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Bioorganic & Medicinal Chemistry Letters 15 (2005) 4632–4635
Bioorganic & Medicinal Chemistry Letters

Synthesis of some pyrazole derivatives and preliminary investigation of their affinity binding to P-glycoprotein

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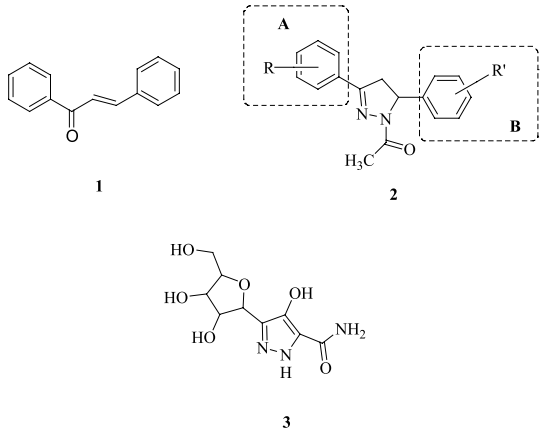
Received 15 March 2005; revised 11 May 2005; accepted 17 May 2005
Available online 15 August 2005

Abstract—A series of substituted pyrazolines were synthesized and evaluated for their anticancer activity and for their ability to inhibit P-glycoprotein-mediated multidrug resistance by direct binding to a purified protein domain containing an ATP-binding site and a modulator interacting region. Compounds **2a** and **e** have been found to bind to P-glycoprotein with greater affinity.

The identification of novel structures that can be potentially useful in designing new, potent selective, and less toxic anticancer agents is still a major challenge to medicinal chemistry researchers.

As reported in the literature,[1] 1,3-diaryl-2-propen-1-ones or chalcones **1** (see Chart 1) are a class of anticancer agents that have shown promising therapeutic efficacy in the management of human cancers. Several chalcones are cytotoxic toward a number of different tumor lines. In particular, they inhibit the proliferation of both established and primary ovarian cells. Moreover, recent studies have shown that chalcones also induce apoptosis in a variety of cell types, including breast cancer.[2,3] Chalcones have shown preferential reactivity toward thiols[4] in contrast to amino and hydroxyl groups. Therefore, because interactions with nucleic acids may be absent, problems of mutagenicity and carcinogenicity that have been associated with certain alkylating agents used in cancer chemotherapy could be eliminated. It is important to note that the resistance of human malignancy to chemotherapeutic agents remains a major obstacle to an efficient cancer therapy. The induction of multiple drug resistance (MDR) is the phenomenon in which the exposure of tumor cells



1 2 3

Chart 1. 1, 1,3-Diaryl-2-propen-1-ones (chalcones); **2**, 1-acetyl-3,5-diphenyl-4,5-dihydro-(1*H*)-pyrazole; **3**, 4-hydroxypyrazole C-glycoside (pyrazofurin).

to a single cytotoxic agent results in cross-resistance to the other structurally unrelated classes of cytotoxic agents. Moreover, on account of MDR many bacteria that cause infections are becoming increasingly difficult to treat.[5–7] The multidrug resistance of cancer cells is often associated with an accelerated efflux of the chemotherapeutic agent by an ATP-dependent process. A family of proteins is responsible for this efflux. In particular, the over-expression of P-glycoprotein (P-gp), an ABC-type plasma membrane transporter that rejects

Keywords: Anticancer; Pyrazoline; P-glycoprotein.
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doi:10.1016/j.bmcl.2005.05.067

