Synthesis and Evaluation of 5-Aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2-(3*H*)-thiones as P-Glycoprotein Inhibitors

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5-Aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thiones 3 were prepared by cyclocondensation of 1-(4-hydroxyphenyl)-2-aroylhydrazines with thiophosgene. All compounds exhibited antiproliferation activity in K562, IC_{50} ranging from 24 to 94 μM comparable efficacy with apigenin and genistein and showed more potent antiproliferation of K562/*adr* cells, highly expressing P-glycoprotein. Compounds 3g, 3e and 3a inhibited the function of P-glycoprotein with the $\alpha_{0.5}$ equal to 10±3 μM, 21±5 μM and 34±7 μM, respectively.

Key words oxadiazolethione; multidrug resistance (MDR); P-glycoprotein; inhibitor

Multidrug resistance (MDR) is a main reason for the cancer chemotherapy failure. One widely studied mechanism of this phenomenon involves the ability of an ATP dependent 170 kDa plasma membrane protein, P-glycoprotein, to act as an efficient efflux pump against anticancer drug molecules.[1] Some compounds such as cyclosporin A,[2—4] verapamil,[5,6] estrogen[7] and genistein[8] have been reported to reverse MDR, resulting in an increased accumulation of antitumor agents in MDR cells.[9,10]

Among different series of oxadiazolethione synthesized in our laboratory, we found that 5-aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thiones **3** potently inhibited the functionality of P-glycoprotein. Interestingly, they also showed growth inhibitory properties in the K562 human leukemia cell line. Herein, we reported the synthesis of 5-aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thiones and preliminary *in vitro* results.

The synthesis of the compounds investigated in this work is outlined in Fig. 1. Condensation of substituted benzoic acid hydrazide with 1,4-benzoquinone in acid conditions allowed to obtain benzoic acid (cyclohexadien-1-ylidene)hydrazide substituted derivatives **1a**—**g** which were reduced with phenylhydrazine[11] in 1-butanol to afford the corresponding substituted benzoic acid 2-(4-hydroxyphenyl)hydrazide **2a**—**g**. Cyclocondensation of **2a**—**g** with thiophosgene[12] in water yielded the 5-aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thiones **3a**—**g** (Caution: reactions involving thiophosgene were achived in a hood).

Results and Discussion

Seven new 5-aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thiones **3** were synthesized and screened for their anticancer and P-glycoprotein inhibitor activities. It was found that all synthesized oxadiazolethiones **3** exhibited anticancer activity against K562 cells with IC_{50} ranging from 24 to 94 μM, comparable efficacy with apigenin and genistein (Table 1). The antiproliferative effects of several symmetrically substituted 5-phenyl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione **3a** derivatives were studied. We introduced various substituents on the *C4* position of the phenyl group: electron donating group, CH_3 (**3b**) and OCH_3 (**3c**), electron withdrawing group, NO_2 (**3d**) and halogen (**3e**, **f**). (Table 1). As compared with unsubstituted phenyl lead compound **3a**, electron donating substituted phenyl oxadiazolethiones (**3b**, **c**) were less cytotoxic. Electron withdrawing (**3d**) or halogen (**3e**, **f**) substitutions did not increase cytotoxicity. The trimethoxy substituted phenyl oxadiazolethione **3g** showed more potent cytotoxicity (IC_{50}=24±4 μM) than the monomethoxy substituted analogue **3c** (IC_{50}=94±10 μM). It has been reported that trimethoxylated chalcone showed greater cytotoxicity against K562 cell line than the corresponding mono and dimethoxylated derivatives.[13] Interestingly, oxadiazolethiones **3** exhibited potent antiproliferation of multidrug resistant K562/*adr* cells with resistance factor (RF) ranging from 0.5 to 1.0. It should be noted that **3a**, **3b**, **3e** and **3g** were at least 2 times more toxic in MDR tumor cells than the parental K562 cells.

