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Research Article

Structural Factors Affecting Cytotoxic Activity of (*E*)-1-(Benzo[*d*][1,3]oxathiol-6-yl)-3-phenylprop-2-en-1-one Derivatives

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Derivatives of (*E*)-1-(5-alkoxybenzo[*d*][1,3]oxathiol-6-yl)-3-phenylprop-2-en-1-one (1) demonstrated exceptionally high *in vitro* cytotoxic activity, with IC_{50} values of the most active derivatives in the nanomolar range. To identify structural fragments necessary for the activity, several analogs deprived of selected fragments were prepared, and their cytotoxic activity was tested. It was found that the activity depends on combined effects of (i) the heterocyclic ring, (ii) the alkoxy group at position 5 of the benzoxathiole ring, and (iii) the substituents in the phenyl ring B. Replacement of the sulfur atom by oxygen does not influence the activity. None of the listed structural fragments alone assured high cytotoxic activity.

Key words: benzoxathiole, chalcone, cytotoxic activity, SAR, structure optimization

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Chalcones are widely explored as potential lead compounds for new therapeutic agents (1–13). Every year, hundreds of new chalcone derivatives are prepared and tested in pursuit of analogs with better pharmacological properties. Concerning compounds being prepared as potential antitumor drugs, *in vitro* cytotoxic activity of most of the new derivatives is in the low micromolar range. Meanwhile, some of the obtained by us oxathiole-fused chalcones **1** (Figure 1) (14) exhibited activity at low nanomolar level, comparable only with activity of 3,4,5-trimethoxy derivatives of chalcone (11).

Cytotoxic chalcones are generally believed to act as inhibitors of microtubule polymerization, interacting with tubulin at colchicine-binding site (11, 15). For this reason, and based on preliminary studies of mechanism (16), it seems that oxathiole-fused chalcones **1** act similarly. However, taking into account the diversity of chalcones activity and mechanisms of action (e.g., 1, 13), other possibilities could not be ruled out.

The outstanding cytotoxic activity of chalcones **1** prompted us to carry out detailed studies on structural factors essential for the high cytotoxicity.

As a part of these studies, dioxolone derivatives **2**, ring-opened chalcones **3** and OR-group-deprived oxathiole derivative **4** (Figure 1) were prepared to establish significance of (i) the presence of sulfur atom, (ii) the presence of the heterocyclic ring, (iii) the presence of the OR substituent, (iv) structure of substituents OR and X. The information should be useful in optimization of activity and pharmacological properties of the compounds.

Methods and Materials

Chemistry

Melting points were determined using a Stuart SMP3 instrument (Bibby Scientific Ltd., Staffordshire, UK) and were uncorrected. Infrared spectra were obtained from

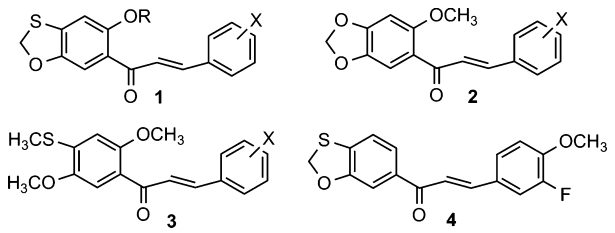

Figure 1: Structures of the studied compounds.

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KBr pellets on a Thermo Mattson Satellite instrument (Mattson Technology, Inc., Fremont, CA, USA). The ^{1}H NMR spectra were recorded on 200 MHz (Varian Gemini) or 500 MHz (Varian Unity Plus) spectrometers. Elemental analyses were performed using a Carlo-Erba 1108 instrument (CE Instruments Ltd., Hindley Green, Wigan, UK), and results were within 0.4% of theoretical values. TLC was carried out on Merck 0.2 mm silica gel 60 F254 aluminum plates. The described reactions are unoptimized.

Methods of synthesis and analytical data of the described compounds are presented in the Appendix S1.