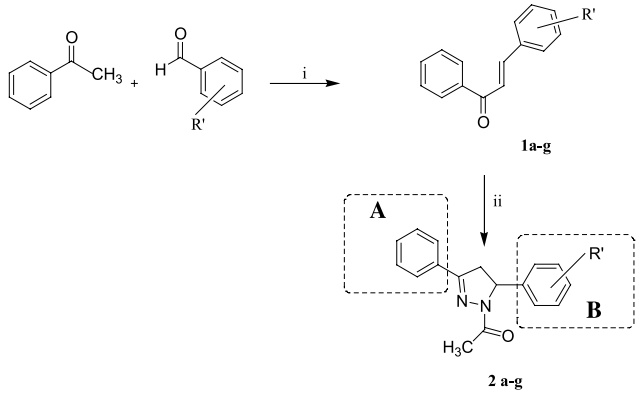
chemotherapeutic drugs out of the cell using ATP hydrolysis as an energy source.[8] On account of the deleterious effect of P-glycoprotein on chemotherapeutic efficiency, compounds that modify its function have a potential by clinical value. Recently, several inhibitors of P-glycoprotein-mediated drug efflux have been identified. These so-called MDR modulators lead to resensitization of multidrug resistant tumor cells to chemotherapeutic agents. They include a variety of structural classes, such as calcium channel blockers (such as verapamil, anthracyclines, vinca alkaloids, taxanes, and epipodophyllotoxins) and immunosuppressors (such as cyclosporine A that usually acts by competing with cytotoxic drugs to bind to P-glycoprotein).[9,10]

It is therefore of high priority to develop cytotoxic agents that show good anticancer activity and can inhibit P-gp activity. Since chalcones **1** (see Chart 1) are intermediates in the synthesis of pyrazoline, pursuing our studies[11,12] on these moieties we have synthesized and evaluated the in vitro activity of derivatives **2** (see Chart 1) to investigate and evaluate their in vitro anticancer activity, following literature reports on the discovery of natural antibiotic pyrazofurin **3** (see Chart 1).[13]

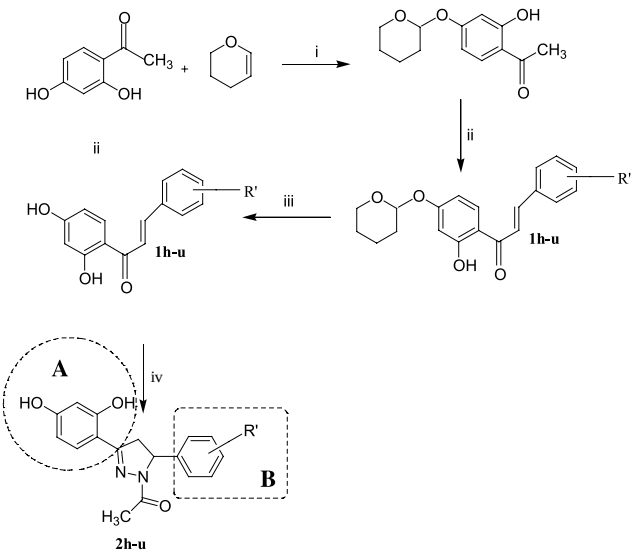
Pyrazofurin has received considerable attention as a result of its many biological effects, including potent antimicrobial and broad spectrum antiviral activities against many DNA and RNA viruses. It has been reported that pyrazofurin has been evaluated against several cell lines. Consequently, it has only been used clinically as an anticancer agent. Lastly, we investigated the ability of derivatives **2** to bind P-glycoprotein, since, to our knowledge, there are no reports investigating the antitumor activity of pyrazolines on multidrug resistant (MDR) cells or their potential to modulate the protein activity of P-glycoprotein (P-gp).

Pyrazolines **2a**–**u** were prepared, according to the one-pot synthesis methods shown in Schemes 1 and 2.

Starting from chalcone **1**, the 1-acetyl-3,5-diphenyl-4,5-dihydro-(1*H*)-pyrazole derivatives **2a**–**u** were obtained by the addition of hydrazine hydrate to acetic acid.[14a]



Scheme 1. Reagents and conditions: (i) $Ba(OH)_2$, EtOH, 25 °C; (ii) N_2H_4, CH_3COOH, refluxed.



Scheme 2. Reagents and condition: (i) PPTS, CH_2Cl_2; (ii) $Ba(OH)_2$, EtOH, 30 °C; (iii) HCl 3 N, EtOH; (iv) N_2H_4, CH_3COOH, refluxed.