All oxadiazolethiones **3** used in our experiments inhibited the functionality of P-glycoprotein except **3f**. Compounds **3g**, **3e** and **3a** showed $\alpha_{0.5}$ equal to 10±3 μM, 21±5 μM and 34±7 μM, respectively. Compounds **3b**, **3c**, and **3d** gave maximal inhibition only about 20% of the functionality of P-

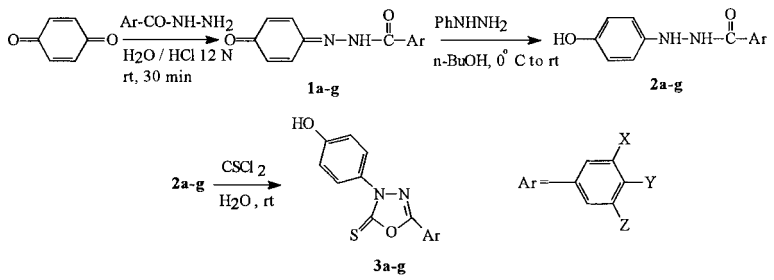

Fig. 1. Synthetic Route to 1,3,4-Oxadiazole-2(3*H*)-thione Derivatives **3a**—**g**

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Table 1. *In Vitro* Cytotoxicity against K562 and K562/*adr* Cells and Inhibitory P-Glycoprotein Activity of Oxadiazolethiones **3**

Compounds	Substitutions X	Y	Z	IC_{50}(S) μM	RF	$\alpha_{0.5}$ μM
3a	H	H	H	34.0±2.0	0.6	34.0±7.0
3b	H	Me	H	48.0±1.0	0.6	A
3c	H	OMe	H	94.0±10.0	0.7	A
3d	H	NO_2	H	37.0±7.0	0.8	A
3e	H	Cl	H	29.0±6.0	0.6	21.0±5.0
3f	H	Br	H	35.0±2.0	1.0	B
3g	OMe	OMe	OMe	24.0±4.0	0.5	10.0±3.0
Genistein				50.0±9.0	0.9	B
Apigenin				27.0±2.0	0.9	B

A: 20% of maximum inhibition and B: not active

glycoprotein. Trimethoxylated oxadiazolethione **3g** also showed more potent P-glycoprotein inhibition than the monomethoxylated analogue **3c**. The results seem to suggest that electronic effect do not play an important role. However, they suggest that 3, 4, 5 trilipophilic substituents on the phenyl ring are important for demonstrating cytotoxicity and P-glycoprotein inhibition. In fact that apigenin and genistein, up to 100 μM, did not inhibit the function of P-glycoprotein.

The present study clearly indicated that: (a) oxadiazolethiones **3** exhibited anticancer properties and were more cytotoxic in multidrug resistant K562/*adr* cells than the parental K562 cells, (b) they inhibited the functionality of P-glycoprotein and (c) both activities were improved by trisubstitution of lipophilic methoxy group on the 3, 4 and 5 positions of the phenyl ring.

Experimental

Chemistry ^{1}H nuclear magnetic resonance spectra were recorded with a Bruker AC200 spectrometer. Chemical shifts (δ) are reported as ppm using tetramethylsilane as internal standard. IR spectra were recorded in KBr pellets on a Perkin-Elmer Spectrum BX I spectrophotometer. Elemental analyses (C, H, N) were performed by the Service de microanalyse at the Université Pierre et Marie Curie and were within ±0.4% of the theoretical values. Melting points were determined using a Kofler hotstage apparatus and are uncorrected.