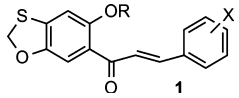
Determination of cytotoxic activity

A549 (human lung carcinoma, NSCLC), HCT116 (human colorectal carcinoma) and HeLa (human cervix adenocarcinoma) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 cells were maintained in RPMI 1640 medium, HCT116 in McCoy's 5A medium, and HeLa cells were maintained in Minimum Essential Medium (all media from Life Technologies). The media were supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 μg/mL streptomycin. Cultures were grown in standard conditions: 37 °C, 5% CO_2, 95% humidity.

Cell viability assays

Cells were seeded in 96-well plates in a total volume of 150 μL medium per well, 1 day prior to start of the experiment. Then, 50 μL of medium containing different concentrations of tested compounds (seven concentrations, each in triplicates) was added to cells. Fifty microliters of medium with 0.25% DMSO was added to the control cells. Next, cells were incubated with tested compounds for the following 72 h. After incubation, 20 μL of MTT working solution in PBS (5 mg/mL) was added and incubated for 3 h in 37 °C in 5% CO_2. Finally, medium with MTT solution was removed, and formazan crystals were dissolved by adding 100 μL of DMSO. After mixing, absorbance was measured at 570 nm wavelength (690 nm reference filter). MTT reduction occurs only in living cells. Data analysis consisted of determining the IC_{50} concentration of the compound (in μM), at which the 50% reduction in the number of cells occurs in the population treated, compared with the control cells. Results were analyzed

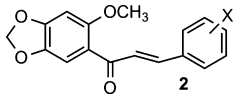
Table 1: *In vitro* cytotoxicity of the prepared chalcones of general structure **1**