Chalcones **1a**–**g** were obtained by the direct Claisen–Schmidt procedure, according to which the condensation of benzaldehyde derivatives with the substituted acetophenone is effected using barium hydroxide as a catalyst in ethanol.[14b]

The hydroxyl groups present in derivatives **1h**–**u** were protected with 3,4-dihydro-α-pyrane and then hydrolyzed.[14c] Structural assignment for compounds is based on UV and ^1H NMR, and is according to the literature data. The chemical and physical data are reported in Table 1. The microanalyses of derivatives **2a**–**u** are reported in Table 2.

All compounds **2a**–**u** were tested to evaluate their cytotoxicity against human tumor cell lines. Pyrazoline derivatives were dissolved in DMSO (stock solution,

Table 1. Chemical and physical data of compounds **2a**–**u**

Compound	R	R′	Yield (%)	Mp (°C)
2a	H	H	64	122–124
2b	H	2-CH_3	44	110–112
2c	H	3-CH_3	54	78–80
2d	H	4-CH_3	43	110–111
2e	H	2-OCH_3	53	157–159
2f	H	3-OCH_3	49	162–164
2g	H	4-OCH_3	52	105–107
2h	4-OH	H	91	170–173
2i	4-OH	2-CH_3	74	183–186
2j	4-OH	3-CH_3	68	157–159
2k	4-OH	4-CH_3	50	195–198
2l	4-OH	2-OCH_3	68	154–156
2m	4-OH	3-OCH_3	80	146–149
2n	4-OH	4-OCH_3	92	184–187
2o	2,4-OH	H	62	126–128
2p	2,4-OH	2-CH_3	67	130–133
2q	2,4-OH	3-CH_3	71	160–161
2r	2,4-OH	4-CH_3	84	185–188
2s	2,4-OH	2-OCH_3	71	140–143
2t	2,4-OH	3-OCH_3	81	154–156
2u	2,4-OH	4-OCH_3	89	149–151

Table 2. Table of microanalyses of derivatives **2a**–**u**

Compound		C%	H%	N%
2a	Calcd	84.53	6.08	9.39
	Found	85.50	6.10	9.40
2b	Calcd	84.58	6.45	8.97
	Found	84.57	6.44	8.99
2c	Calcd	84.58	6.45	8.97
	Found	84.60	6.44	8.99
2d	Calcd	84.58	6.45	8.97
	Found	84.55	6.47	8.98
2e	Calcd	80.46	6.14	8.53
	Found	80.49	6.13	8.55
2f	Calcd	80.46	6.14	8.53
	Found	80.46	6.15	8.52
2g	Calcd	80.46	6.14	8.53
	Found	80.44	6.15	8.55
2h	Calcd	80.23	5.77	8.91
	Found	80.20	5.80	8.92
2i	Calcd	80.46	6.14	8.53
	Found	80.49	6.12	8.55
2j	Calcd	80.46	6.14	8.53
	Found	80.45	6.12	8.56
2k	Calcd	80.46	6.14	8.53
	Found	80.48	6.13	8.54
2l	Calcd	76.72	5.85	8.13
	Found	76.75	5.81	8.15
2m	Calcd	76.72	5.85	8.13
	Found	76.70	5.83	8.16
2n	Calcd	76.72	5.85	8.13
	Found	76.69	5.86	8.16
2o	Calcd	76.34	5.49	8.48
	Found	76.34	5.45	8.51
2p	Calcd	76.72	5.85	8.13
	Found	76.70	5.82	8.19
2q	Calcd	76.72	5.85	8.13
	Found	76.75	5.90	8.10
2r	Calcd	76.72	5.85	8.13
	Found	76.71	5.84	8.16
2s	Calcd	73.32	5.59	7.77
	Found	73.30	5.57	7.79
2t	Calcd	73.32	5.59	7.77
	Found	73.31	5.61	7.80
2u	Calcd	73.32	5.59	7.77
	Found	73.35	5.58	7.75

10 mM) and used within 7 days. The final DMSO concentration never exceeded 0.1% (v/v) either in controls or in treated samples.

