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3-(3,4,5-Trimethoxyphenyl)-1-oxo-2-propene: A novel pharmacophore displaying potent multidrug resistance reversal and selective cytotoxicity

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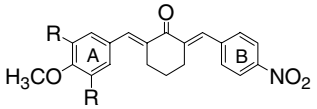
Abstract—This study revealed that various alicyclic and acyclic compounds containing the 3-(3,4,5-trimethoxyphenyl)-2-propenoyl group displayed potent MDR reversal properties. In particular, a concentration of 4 μg/ml of 2,5-bis(3,4,5-trimethoxyphenylmethylene)cyclopentanone was 31 times more potent than verapamil as a MDR revertant. In general, they were selectively toxic to malignant rather than normal cells. Two representative compounds induced apoptosis in human HL-60 cells and markedly activated caspase-3.

1. Introduction

A major problem in cancer chemotherapy is multidrug resistance (MDR) which is caused principally by the increased efflux of drugs from malignant cells due to increased expression of P-glycoprotein. A number of structurally divergent drugs used in treating a variety of pathological conditions possess MDR-reversing properties such as verapamil,[1] trifluoperazine,[2] and quinine[3] as well as molecules designed as MDR-reversing agents such as biricodar.[4] A preliminary communication from our laboratory disclosed that 6-(4-nitrophenylmethylene)-2-(3,4,5-trimethoxyphenylmethylene)cyclohexanone **1** reversed MDR in murine L-5178 cells and demonstrated greater toxicity toward various neoplasms than non-malignant cells (Fig. 1 and Table 1).[5] In this series of compounds, the 3,4,5-trimethoxyphenyl group was optimal, for example, under the test conditions utilized, the 4-methoxy analog **2** was bereft of MDR-reversing and cytotoxic properties.[5] A subsequent study confirmed the good potency of **1** (but not **2**) towards additional neoplastic cell lines.[6] Clearly **1** is a novel lead compound and the objective of the present study was to prepare a limited number of prototypic molecules in order to develop guidelines for a major expansion in this area.

The MDR-reversing properties of **1** are likely influenced, inter alia, by hydrophobic bonding between the aryl rings A and B and a complementary area on a binding site. In addition, bioactivity is likely mediated by interactions between the olefinic carbon atoms of **1** with cellular nucleophiles such as thiols. Hence the alicyclic and acyclic analogs of **1**, namely **3** and **4**, were proposed



1 R = OCH_3
2 R = H

Figure 1. Structures of compounds **1** and **2**.

Keywords: Unsaturated ketones; 3-(3,4,5-Trimethoxyphenyl)-2-propenoyl pharmacophore; Multidrug resistance; Cytotoxicity.
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Table 1. Evaluation of **1**–**10** for MDR reversal

Compound	FAR values[a]	
	4 μg/mL	40 μg/mL
1[b]	15.6	75.1
2[b]	0.76	0.82
3	1.10	1.36
4	0.82	1.89
5	1.38	1.34
6	13.3	69.3
7	4.30	13.8
8	96.3	107
9	1.15	39.0
10	1.37	48.3

[a] FAR, fluorescence activity ratio. The FAR values of verapamil are 3.08 and 37.20 when 4 and 40 μg/mL of drug were employed.[28]
[b] The data were previously reported in Ref. 5 copyright (2005) with permission of Elsevier.

in which the relative positions of the aryl rings and olefinic carbon atoms of these three molecules should differ which may be reflected by variations in MDR-reversing potencies. For similar reasons, a comparison of the bioactivities of **2** and the related acyclic analog **5** was planned.

The compounds related to **1** which had previously shown MDR-reversing properties all contained a 4-nitrophenylmethylene group.[5] However, aryl nitro groups can give rise to unwanted toxicity[7–9] and the ability of **1** to cause reversal of MDR may have been due, either wholly or in part, to the presence of the 3,4,5-trimethoxyphenylmethylene group. Hence removal of the nitro group from ring B of **1** and the introduction of multiple methoxy substituents to form **6** were suggested. In order to explore whether the mounting of both 3,4,5-trimethoxyphenylmethylene groups on different cyclic scaffolds would influence MDR-reversing potencies, the preparation of two analogs of **6**, namely **7** and **8**, was planned. Finally the presence of one 3,4,5-trimethoxyphenylmethylene group could be sufficient to reverse MDR and in order to explore this possibility, the synthesis and bioevaluation of **9** and **10** were proposed.

In addition, an assessment of whether the analogs of **1** would demonstrate cytotoxic activity was planned and, if so, whether selective toxicity to malignant cells occurred. The final phase of the study was the evaluation of the biodata in the light of molecular modeling techniques as well as probing for the mode of action of representative compounds.