Cmpd no (cmpd code), yield	Substituent R	Substituent X	Cytotoxicity, IC_{50} [μM] ± SD*		
			A549	HCT116	HeLa
24 (**AMG-173**), 60%	CH_3	–	3.79 ± 0.21	1.85 ± 0.30	1.85 ± 0.456
25 (**AMG-190**), 70%	CH_3	4-OCH_3	0.369 ± 0.029	0.168 ± 0.009	0.061 ± 0.008
26 (**AMG-303**), 76%	CH_3	3-OH-4-OCH_3	0.050 ± 0.008	0.037 ± 0.007	0.012 ± 0.002
27 (**AMG-301**), 84%	CH_3	3-F-4-OCH_3	0.269 ± 0.069	0.454 ± 0.120	0.078 ± 0.015
28 (**AMG-305**), 47%	CH_3	2,4,6-triOCH_3	0.309 ± 0.113	0.401 ± 0.011	0.103 ± 0.004
29 (**AMG-377**), 41%	CH_2CH_3	3-OH-4-OCH_3	0.009 ± 0.001	0.006 ± 0.002	0.001 ± 0.000
30 (**AMG-379**), 60%	CH_2CH_3	3-F-4-OCH_3	0.012 ± 0.003	0.014 ± 0.002	0.006 ± 0.002
31 (**AMG-378**), 30%	CH_2CH_3	3,4,5-triOCH_3	2.84 ± 0.608	1.40 ± 0.141	2.95 ± 0.771
32 (**AMG-310**), 22%	$CH(CH_3)_2$	–	7.00 ± 0.245	4.68 ± 0.360	3.11 ± 0.030
33 (**AMG-300**), 55%	$CH(CH_3)_2$	4-OCH_3	0.091 ± 0.015	0.037 ± 0.019	0.027 ± 0.008
34 (**AMG-299**), 76%	$CH(CH_3)_2$	3-F-4-OCH_3	0.093 ± 0.007	0.048 ± 0.016	0.035 ± 0.010
35 (**AMG-296**), 45%	$CH_2CH_2CH_3$	–	6.69 ± 1.62	3.34 ± 0.08	3.02 ± 0.74
36 (**AMG-336**), 14%	$CH_2CH_2CH_3$	3-OH-4-OCH_3	0.022 ± 0.005	0.010 ± 0.003	0.004 ± 0.001
37 (**AMG-349**), 65%	$CH_2CH_2CH_3$	3-OPO_3Na_2-4-OCH_3	0.121 ± 0.026	0.097 ± 0.001	0.009 ± 0.001
38 (**AMG-350**), 58%	$CH_2CH_2CH_3$	3-OCH_2CH_2OH-4-OCH_3	2.58 ± 0.064	1.76 ± 0.537	1.72 ± 0.049
39 (**AMG-338**), 75%	$CH_2CH_2CH_3$	3-OTHP-4- OCH_3	2.31 ± 0.244	3.54 ± 0.872	1.78 ± 0.145
40 (**AMG-295**), 89%	$CH_2CH_2CH_3$	3-F-4-OCH_3	0.142 ± 0.032	0.046 ± 0.008	0.044 ± 0.002
41 (**AMG-334**), 85%	$CH_2CH_2CH_3$	2,4,6-triOCH_3	5.43 ± 2.38	2.05 ± 0.423	2.12 ± 0.215
42 (**AMG-322**), 67%	$CH_2CH_2CH_2CH_3$	3-F-4-OCH_3	0.331 ± 0.069	0.161 ± 0.023	0.130 ± 0.028
43 (**AMG-333**), 62%	$CH_2CH_2CH_2CH_3$	2,4,6-triOCH_3	3.37 ± 1.92	2.43 ± 0.628	1.80 ± 0.527
44 (**AMG-337**), 91%	$CH_2CH_2CH_2CH_3$	3,4,5-triOCH_3	12.0 ± 4.810	3.52 ± 0.330	15.0 ± 4.495
45 (**AMG-308**), 49%	$CH_2CH_2CH_2NHBoc$	3-F-4-OCH_3	3.52 ± 0.940	1.10 ± 0.080	0.970 ± 0.156
46 (**AMG-353**), 60%	$CH_2CH_2CH_2NH_2$	3-F-4-OCH_3	1.20 ± 0.280	1.04 ± 0.200	1.20 ± 0.090
Doxorubicin			0.124 ± 0.006	0.069 ± 0.027	0.154 ± 0.017
Combretastatin A4			0.008 ± 0.003	0.004 ± 0.002	0.003 ± 0.000
Paclitaxel			0.006 ± 0.002	0.004 ± 0.001	0.008 ± 0.004

*SD – standard deviation.

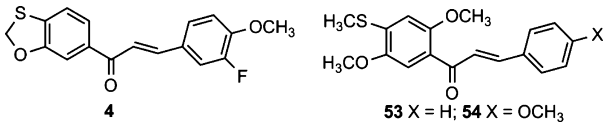
Table 2: *In vitro* cytotoxicity of the prepared chalcones of general structure **2**



Cmpd no (cmpd code), Yield	Substituent X	Cytotoxicity, IC_{50} [μM] ± SD*		
		A549	HCT116	HeLa
47 (**AMG-263**), 33%	–	6.00 ± 0.08	2.95 ± 0.13	4.10 ± 0.37
48 (**AMG-260**), 76%	4-Br	4.98 ± 0.25	2.72 ± 0.06	4.03 ± 0.88
49 (**AMG-261**), 92%	2-Cl	8.75 ± 1.24	2.55 ± 0.30	6.27 ± 0.64
50 (**AMG-249**), 80%	4-OCH_3	0.199 ± 0.003	0.063 ± 0.004	0.043 ± 0.005
51 (**AMG-250**), 68%	3-F-4-OCH_3	0.213 ± 0.001	0.066 ± 0.004	0.049 ± 0.004
52 (**AMG-251**), 81%	3,4,5-triOCH_3	6.30 ± 0.01	3.06 ± 0.33	4.40 ± 0.25

*SD – standard deviation.