The cytotoxic activities of the synthesized compounds were evaluated in vitro against A2780, A2780-CIS, and ECACC.

To perform growth experiments, cells were seeded (20,000 cells/well) in 96-well flat-bottomed plates (Cultureplates, Perkin-Elmer Life Science). After 24 h, the media were replaced, and after one wash those containing the drugs were added. Three independent experiments were performed in quadruplicate. After 72 h of culture in the presence of the tested compounds, the plates were harvested and the number of viable cells was estimated by dosing ATP using the ATPlite kit (Perkin-Elmer Life Science). The kit was employed following manufacturer's suggestions. For each drug/cell line, a dose–response curve was plotted and IC_{50} values were then calculated by fitting the concentration–effect curve data obtained in the three experiments with the sigmoid-E_{max} model using non-linear regression, weighted by the reciprocal of the concentration effect.[15] All tested compounds showed non-significant anticancer activity at IC_{50} values higher than 10,000 nM.

All compounds **2a**–**u** were also tested to investigate their affinity binding to P-glycoprotein. A Rhodamine 123 (Rh 123) fluorescent probe (molecular probes) was used to measure the functionality of the P-gp efflux pump according to the protocol of the National Cancer Institute Drug Screen[16], except for a minor modification. Briefly, MCF-7 ADRr cells were loaded at 37 °C with 0.5 μg/ml of the dye in PBS supplemented by 0.2% BSA. After 15 min, the cells were transferred onto ice and washed twice to remove free Rh 123 from the medium. After washing, 10 μM of the potential P-gp inhibitors was added and the cells were kept at 37 °C for 30–120 min. The positive control was Quercetin, a well-known inhibitor of the P-gp function. An aliquot of cells was maintained on ice to prevent dye efflux (control at 4 °C), and maximal efflux was effected by adding the vehicle DMSO 0.1% and permitting efflux at 37 °C. As regards flow cytometric acquisition, a minimum of 20,000 cells were acquired using an Epics-XL flow cytometer with standard collection filters and electronics. The mean channel of Rh 123 fluorescence was calculated for each condition and time point. The ratio of mean channel between control at 37 °C and control at 4 °C was considered as the control dye efflux. Similarly, the mean channel of Rh 123 fluorescence of treated cells was divided by the control at 4 °C. This ratio was divided by the control dye efflux to establish the potency of P-gp inhibition. The results from two independent experiments were averaged.

The in vitro results of tests of P-gp inhibition are reported in Figure 1. The data representation of derivatives, **2l**, **o**, **p**, **t**, and **u**, is not reported, because they are totally inactive (0%) at a concentration value of 10 μM. Figure 1 shows the percentage of inhibition of Quercetin used as control (100%, dashed line). Derivatives **2b**, **c**, **d**, **g**, **i**, **j**, **k**, **m**, and **q** are comparable to or less active than the control (⩽100%); derivatives **2f**, **h**, **n**, and **r** show a

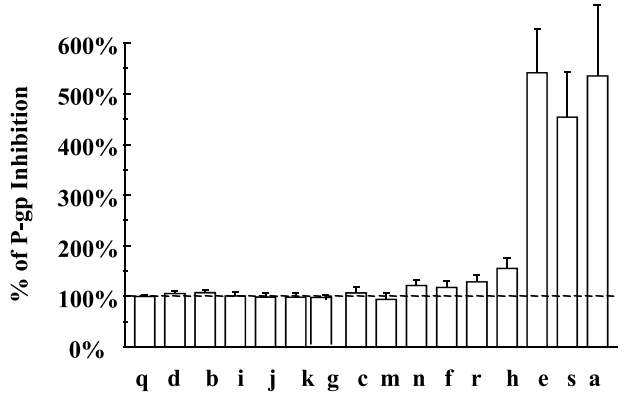


Figure 1. Percentage of P-glycoprotein inhibition of derivatives **2a**–**k**, **m**, **n**, **q**, **r**, and **s** in comparison to Quercetin (P-gp inhibition 100% dashed line).