2. Results

Compounds **3**–**10** were prepared by the synthetic chemical pathways indicated in Figure 2. The ability of these compounds to reverse MDR was assessed using murine lymphoma L-5178 cells transfected with the human MDR1 gene and the data are presented in Table 1. In addition, two representative compounds **7** and **8** were evaluated for reversal of MDR using human colo 320 colon cancer cells. The quest for identifying compounds with preferential cytotoxicity for malignant cells used HSC-2, HSC-3, and HSC-4 squamous cell carcinomas and HL-60 promyelocytic leukemia cells as well as the following non-malignant cells viz. HGF gingival fibroblasts, HPLF periodontal ligament fibroblasts, and HPC pulp cells. The results for these assays are portrayed in Table 2. The unsaturated ketones **1** and **4** induced bimodal changes in the caspase activation in HL-60 and HSC-2 cells. They induced caspase activation more potently in HL-60 cells than in HSC-2 cells (Fig. 4). They induced internucleosomal DNA fragmentation in HL-60 cells, while in HSC-2 cells they induced a large DNA fragment (indicated by an arrow in Fig. 5) without the induction of internucleosomal DNA fragmentation.

3. Discussion

The ^{1}H NMR spectra of the unsaturated alicyclic ketones revealed that the compounds possess the *E* stereochemistry based on the absorptions of the olefinic protons[10–13], while the acyclic molecules **4**, **5**, and **10** were the *E* isomers as revealed by the coupling constants of the olefinic protons.[14] These conclusions are in accord with X-ray crystallographic determinations of related compounds.[15–17]

The evaluation of two concentrations of the enones **1**–**10** as MDR reversing agents is summarized in Table 1. In this assay, rhodamine 123 is employed and its concentration in treated and untreated cells is compared from which data, fluorescence activity ratio (FAR) values are computed. Compounds with a FAR value of 1 or more indicate that reversal of MDR has taken place. MDR modulators likely bind in the transmembrane domains of Pgp thereby inducing a conformational change in Pgp which inhibits the action of ABC transporters.[18] The following conclusions may be drawn from the biodata. First, the 4-nitrophenylmethylene group is not essential for inducing MDR-reversing properties. Thus, replacement of this functionality in **1** by a 3,4,5-trimethoxyphenylmethylene group led to **6** having a similar MDR reversing potency. In fact, on occasions the 4-nitrophenylmethylene substituent exerted a dystherapeutic effect, for example, excision of this group from the inactive compounds **3** and **4** led to **9** and **10**, respectively, which possess MDR reversing properties. Second, an attempt was made to find reasons for the differences in MDR-reversing properties between **1** which is an active compound and **3** and **4** which are devoid of this property. Figure 3 reveals the differences in the relative locations of not only the aryl rings A and B but the olefinic carbon atoms as well, suggesting that the topography of these molecules impacts considerably on the magnitude of the biological responses. Third, both compounds containing a sole methoxy group in aryl ring A viz. **2** and **5** were devoid of MDR-reversing properties. Fourth, all three bis compounds **6**–**8** reverse MDR. In particular, the remarkable potency of **8** establishes it as a very useful lead compound which is likely due, at least in part, to the favorable spatial arrangement of