Table 3: *In vitro* cytotoxicity of compounds **4**, **53,** and **54**



4 **53** X = H; **54** X = OCH_3

Cmpd no (cmpd code), Yield	Cytotoxicity, IC_{50} [μM] ± SD*		
	A549	HCT116	HeLa
4 (**AMG-470**), 87%	22.8 (0.97)	16.8 (5.68)	–
53 (**AMG-180**), 67%	4.51 (0.18)	2.68 (0.84)	4.07 (0.69)
54 (**AMG-181**), 64%	>10	>10	>10

*SD – standard deviation.

using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

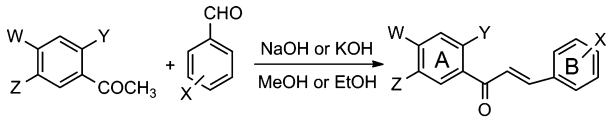
Results are presented in Tables 1, 2 and 3.

Results

Chemistry

Chalcones **1**-**4** were prepared by condensation of suitable acetophenones with benzaldehydes in alkaline alcoholic solution (Scheme 1).

The starting 6-acetyl-5-methoxybenzo[*d*][1,3]oxathiole (**6**) was obtained in one step from 6-acetyl-5-methoxybenzo



6, **9**-**13**	W,Z = SCH_2O, Y = OR	**1**
15	W,Z = OCH_2O, Y = OCH_3	**2**
17	W = SCH_3, Z = OCH_3, Y = OCH_3	**3**
23	W,Z = SCH_2O, Y = H, X = 3-F-4-OCH_3	**4**

Scheme 1: The synthetic route employed to access the described chalcones.

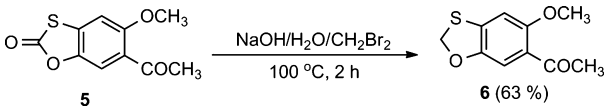
[*d*][1,3]oxathiol-2-one (**5**) (17) by alkaline ring opening and recyclization with methylene bromide (Scheme 2).

Other 6-acetyl-5-alkoxybenzo[*d*][1,3]oxathioles (**9**-**13**) were prepared from 6-acetyl-5-hydroxybenzo[*d*][1,3]oxathiol-2-one (**7**) (17) by its transformation into oxathiole **8**, followed by alkylation with a suitable halide (Scheme 3).

6-Acetyl-5-methoxybenzo[*d*][1,3]dioxole (**15**), required as substrate for chalcones **2**, was prepared by alkylation of phenol **14** (Scheme 4) (18,19).

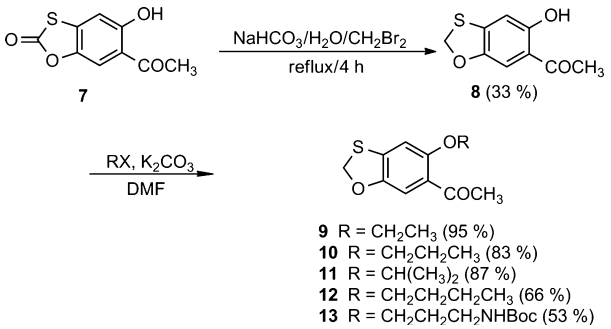
2,5-dimethoxy-4-(methylthio)acetophenone (**17**) required as substrate for chalcones **3**, was prepared by alkaline hydrolysis of oxathiolone **5** to 5-hydroxy-2-methoxy-4-mercaptoacetophenone (**16**), and followed by methylation with methyl iodide (Scheme 5).

6-acetylbenzo[*d*][1,3]oxathiole (**23**) required as substrate for synthesis of chalcone **4**, was prepared starting from



5 **6** (63 %)

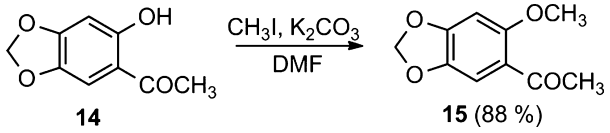
Scheme 2: Synthesis of 6-acetyl-5-methoxybenzo[*d*][1,3] oxathiole (**6**).