General Procedure for the Preparation of Benzoic Acid (Cyclohexadien-1-ylidene)hydrazide Substituted Derivatives (1) 1,4-Benzoquinone (5.40 g, 50 mmol) was suspended in water (350 ml) at room temperature and benzoic acid hydrazide derivative (55 mmol) dissolved in HCl 10% (35 ml) was added. The mixture was then stirred 15 min. The precipitate was collected, washed and dried. It was used without further purification. (**1a**) mp 148 °C (143—144 °C).[11] IR (KBr) cm^{-1}: 3280, 1675, 1620, 1595. (**1b**) mp 124 °C. IR (KBr) cm^{-1}: 3320, 1706, 1672, 1630. (1c) mp 80 °C. IR (KBr) cm^{-1}: 3445, 1665, 1620, 1590. (**1d**) mp 90 °C. IR (KBr) cm^{-1}: 3430, 1683, 1607, 1582. (**1e**) mp 165 °C. IR (KBr) cm^{-1}: 3270, 1699, 1681, 1638. (**1f**) mp 172 °C. IR (KBr) cm^{-1}: 3305, 1681, 1633, 1586. (**1g**) mp 240—242 °C. IR (KBr) cm^{-1}: 3108, 1670, 1643, 1602.

General Procedure for the Preparation of Benzoic Acid 2-(4-Hydroxyphenyl)hydrazide Substituted Derivatives (2) To a stirred suspension of (**1**) (35 mmol) in 1-butanol (75 ml) was added dropwise at 0 °C pure phenylhydrazine (7.57 g, 70 mmol). The mixture was then allowed to rise to room temperature and remained for 1 h. Excess of petroleum ether was added and after 15 min stirring, the resulting precipitate was collected by filtration and washed with petroleum ether. (**2a**) Yield: 48%; mp 172 °C (AcOEt–petroleum ether) (170—171 °C).[14] IR (KBr) cm^{-1}: 3225, 1730, 1630. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.64, 7.52, 7.90 (3m, 9H), 8.68 (br s, 1H), 10.32 (br s, 1H). (**2b**) Yield: 47.50%; mp 184 °C (AcOEt–petroleum ether) (178—179 °C).[14] IR (KBr) cm^{-1}: 3220, 1610. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 2.35 (s, 3H), 6.57, 6.64, 7.28, 7.36, 7.78 (5d, 8H), 8.68 (br s, 1H), 10.22 (br s, 1H). (**2c**) Yield: 57%; mp 202 °C (AcOEt–petroleum ether) (195—196 °C).[14] IR (KBr) cm^{-1}: 3220, 3230, 1620, 1600. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.81 (s, 1H), 6.65 (m, 4H), 7.01, 7.89 (2d, 4H), 8.67 (br s, 1H), 10.19 (br s, 1H). (**2d**) Yield: 71%; mp 204 °C (EtOH–H_2O). IR (KBr) cm^{-1}: 3260, 1639. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.69, 3.82 (2S, 9H), 6.58, 6.64 (2m, 3H), 7.23, 7.38 (2d, 3H), 8.69 (br s, 1H), 10.27 (br s, 1H). (**2e**) Yield: 41.50%; mp 202 °C (EtOH–H_2O). IR (KBr) cm^{-1}: 3274, 1642. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.64 (m, 4H), 7.56, 7.92 (2d, 4H), 8.69 (br s, 1H), 10.40 (br s, 1H). (**2f**) Yield: 60%; mp 194 °C (EtOH–H_2O) (191—192 °C).[14] IR (KBr) cm^{-1}: 3245, 1664. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.64 (m, 4H), 7.71, 7.84 (2d, 4H), 8.70 (br s, 1H), 10.40 (br s, 1H). (**2g**) Yield: 67.50%; mp 203 °C (AcOEt–petroleum ether). IR (KBr) cm^{-1}: 3242, 3109, 1668. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.60 (m, 4H), 7.52 (br s, 1H), 8.12, 8.33 (2d, 4H), 8.72 (br s, 1H), 10.63 (br s, 1H).

General Procedure for the Preparation of 5-Aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (3) To a stirred suspension of (**2**) (10 mmol) in water (100 ml) was added at room temperature pure thiophosgene (1.53 ml, 20 mmol). The mixture was stirred 30 min and filtered. The precipitate was washed with water, dried and recristallized using suitable solvent.