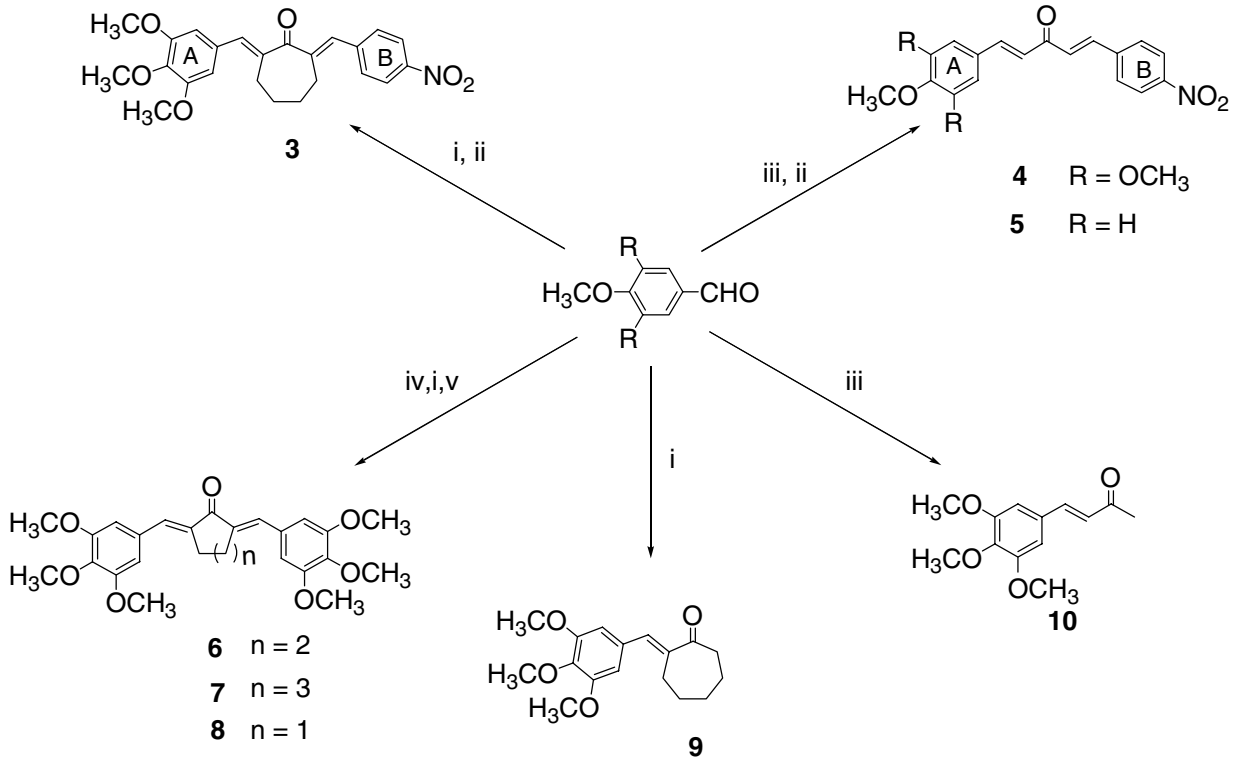

Figure 2. The synthetic chemical scheme used in preparing **3**–**10**. The reagents used were as follows, i, cycloheptanone/NaOH; ii, 4-nitrobenzaldehyde/NaOH; iii, acetone/NaOH; iv, cyclohexanone/HCl; v, cyclopentanone/HCl.

Table 2. Evaluation of **1**–**10** for reversal of MDR and cytotoxic properties

Compound	CC_{50}[a] (μM)								
	HSC-2	HSC-3	HSC-4	HL-60	Ave[b]	HGF	HPLF	HPC	SI[c]
1[d]	0.76	4.7	1.5	1.1	< 2.0	336	378	347	>176
2[d]	>400	>400	>400	>400	>400	>400	>400	>400	~1.0
3	116	279	100	266	190	256	370	198	1.4
4	<3.1	5.7	3.8	6.4	<4.8	16	106	94	>15
5	6.3	16	12	25	15	81	193	141	9.3
6	34	62	36	46	45	335	388	285	7.5
7	113	132	67	46	90	229	250	276	2.8
8	19	89	58	21	47	240	281	247	5.5
9	214	200	269	31	179	262	291	277	1.6
10	55	72	41	47	54	159	263	188	3.8
Melphalan[e]	35	115	81	6	59	>200	>200	>200	>3.4

[a] The CC_{50} figure is the concentration of compound required to kill 50% of the cells and is the average of two independent determinations.
[b] Average CC_{50} value of the compound toward HSC-2, HSC-3, HSC-4, and HL-60 neoplastic cells.
[c] The letters SI refer to the selectivity index.
[d] With the exception of the figure of HSC-4 cells, the data were previously reported in Ref. 5 copyright (2005) with permission of Elsevier.
[e] Solubility problems of melphalan in dimethylsulfoxide precluded the use of concentrations above 200 μM.

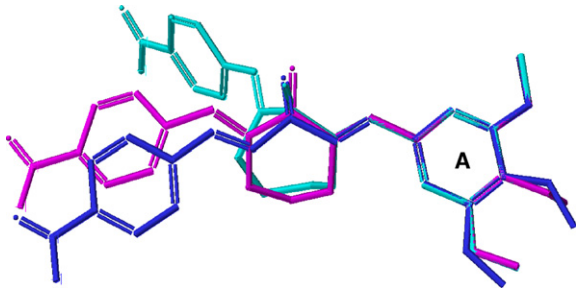

Figure 3. Overlap of ring A in **1** (pink), **3** (green), and **4** (blue). Hydrogen atoms were removed for clarity.

the 3,4,5-trimethoxyphenylmethylene groups. Fifth, removal of one of the 3,4,5-trimethoxyphenylmethylene groups of **7** yielding **9** led to an increase in potency suggesting that only one of these structural moieties may be required to confer MDR reversing properties in these compounds. Finally in considering the possible structural features which may influence the magnitude of MDR reversal in mouse L-5178 cells, two other physicochemical parameters were considered. The ability of the 3-(3,4,5-trimethoxyphenyl)-2-propenoyl group to align at a binding site will likely be influenced by the torsion angle θ between aryl ring A and the adjacent enone

group. Hence the torsion angles of **1**–**10** were obtained and are listed in Table 3. In addition, the olefinic carbon atom adjacent to ring A may well undergo electrophilic attack with cellular nucleophiles and hence the varying charges on these atoms may influence bioactivity. These atomic charges were obtained and are presented in Table 2. Linear and semilogarithmic plots were made between the FAR figures generated using 40 μg/ml of the compounds **1**–**10** and both the θ values and atomic charges. No significant correlation was noted between the FAR values and either the torsion angles or atomic charges ($p > 0.05$).

The potential of these compounds as MDR revertants will be enhanced if similar results were observed using a different species and another neoplastic disease. In order to explore this avenue, two representative compounds **7** and **8** with substantially different potencies in the murine L-5178 lymphoma assay were examined using human colo 320 colon cancer cells. When a concentration of 4 μg/ml was employed, the FAR values of **7** and **8** are 3.65 and 8.26, respectively, which compares favorably with verapamil (a concentration of 10 μg/ml of this compound in this assay led to a FAR value of 3.84). These results provide additional evidence of the value of exploring further the 3-(3,4,5-trimethoxyphenyl)-2-propenoyl pharmacophore in the design of MDR revertants.