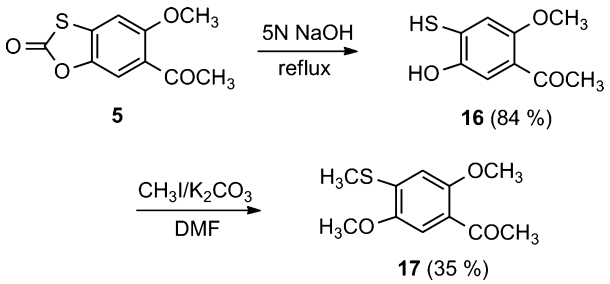
7 **8** (33 %)

9 R = CH_2CH_3 (95 %)
10 R = $CH_2CH_2CH_3$ (83 %)
11 R = $CH(CH_3)_2$ (87 %)
12 R = $CH_2CH_2CH_2CH_3$ (66 %)
13 R = $CH_2CH_2CH_2NHBoc$ (53 %)

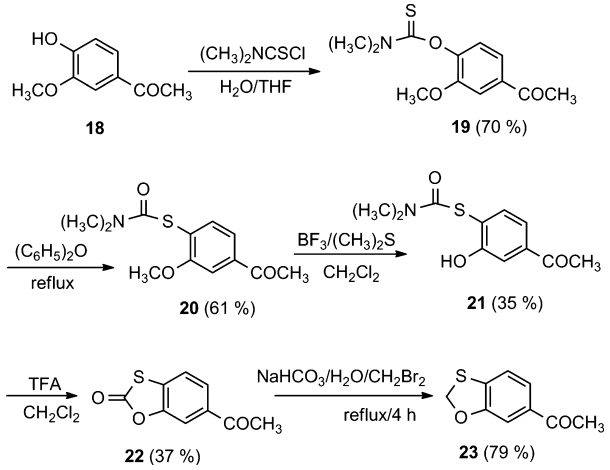
Scheme 3: Synthesis of 6-acetyl-5-alkoxybenzo[*d*][1,3]oxathioles (**9**-**13**).



Scheme 4: Synthesis of 6-acetyl-5-methoxybenzo[*d*][1,3]dioxole (**15**).



Scheme 5: Synthesis of 2,5-dimethoxy-4-(methylthio)acetophenone (**17**).



Scheme 6: Synthesis of 6-acetylbenzo[*d*][1,3]oxathiole (**23**).

4-hydroxy-3-methoxyacetophenone (**18**) (Scheme 6), by reaction with dimethylthiocarbamoyl chloride, and followed by Newman–Kwart rearrangement of the formed thiocarbamate **19** to thiophenol derivative **20** (20). Deprotection of methoxy group to 3-hydroxyacetophenone **21** and subsequent cyclization gave oxathiolone derivative **22**, which was transformed into the desired oxathiole **23** by ring-opening ring-closure reaction with methylene bromide under alkaline conditions.

Cytotoxicity in vitro

The cytotoxicity of the prepared compounds was evaluated against three tumor cell lines using the MTT test. Most of the compounds of general formula **1** (Table 1) demonstrated significant activity, with IC_{50} values of the most active compounds (**29**, **30**, **36**) in the nanomolar range (9, 12 and 22 nM, respectively) (all IC_{50} values used in this discussion were measured in A549 cells).

Cytotoxic activity of compounds deprived of oxathiole ring is given in Tables 2 and 3.