5-Phenyl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**3a**): Yield: 72%; mp 232 °C (EtOH). IR (KBr) cm^{-1}: 3300, 1590, 1517. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.93, 7.93 (2d, 4H), 7.65 (m, 5H), 10.03 (br s, 1H). *Anal.* Calcd for $C_{14}H_{10}N_2O_2S$: C, 62.20; H, 3.72; N, 10.36. Found: C, 62.25; H, 3.68; N, 10.42.

5-(4-Methylphenyl)-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**3b**): Yield: 71.50%; mp 210 °C (*n*-PrOH). IR (KBr) cm^{-1}: 3420, 1606, 1569. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 2.40 (s, 3H), 6.92, 7.42, 7.71, 7.82 (4d, 8H), 10.02 (br s, 1H). *Anal.* Calcd for $C_{15}H_{12}N_2O_2S$: C, 63.36; H, 4.25; N, 9.85. Found: C, 63.40; H, 4.31; N, 9.90.

5-(4-Methoxyphenyl)-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**3c**): Yield: 52%; mp 249 °C (EtOH–H_2O). IR (KBr) cm^{-1}: 3321, 1614, 1621. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.85 (s, 3H), 6.91, 7.15, 7.71, 7.88 (4d, 8H), 10.01 (br s, 1H). *Anal.* Calcd for $C_{15}H_{12}N_2O_3S$: C, 59.99; H, 4.02; N, 9.32. Found: C, 60.08; H, 4.10; N, 9.40.

5-(3,4,5-Trimethoxyphenyl)-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**3d**): Yield: 49.50%; mp 212 °C (EtOH–H_2O). IR (KBr) cm^{-1}: 3323, 1577, 1502. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.75, 3.87 (2s, 9H), 6.92, 7.71 (2d, 4H), 7.16 (s, 2H), 10.02 (br s, 1H). *Anal.* Calcd for $C_{17}H_{16}N_2O_5S$: C, 56.65; H, 4.47; N, 7.77. Found: C, 56.70; H, 4.52; N, 7.85.

5-(4-Chlorophenyl)-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**3e**): Yield: 54.50%; mp 226 °C (*n*-PrOH). IR (KBr) cm^{-1}: 3413, 1610, 1515. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.92, 7.93 (2d, 4H), 7.68 (m, 4H), 10.03 (br s, 1H). *Anal.* Calcd for $C_{14}H_9ClN_2O_2S$: C, 55.17; H, 2.97; N, 9.19. Found: C, 55.20; H, 3.02; N, 9.25.

5-(4-Bromophenyl)-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**3f**): Yield: 29%; mp 226 °C (*n*-PrOH). IR (KBr) cm^{-1}: 3414, 1608, 1587. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.91, 7.70 (2d, 4H), 7.84 (m, 4H), 10.03 (br s, 1H). *Anal.* Calcd for $C_{14}H_9BrN_2O_2S$: C, 48.15; H, 2.59; N, 8.02. Found: C, 48.17; H, 2.63; N, 8.10.

5-(4-Nitrophenyl)-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thione (**3g**): Yield: 49.50%; mp 246 °C (*n*-PrOH). IR (KBr) cm^{-1}: 3438, 1593, 1513. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 6.93, 7.71, 8.18, 8.41 (4d, 8H), 10.06 (br s, 1H). *Anal.* Calcd for $C_{14}H_9N_3O_4S$: C, 53.33; H, 2.87; N, 13.32. Found: C, 53.38; H, 2.98; N, 13.38.

Cytotoxicity Assay Anticancer and P-glycoprotein inhibitor activities of oxadiazolethiones **3a**—**g** were performed by using human myelogenous leukemia K562 and K562/*adr* cell line with overexpression of the P-glycoprotein.[9,10] *In vitro* cytotoxic activity was measured by incubation of 5×10^4 cells/ml in the presence of various concentrations of compounds **3a**—**g** for 72 h in 24-well plates. IC_{50} (50% inhibitory drug concentration) was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay. RF represents the ratio of the IC_{50} in the resistant cells to the IC_{50} level in the corresponding sensitive cells (IC_{50}(S)).