The evaluation of compounds **1**–**10** for cytotoxic properties and selective toxicity to malignant cells is summarized in Table 2. Comments will be made on the new biodata generated for **3**–**10**. All of the enones display cytotoxic properties to the malignant HSC-2, HSC-3, HSC-4, and HL-60 cells. Apart from **2**, **3**, and **9**, the average CC_{50} values were lower than that of melphalan and the most potent compounds are the acyclic enones **4** and **5**. In order to identify those compounds displaying selective toxicity to neoplastic cells, selective index (SI) figures were calculated. These values reveal that the compounds demonstrate greater cytotoxicity toward tumorous cells, particularly **4** and **5**. Thus in terms of both cytotoxic potencies and selective toxicity for neoplastic cells, both **4** and **5** emerged as lead molecules.

Table 3. Torsion angles and charge densities in compounds **1**–**10**

Compound	θ[a] (°)	Atomic charge[b] (esu)
1	−42.5	−0.052
2	−42.9	−0.037
3	−54.0	−0.098
4	−0.01	−0.030
5	−0.11	−0.015
6	−0.02	−0.050
7	−52.1	−0.101
8	8.15	−0.053
9	−51.8	−0.053
10	−0.05	−0.029

[a] θ is the torsion angle between aryl ring A and the adjacent olefinic group.

[b] The atomic charge is the electron density on the olefinic carbon atom adjacent to ring A.

Studies were undertaken to determine the manner whereby cytotoxicity was caused by two of the lead molecules **1** and **4**. Most if not all cytotoxic agents induce apoptosis.[19] Incubation of **1** and **4** revealed that DNA laddering, a hallmark of apoptosis, occurred only in HL-60 cells, while they did not induce DNA laddering in HSC-2 cells, possibly due to the lack of activation of endonuclease or change in the chromatin structure (Fig. 5). Death stimuli activate two principal pathways which lead to apoptosis; both biochemical mechanisms are dependent on various cysteine proteases or caspases. First, the mitochondrial pathway involves the activation of caspase-9 while second, the production of caspase-8 is increased in the death receptor pathway.[20] Both processes lead to activation of downstream effector caspases such as caspase-3.[21] Hence the ability of **1** and **4** to activate caspases-3, -8, and -9 in HL-60 and HSC-2 cells was investigated.

These data are presented in Figure 4. In the case of HL-60 cells, at the lowest concentration utilized viz. 4 μM, caspase-3 was activated markedly by **1**, while **4** increased the activity of caspases-3, -8, and -9 above 8 μM. Some increases in the activations of caspases-8 and -9 were noted when 8 μM of **1** was employed. It should be noted that the extent of caspase activation was comparable with that achieved with the positive control actinomycin D. These data reveal that the mode of action of **1** and **4** in this cell line is due, at least in part, to activation of caspases-3, -8, and -9. On the other hand, while **1** and **4** were more cytotoxic toward HSC-2 cells than the HL-60 neoplasm, activation of caspases was appreciably less noticeable as compared with that attained by actinomycin D. Thus while **4** activated caspase-3 at 8 μM, only at the highest concentration of 32 μM were there increases in the activation of both caspases-3 and -8 by **1** and in caspase-8 and -9 by **4**, while **1** failed to activate caspase-9. Thus the modes of action of **1**–**10** likely vary depending on the cell line. These compounds may induce apoptosis in HL-60 cells while they may induce incomplete apoptosis in other type of cells. This capacity to cause toxicity by different biochemical mechanisms depending on the cells under consideration may contribute to the selective damage to malignant cells observed on occasions vide supra thereby enhancing the potential of these compounds as lead molecules in developing agents for use in cancer chemotherapy.

4. Conclusions

Compounds **6**–**10** are novel MDR-reversing agents which have been developed from the lead compound **1**. In particular the remarkable potency of **8** was discovered in this study. Future work will involve placing one or more 3-(3,4,5-trimethoxyphenyl)-2-propenoyl groups onto a variety of acyclic, alicyclic, aryl, and heteroaryl scaffolds in an attempt to find correlations between the topography of these molecules and potencies in this assay. Most of the compounds possess noteworthy cytotoxic potencies and display greater lethality to some neoplasms than normal cells. Activation of different caspases which induced apoptosis was shown in repre-