better binding affinity to P-glycoprotein than the control (>100%). The most active derivatives are **2a**, **e**, and **s**, which are three (**2s**) or four times (**2a** and **e**) more potent than Quercetin. Considering the data in Figure 1 of this preliminary study, the following point on the structure–activity relationship needs to be highlighted. The highest affinity to P-gp was observed with compounds **2a** and **e**, where ring A remains unsubstituted, and which appears to be the most important prerequisite for affinity when comparing with other compounds. The presence of an R′ substituent, such as hydrogen or 2-OCH_3 group, in ring B also appears to be an important factor. In fact, when R = H in ring A, the presence of a methyl group and of a 3- or 4-OCH_3 group in R′ gives compounds with no (**2b**–**d**, **g**) or non-significant affinity (**2f**). The only exception was that observed with compound **2s**, which bears 2,4-OH in ring A and a 2-OCH_3 group in ring B, and whose affinity to P-gp is one time lower than those of **2a** and **e**. All compounds with a 4-OH or 2,4-OH in ring A (**2h**–**n**) showed no or very weak affinity to P-gp, except for the above-mentioned compound **2s**.

Acknowledgments

This work is supported by a grant from MURST 60%. We also acknowledge Mr. Anton Gerada, a professional translator, Fellow of the Institute of Translation and Interpreting of London and Member of AIIC (Association Internationale des Interprêtes de Conférences—Geneva) for revising the manuscript.

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14. (a) A solution of chalcones **1a**–**u** (0.03 mol) in 30 mL of acetic acid was added dropwise to a solution of hydrazine hydrate (0.06 mol) in 10 mL absolute ethanol and stirred with refluxing for 24 h. The mixture was then poured into ice to obtain the crude products **2a**–**u**, which were crystallized from ethanol; (b) Barium hydroxide octahydrate (0.03 mol) was added to a solution of the appropriate acetophenone (0.03 mol) and the appropriate aryl aldehyde (0.03 mol) in 96% ethanol. The suspension was stirred overnight at room temperature. The reaction mixture was then poured into ice and extracted with chloroform after neutralization. The organic layer was washed with water (3× 20 mL), dried (Na_2SO_4), and concentrated in vacuo to obtain the crude product. Chalcones **1a**–**g** were crystallized from methanol; (c) A solution of 3,4-dihydro-α-pyran in methylene chloride (30 mL) was added dropwise to a suspension of suitable acetophenone (0.03 mol) and pyridinium *p*-toluene sulfonate (PPTS) (0.015 mol) in methylene chloride (20 mL), and stirred at room temperature for one night. The reaction mixture was then poured into ice and extracted with chloroform (4× 20 mL). The organic layer was washed with water (3× 20 mL), dried (Na_2SO_4), and concentrated in vacuo to obtain the protected acetophenone. Barium hydroxide octahydrate (0.03 mol) was added to a solution of 4-hydroxy-4-(tetrahydropyran-2-yloxy) acetophenone (0.03 mol) or 2,4-dihydroxy-4-(tetrahydropyran-2yloxy)acetophenone (0.03 mol) and appropriate benzaldehyde (0.03 mol) in dry ethanol and stirred for 24 h at 30 °C. The reaction mixture was then poured into ice and extracted with chloroform after neutralization. The organic layer was washed with water (3× 20 mL), dried (Na_2SO_4), and concentrated in vacuo to obtain the crude 4-hydroxy-4-(tetrahydropyran-2-yloxy) chalcone or 2,4-dihydroxy-4-(tetrahydropyran-2-yloxy) chalcone. The crude products were suspended in ethanol and HCl 3 N (15 mL) was added. The reaction suspension was stirred for one night at room temperature, then poured into ice, neutralized with Na_2CO_3, and extracted with chloroform (3× 30 mL). The organic layer was dried over sodium sulfate and evaporated in vacuo to give the crude product. Chalcones **1h**–**u** were crystallized from methanol.
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