Discussion

The importance of the heterocyclic ring was verified by comparison of activities of 4-OCH_3 derivative of oxathiole **25** (0.369 μM) (Table 1) with activity of ring-opened compound **54** (>10 μM) (Table 3). The last derivative was found to be about 40 times less active than its cyclic analog, which indicated a crucial role of the heterocyclic ring. However, differences in cytotoxicity of analogs, ring B-unsubstituted pair of compounds oxathiole **24** (3.79 μM) and ring-opened derivative **53** (4.51 μM), were negligible. The result suggested that cytotoxic effects of the compounds resulted from a combined influence of the both factors, and the heterocyclic ring had to be accompanied by suitable substituents X.

Replacement of sulfur by oxygen in the heterocyclic ring did not affect the activity, as indicated by comparison of values of IC_{50} of oxathiole–chalcones **25** (0.369 μM) and **27** (0.269 μM) with activities of related dioxole derivatives **50** (0.199 μM) and **51** (0.213 μM).

The presence of ring A 5-OR substituent and structure of the R group were very important. Activity of ring B 3-F-4-OCH_3-substituted compound **4** (22.8 μM) (Table 3) deprived of the 5-OR substituent was significantly lower than activity of related 5-OCH_3 (**27**) (0.264 μM), 5-OC_2H_5 (**30**) (0.012 μM), 5-$OCH(CH_3)_2$ (**34**) (0.093 μM), 5-$OCH_2CH_2CH_3$ (**40**) (0.142 μM) and 5-$OCH_2CH_2CH_2CH_3$ (**42**) (0.331 μM) analogs (Table 1). As can be seen, the best activity was found for 5-OC_2H_5 compound, whereas both 5-shorter and 5-longer alkoxy chain analogs were significantly less active. Similar result was obtained for ring B 3-OH-4-OCH_3-substituted compounds: The 5-OC_2H_5 derivative (**29**) (0.009 μM) was more active than related 5-OCH_3 (**26**) (0.050 μM) and $OCH_2CH_2CH_3$ (**36**) (0.022 μM) analogs.

Increase in polarity of the 5-OR group decreased the cytotoxic activity *in vitro*, as evidenced by comparison of activities of 5-$OCH_2CH_2CH_2CH_3$ (**42**) (0.331 μM) and 5-$OCH_2CH_2CH_2NH_2$ (**46**) (1.2 μM) derivatives. Similarly, 3-OH-4-OCH_3 compound **36** (0.022 μM) was more active than its polar phosphate prodrug **37** (0.121 μM). However, although the polar compounds were less active *in vitro*, their water solubility could result in much better pharmacokinetic parameters.

The differences in *in vitro* activity of phosphate prodrug **37** and its precursor **36** are intriguing, as when tested toward HCT116 cells, **37** is about 10-fold less cytotoxic than **36**

(0.097 and 0.010 μM, respectively), whereas for HeLa cells the activities are comparable (0.009 versus 0.004 μM). The phosphate derivative is activated in the cytoplasm by intracellular phosphatases, and possible differences in the activity of these enzymes could be responsible for cellular response to phosphate prodrugs of chalcones. However, this is not a unique property of our compounds as similar relation was described by Pettit *et al.* for combretastatin A4 and its analogs (21). In addition, it was noted that changing the counter-cation in the phosphates of combretastatin A4 derivatives also influences their cytotoxicity. Together, it follows that the activity of cellular phosphatases is probably not a sole reason for the different cytotoxicities of chalcones and their phosphate salts.

Conclusion

The studies identified structural requirements for very high cytotoxic activity *in vitro* of oxathiole-fused chalcones **1**. The results revealed that the nanomolar level cytotoxic activity depends on combination of three factors: (i) the presence of the heterocyclic ring, (ii) the presence and structure of 5-OR group in ring A, and (iii) substituents in ring B of chalcones. Concerning the cytotoxic activity *in vitro*, the oxathiole ring in combination with 2 -3 carbon alkoxy group OR, and (3-OH-4-OCH_3) or (3-F-4-OCH_3) substituents in ring B, seemed to be optimal.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Synthesis and analytical data of the described compounds.