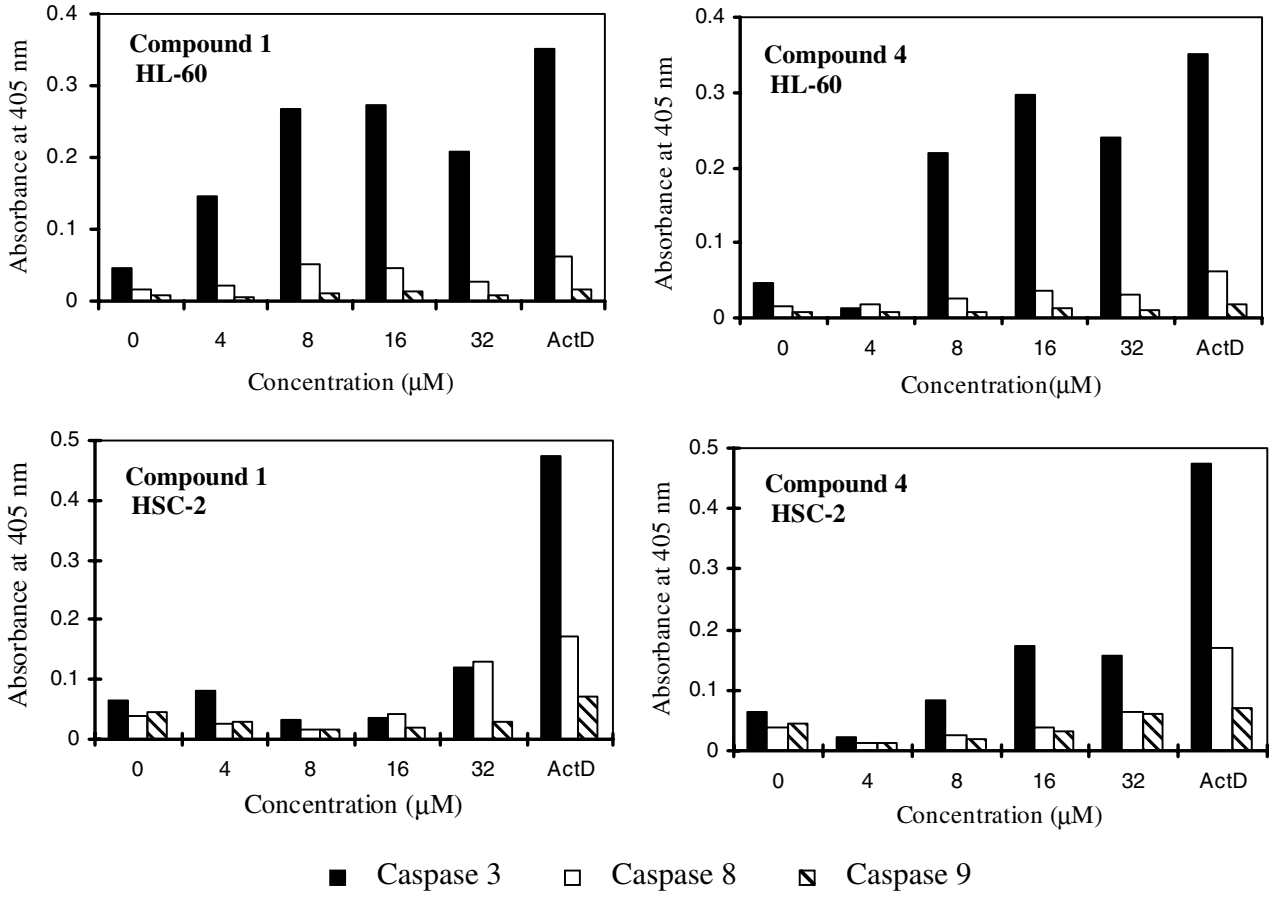

Figure 4. The effect of **1** and **4** on the activation of caspases-3, -8, and -9 in HL-60 and HSC-2 cells. HL-60 cells (4×10^6 cells) and HSC-2 cells (2×10^6 cells) were incubated for 4 h with the indicated concentrations of **1** or **4** or actinomycin D (1 μg/mL), and caspase activity was measured by the substrate cleavage assay. Each value represents the mean from 2 to 3 experiments.

sentative molecules to be at least one way whereby cytotoxicity was mediated.

5. Experimental

The melting point (mp) of each compound was determined on a Gallenkamp melting point apparatus and are uncorrected. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Bruker AM 500 FT NMR instrument. The letters s, br s, d, t, and q refer to singlet, broad singlet, doublet, triplet, and quartet, respectively. Elemental analyses were undertaken on **3–5** (C, H, N) and **6–10** (C, H) by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan. Column chromatography was carried out using silica gel 60 (70–230 mesh).

5.1. Synthesis of 3–10

5.1.1. 2-(4-Nitrophenylmethylene)-7-(3,4,5-trimethoxyphenylmethylene)-cycloheptanone (3). Aqueous sodium hydroxide solution (10%, 3 ml) was added dropwise over a period of 10 min to a cooled solution of **9** (2.9 g, 0.01 mol) vide infra and 4-nitrobenzaldehyde (1.51 g, 0.01 mol) in ethanol (25 ml). The mixture was stirred for 8 h. The yellow solid separated out was filtered, washed with water, dried, and crystallized from chloroform/ethanol to give **3**. Mp 157 °C; yield 61%. ^{1}H NMR ($CDCl_3$): 2.04 (br s, 4H, $2 \times CH_2$), 2.70 (s, 2H, CH_2), 2.76 (s, 2H, CH_2), 3.90 (s, 9H, $3 \times OCH_3$), 6.71 (s, 2H, Ar–H), 7.38 (s, 1H, =CH), 7.42 (s, 1H, =CH), 7.59 (d, 2H, Ar–H, $J = 8.50$ Hz), 8.28 (d, 2H, Ar–H, $J = 8.55$ Hz). Anal. Calcd for $C_{24}H_{25}NO_6$: C, 68.07; H, 5.95; N, 3.31. Found C, 68.21; H, 5.82; N, 2.99%.