Inhibition of P-Glycoprotein Functionality The efficacy of molecules to inhibit P-glycoprotein activity was evaluated by following an increase in intracellular concentration of pirarubicin in K562/*adr* cells in the presence of inhibitor, has been extensively described.[15—17] Briefly, 2×10^6 cells were incubated in 2 ml of HEPES-Na^+ in 1 cm quartz cuvettes, vigorously stirred at 37 °C, then 1 μM pirarubicin was added to the cells. The fluorescence intensity of pirarubicin at 590 nm (excited at 480 nm) was followed as a function of time (Perkin-Elmer model LS 50B spectrofluorometer). At the steady state, the addition of MDR modulators verapamil or compound **3** yielded a decrease in fluorescence intensity to reach a new steady state F^i_n. The ability of compound **3** (**a**) to inhibit the P-gp-mediated efflux of a drug can be determined using the ratio

$$k_a^i/k_a^0$$

where k_a^i and k_a^0 stand for the P-gp-mediated active efflux of drug in the presence and absence of modulator, respectively. k_a^i/k_a^0 is equal to 1 when there is no inhibition of the active efflux, and to 0 when the P-gp-mediated active efflux is completely blocked. $\alpha_{0.5}$ was defined as a concentration of 5-aryl-3-(4-hydroxyphenyl)-1,3,4-oxadiazole-2(3*H*)-thiones **3** required to inhibit the P-glycoprotein function 50%.

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References

1) Gottesman M. M., Pastan I., *Annu. Biochem.*, **62**, 385—427 (1993).
2) Slater L. M., Sweet P., Stupecky M., Gupta S., *J. Clin. Invest.*, **77**, 1405—1408 (1986).
3) Safa A. R., *Cancer Invest.*, **10**, 295—305 (1992).
4) Beck W. T., Qian, X. D., *Biochem. Pharmacol.*, **43**, 89—93 (1992).
5) Tsuruo T., Lida H., Tsukagoshi S., Sakurai Y., *Cancer Res.*, **41**, 1967—1972 (1981).
6) Watanabe T., Tsuge H., Oh-Hara T., Naito M., Tsuruo T., *Acta Oncol.*, **34**, 235—241 (1995).
7) Zalcberg J. R., Hu X. F., Ching M., Wakeling A., Wall D. M., Marschner I. C., de Luise M., *Cancer Chemother. Pharmacol.*, **33**, 123—129 (1993).
8) Castro A. F., Altenberg G. A., *Biochem. Pharmacol.*, **53**, 89—93 (1997).
9) Mankhetkorn S., Garnier-Suillerot A., *Eur. J. Pharmacol.*, **343**, 313—321 (1998).
10) Mankhetkorn S., Teodori E., Scapecchi S., Garnier-Suillerot A., *Biochem. Pharmacol.*, **52**, 213—217 (1996).
11) Borsche W., Ockinga K. A., *Lieb. Ann. der Chem.*, **340**, 85—109 (1905).
12) Sherman W. R., *J. Org. Chem.*, **26**, 88—95 (1961).
13) Ducki S., Hadfield J. A., Hepworth L. A., Lawrence N. J., Liu C. Y., McGown A. T., *Bioorg. Med. Chem. Lett.*, **24**, 3091—3094 (1997).
14) Burmistrov K. S., Belov V. V., Burmistrov S. I., *Ukr Khim. Zh.*, **49**, 761—763 (1983).
15) Frezard F., Garnier-Suillerot A., *Eur. J. Biochem.*, **196**, 483—491 (1991).
16) Mankhetkorn S., Teodori E., Garnier-Suillerot A., *Chemico-Biological Interactions*, **121**, 125—140 (1999).
17) Frézard F., Garnier-Suillerot A., *Biochem. Biophys. Acta*, **1091**, 29—35 (1991).