50}) that causes a 50% reduction in fluorescence compared with the controls.[41]

MCF-7 Cytotoxicity Studies. MCF-7 cells (human breast cancer cells) were maintained in asynchronous logarithmic growth at 37 °C in phenol-red free Iscoves modified Dulbecco's medium with L-glutamine supplemented with 10% (v/v) FBS. The population doubling time was 24−36 h. Every 48 h, the old media was replaced by fresh media. All stock solutions and dilutions were made in unsupplemented Dulbecco's medium. The procedures utilized to determine the IC_{50} values for the various phenols have been previously described.[42]

Determination of Caspase Activity. A fluorimetric, homogeneous caspase assay (Roche Molecular Biochemicals, Mannheim, Germany) was utilized for the quantitative in vitro determination of caspase activity in L1210 cells. Cells (4 × 10^4 cells/mL) were seeded in 96-well plates and incubated at 37 °C for 12 h in the presence or absence of varying concentrations of each substituted phenol. Then the fluorescent caspase substrate DEVD-R110 (Asp-Glu-Val-Asp-rhodamine 110) prediluted in incubation buffer was added and the microplates were incubated at 37 °C for 2 h. The degree of fluorescence was measured at 521 nm after excitation at 490 nm. The concentration of X-phenol that induced caspase activity by 50% (I_{50}) was calculated and used to derive the appropriate QSAR equation.

QSAR Analysis. The CQSAR suite of programs (BioByte, Inc., Claremont, CA) was used to derive the various models.[43] P represents the octanol−water partition coefficients of the phenols. In this study, calculated log P (CLogP) values were used to represent hydrophobicity. σ^+ is Brown's refinement of the Hammett electronic constant σ.[44] In all equations, n represents the number of data points, r is the correlation coefficient, s is the standard deviation of the regression equation, and q^2 represents the cross-validated r^2. The 95% confidence intervals for the terms in the equations are listed in parentheses. The cross-validated r^2 (q^2) is obtained by using the leave-one-out (LOO) procedure of Cramer et al.[14]

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