5.1.2. 1-(4-Nitrophenyl)-5-(3,4,5-trimethoxyphenyl)-1,4-pentadien-3-one (4). Aqueous sodium hydroxide solution (10%, 3 ml) was added dropwise over a period of 10 min to a cooled solution of **10** (2.36 g, 0.01 mol) vide infra and 4-nitrobenzaldehyde (1.51 g, 0.01 mol) in ethanol (25 ml). A yellow solid separated after the addition of sodium hydroxide was completed. The mixture was stirred for additional 1 h, filtered, washed with water, and dried. The crude compound was purified by column chromatography [eluent/ethyl acetate and hexane (2:8)] and finally crystallized from ethanol to give **4**. Mp 169 °C; yield 42%. ^{1}H NMR ($CDCl_3$): 3.93 (s, 3H, OCH_3), 3.94 (s, 6H, $2 \times OCH_3$), 6.87 (s, 2H, Ar–H), 6.97 (d, 1H, =CH, $J = 15.85$ Hz), 7.23 (d, 1H, =CH, $J = 15.87$ Hz), 7.71 (d, 1H, =CH, $J = 15.88$ Hz), 7.76 (d, 1H, $J = 16.15$ Hz), 7.78 (d, 2H, $J = 8.68$ Hz), 8.29 (d, 2H, $J = 8.7$ Hz). Anal. Calcd for $C_{20}H_{19}NO_6$: C, 65.03; H, 5.18; N, 3.79. Found C, 64.82; H, 4.98; N, 3.61%.

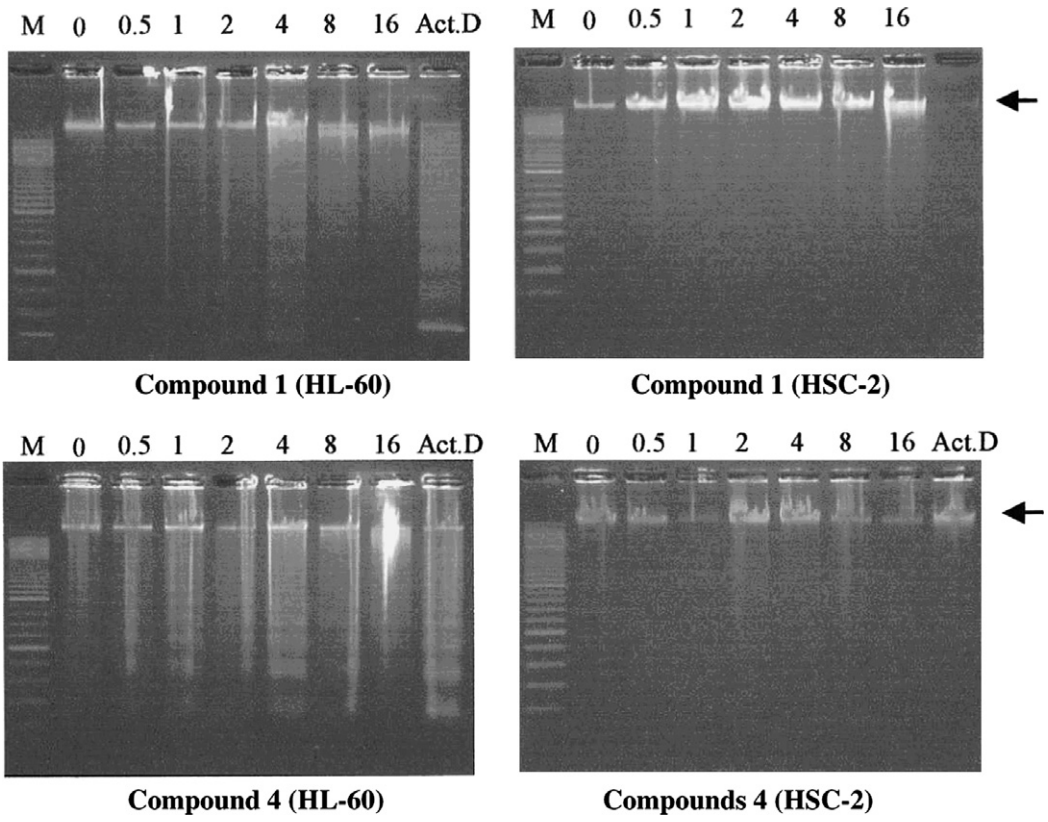

Figure 5. HL-60 or HSC-2 cells (5×10^5 cells) were incubated for 6 hours with the indicated concentrations of **1** or **4**, or actinomycin D (1 μg/mL). DNA fragmentation was detected above 4 μM in HL-60 cells. No DNA fragmentation was observed in HSC-2 cells. M is the molecular weight marker of DNA. The arrow indicates a large DNA fragment. Reproducible results were obtained in another experiment.

5.1.3. 1-(4-Methoxyphenyl)-5-(4-nitrophenyl)-1,4-pentadien-3-one (5). 4-Methoxybenzaldehyde was condensed with acetone using the same method employed in the presence of **10** to give 4-(4-methoxyphenyl)-3-buten-2-one. Mp 73 °C (lit.[22] 72 °C); yield 62%. ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H, $COCH_3$), 3.86 (s, 3H, OCH_3), 6.63 (d, 2H, Ar–H, $J = 8.64$ Hz), 7.48 (d, 1H, =CH, $J = 15.0$ Hz), 7.51 (d, 2H, Ar–H, $J = 8.08$ Hz). This intermediate was reacted with 4-nitrobenzaldehyde using the same procedure as employed in the synthesis of **4** to give the desired product which was purified by crystallization from chloroform/ethanol. Mp 182 °C; yield 56%. ^{1}H NMR ($CDCl_3$): 3.87 (s, 3H, OCH_3), 6.96 (m, 3H, =CH and Ar–H), 7.20 (d, 1H, =CH, $J = 15.85$ Hz), 7.61 (d, 1H, $J = 6.9$ Hz), 7.75 (m, 4H, 2 × =CH and Ar–H), 8.28 (d, 2H, Ar–H, $J = 8.75$ Hz). Anal. Calcd for $C_{18}H_{15}NO_4$: C, 69.89; H, 4.89; N, 4.53. Found C, 69.74; H, 4.97; N, 4.49%.

5.1.4. 2,6-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclohexanone (6). Dry hydrogen chloride was bubbled through a solution of cyclohexanone (1.96 g, 0.01 mol) and 3,4,5-trimethoxybenzaldehyde (3.92 g, 0.02 mol) in ether (30 ml) for 10 min. The solution was stirred at room temperature for 2 h. The solid obtained was filtered and crystallized from chloroform/ethanol to give **6**. Mp 208 °C; yield 68%. ^{1}H NMR ($CDCl_3$): δ 2.05 (br s, 4H, 2 × CH_2), 2.75 (br s, 4H, 2 × CH_2), 3.88 (s, 18H, 6 × OCH_3), 6.72 (s, 4H, Ar–H), 7.35 (s, 2H, 2 × =CH). Anal. Calcd for $C_{26}H_{30}O_7$: C, 68.71; H 6.65. Found C, 68.58; H 6.67%.

5.1.5. 2,7-Bis-(3,4,5-trimethoxyphenylmethylene)-cycloheptanone (7). A mixture of cycloheptanone (1.12 g, 0.01 mol), 3,4,5-trimethoxybenzaldehyde(3.92 g, 0.02 mol), and aqueous sodium hydroxide solution (35%, 2 ml) in ethanol (30 ml) was stirred at room temperature for 3 h and then heated under reflux for 4 h. The reaction mixture was cooled to room temperature and stirred overnight. The solid obtained was filtered, washed with water, and crystallized from chloroform/ethanol to give **7**. Mp 182 °C; yield 63%. ^{1}H NMR ($CDCl_3$): δ 1.84 (m, 2H, CH_2), 2.97 (t, 4H, 2 × CH_2), 3.90 (s, 18H, 6 × OCH_3), 6.73 (s, 4H, Ar–H), 7.74 (s, 2H, =CH). Anal. Calcd for $C_{27}H_{32}O_7$: C, 69.21; H 6.88. Found C, 69.21; H 7.10%.

5.1.6. 2,5-Bis-(3,4,5-trimethoxyphenylmethylene)-cyclopentanone (8). Compound **8** was prepared from cyclopentanone by the same procedure which was employed in synthesizing **6**. The crude product was recrystallized from chloroform/ethanol. Mp 205 °C; yield 71%. ^{1}H NMR ($CDCl_3$): δ 3.16 (s, 4H, 2 × CH_2), 3.92 (s, 18H, 6 × OCH_3), 6.87 (s, 4H, Ar–H), 7.54 (s, 2H, 2 × =CH). Anal. Calcd for $C_{25}H_{28}O_7$: C, 68.17; H, 6.41. Found C, 68.12; H 6.45%.

5.1.7. 2-(3,4,5-Trimethoxyphenylmethylene)-cycloheptanone (9). A solution of cycloheptanone (3.36 g, 0.03 mol), 3,4,5-trimethoxybenzaldehyde (3.92 g, 0.02 mol), and aqueous sodium hydroxide solution (10%, 3 ml) in ethanol (30 ml) was stirred at room temperature for 12 h. The solution was acidified with dilute hydrochloric acid and the solvents removed in vacuo. The residue remaining was extracted with chloroform, the organic extracts were washed with water, dried over sodium sulfate, and evaporated under vacuum. The residue obtained was purified by column chromatography using ethylacetate/hexane(1:9) as eluent. Mp 88 °C; yield 61%. ^{1}H NMR ($CDCl_3$): δ 1.80 (m, 6H, $3\times CH_2$), 2.73 (t, 4H, $2\times CH_2$), 3.87 (s, 6H, $2\times OCH_3$), 3.89 (s, 3H, OCH_3), 6.60 (s, 2H, Ar–H), 7.48 (s, 1H, =CH). Anal. Calcd for $C_{17}H_{22}O_4$: C, 70.32; H, 7.64. Found C, 70.59; H, 7.63%.

5.1.8. 4-(3,4,5-Trimethoxyphenyl)-3-buten-2-one (10). A solution of acetone (40 ml), 3,4,5-trimethoxybenzaldehyde (4.9 g, 0.025 mol), and aqueous sodium hydroxide solution (4%, 10 ml) was stirred at room temperature for 12 h. The solution was neutralized with dilute hydrochloric acid, extracted with chloroform, the combined organic extracts were dried over sodium sulfate and evaporated to dryness. The residue obtained was purified by column chromatography (ethyl acetate/hexane 1:9) to obtain the desired product. Mp 91 °C; yield 54%. ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, $COCH_3$), 3.90 (s, 9H, $3\times OCH_3$), 6.63 (d, 1H, =CH, J = 16.16 Hz), 6.81 (s, 2H, Ar–H),7.44 (d,1H, =CH, J = 16.17 Hz). Anal. Calcd for $C_{13}H_{16}O_4$: C, 66.09; H, 6.83. Found C, 66.23; H, 6.97%.

5.2. Molecular modeling

Models of the compounds **1**–**10** were built using BioMedCache 6.1 for Windows. The lowest energy conformations were obtained from optimized geometry calculations in MOPAC using AM1 parameters. All six carbon atoms comprising ring A in **1**, **3**, and **4** were overlapped when constructing Figure 3.

5.3. MDR-reversal assay

This assay has been reported previously.[23] In brief, L-5178 mouse T-cell lymphoma cells were transfected with the pHa MDR1/A retrovirus using a literature methodology[24] and cultured in the presence of colchicine in order to maintain the expression of the MDR phenotype. Two concentrations of the test compound in dimethylsulfoxide viz. 4 and 40 μg/mL were added to both the parent L-5178 cells and the MDR1 transfected subline. After incubation at room temperature for 10 min, a solution of rhodamine 123 was added to the samples and incubated at 37 °C for 20 min. The fluorescence of the parental cells (designated F1 and F2 for the treated and untreated cells, respectively) and the MDRl subline (designated F3 and F4 for the treated and untreated cells, respectively) was measured by a Beckton–Dickinson FACScan flow cytometer. The FAR value was calculated from the following equation, namely FAR = [F3/F4]/[F1/F2]. The FAR value for dimethylsulfoxide ranged from 1.02 to 2.33. Verapamil was employed as a positive control using a concentration intermediate between 4 and 40 μg/ml, namely 10 μg/ml. A similar method was followed when evaluating MDR in human colo 320 colon cancer cells, except that before flow cytometry was employed, the cells which form a monolayer were treated with 0.25% trypsin solution for 2–3 min in order to produce a suspension of individual cells.

5.4. Evaluation against cancerous and malignant cells

The procedure utilized for evaluating the cytotoxic properties of the enones **3**–**10** using both neoplastic and non-malignant cell lines has been described previously.[25] In brief, the cells were incubated in DMEM supplemented with 10% heat-activated FBS in a humidified 5% carbon dioxide atmosphere for 24 h. Cells in the presence of various concentrations of compounds as well as untreated cells were washed once with phosphate-buffered saline and incubated for 4 h in fresh media containing MTT. The cells were isolated and lysed with dimethylsulfoxide and the relative number of viable cells was determined at 540 nm using a Microplate reader. In the case of HL-60 cells, a slightly different strategy was employed insofar as this cell line was cultured in RPMI1640 media plus 10% FBS and viable cells were measured by trypan blue exclusion. The CC_{50} values were determined from dose–response curves and the figures in Table 1 are means from duplicate experiments. The selectivity index figures were obtained from the following equation, namely $[CC_{50}(HGF) + CC_{50}(HPLF) + CC_{50}(HPC)]/[CC_{50}(HSC\text{-}2) + CC_{50}(HSC\text{-}3) + CC_{50}(HSC\text{-}4) + CC_{50}(HL\text{-}60)] \times 4/3$.

5.5. Effect of 1 and 4 in causing apoptosis and activating caspases in HL-60 and HSC-2 cells

The procedure for determining apoptosis and measuring caspase activation has been described previously.[26] In brief, the cells were cultured in either RPMI1640 media supplemented with 10% heat-inactivated FBS (HL-60 cells) or in DMEM supplemented with 10% FBS (HSC-2 cells) in a humidified 5% carbon dioxide atmosphere. For the DNA fragmentation assay, the cells were incubated at 37 °C for 6 h with varying concentrations of compounds as well as untreated cells. The cells were then removed, lysed, and assayed for internucleosomal DNA fragmentation by agarose gel electrophoresis. The fragmentation pattern was examined in photographs taken under UV illumination. The caspase activation was determined by the substrate cleavage method. Cells were incubated for 4 h with test samples, and then removed and lysed. The lysate (200 μg proteins) was mixed with the substrates for caspase-3 (DEVD-*p*NA(*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*aNA). After incubation for 2 h at 37 °C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured using a microplate reader.[27] The concentrations of compounds used in the DNA fragmentation assay were 0.5, 1, 2, 4, 8, and 16 μM and for caspase activation 4, 8, 16, and 32 μM were employed. In all of these assays, actinomycin D (1 μg/mL) was used as the positive control.

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