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Synthesis of novel 3,5-diaryl pyrazole derivatives using combinatorial chemistry as inhibitors of tyrosinase as well as potent anticancer, anti-inflammatory agents

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ABSTRACT

In the present article, we have synthesized a combinatorial library of 3,5-diaryl pyrazole derivatives using 8-(2-(hydroxymethyl)-1-methylpyrrolidin-3-yl)-5,7-dimethoxy-2-phenyl-4*H*-chromen-4-one (**1**) and hydrazine hydrate in absolute ethyl alcohol under the refluxed conditions. The structures of the compounds were established by IR, ^{1}H NMR and mass spectral analysis. All the synthesized compounds were evaluated for their anticancer activity against five cell lines (breast cancer cell line, prostate cancer cell line, promyelocytic leukemia cell line, lung cancer cell line, colon cancer cell line) and anti-inflammatory activity against TNF-α and IL-6. Out of **15** compounds screened, **2a** and **2d** exhibited promising anticancer activity (61–73% at 10 μM concentration) against all selected cell lines and IL-6 inhibition (47% and 42% at 10 μM concentration) as in comparison to standard flavopiridol (72–87% inhibition at 0.5 μM) and dexamethasone (85% inhibition at 1 μM concentration), respectively. Cytotoxicity of the compounds checked using CCK-8 cell lines and found to be nontoxic to slightly toxic. Out of 15, four 3,5-diaryl pyrazole derivatives exhibiting potent inhibitory activities against both the monophenolase and diphenolase actions of tyrosinase. The IC_{50} values of compounds (**2a**, **2d**, **2h** and **2l**) for monophenolase inhibition were determined to range between 1.5 and 30 μM. Compounds **2a**, **2d**, **2h** and **2l** also inhibited diphenolase significantly with IC_{50} values of 29.4, 21.5, 2.84 and 19.6 μM, respectively. All four 3,5-diaryl pyrazole derivatives were active as tyrosinase inhibitors (**2a**, **2d**, **2h** and **2l**), and belonging to competitive inhibitors. Interestingly, they all manifested simple reversible slow-binding inhibition against diphenolase.

1. Introduction

With the advent of combinatorial chemistry, the number of new chemical entities (NCEs) can be produced in a short space of time by drug discovery teams. Although this has produced a wealth of possible new therapeutic compounds, it has also raised an important question about screening of most therapeutically active ones, from thousands of compounds. One approach to solve this problem has been the use of in vitro screens to identify the characteristics of an NCE, particularly with respect to its drug metabolism. Such information is crucial to the decision-making process of which compounds to progress with and which to discard. Such an approach can also be used to screen smaller sets of structurally related compounds, allowing determination of the chemical structure that is the strongest possible lead candidate.[1,2]

Increased generation of reactive oxygen species (ROS) has been observed in cancer, degenerative diseases and other pathological conditions. ROS can stimulate cell proliferation, promote genetic instability and induce adaptive responses that enable cancer cells to maintain their malignant phenotypes. However, when cellular redox balance is severely disturbed, high levels of ROS might cause various damages leading to cell death. Cancer is the second leading cause of death in the present society after cardiovascular diseases. A great deal of efforts have been underway to treat various forms of cancer for decades; and until recently, chemoprevention of cancer is receiving its due to share of attention. Combinatorial chemistry and high-throughput screening against pure molecular targets and cancer cells are established methods for primary anticancer drug discovery.[3–5]

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Inflammation is the body's way of dealing with infections and tissue damage, but there is a fine balance between the beneficial effects of inflammation cascades and their potential for long-term tissue destruction. If they are not controlled or resolved, inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis. Within many inflammation cascades or pathways, there are often pivotal molecular targets that, when antagonized or neutralized, block the output of the pathway. A relatively small number of pivotal targets has been identified that have yielded many successful anti-inflammatory drugs. These targets include the enzymes (COX 1 and COX 2), cytokines (tumor necrosis factor-α, interleukin-6 and interleukin-2) and the receptor for the Cysteinyl leukotrienes C4 and D4 and nuclear membrane receptors (corticosteroids).[6,7] Therefore, inhibition of these targets has become a major focus of current drug discovery and development, and an important in vitro method for evaluating the bioactivity of drugs.[8,9]

Tyrosinase (EC 1.14.18.1), which is also referred to as polyphenoloxidase (PPO), is most widely associated with the production of melanin for the protection of skin from solar radiation. However, this beneficial trait comes in hand with some severe vices and human maladies since the overproduction of melanin results in skin hypigmentation, characterized by age spots, melasma and chloasama.[10–12] It has also been suggested that tyrosinase may contribute to the neurodegeneration associated with Parkinson's disease.[13] Thus, the unregulated action of tyrosinase is factor in a number of human disease etiologies. Tyrosinase inhibition has thus been avidly explored as an avenue for therapies to these diseases. The inhibition of melanin formation by tyrosinase is also applicable to fruit preservation by the alleviation of browning, rendering inhibitors of even broader importance. In addition, tyrosinase is known to be involved in the molting process of insect and adhesion of marine organisms.[14] In the last decades, a large number of naturally occurring and synthetic compounds acting as tyrosinase inhibitors were reported[15–23] and the most representative tyrosinase inhibitor so far is tropolone. However, only few of them are put into practical use due to their weak individual activities or safety concerns. Undoubtedly, more efforts are still needed to search and develop more effective and safe tyrosinase inhibitors.

Tyrosinase itself is an enzyme containing a binuclear copper active site. It catalyzes two steps in the conversion of tyrosine to melanin. This process proceeds via 3,4-dihydroxy phenylalanine (DOPA), which is formed by tyrosinase monophenolase activity on tyrosine. The next step is the oxidation of DOPA into DOPA quinine, which is a process catalysed by active diphenolase.[24–26] These quinines spontaneously polymerize to high molecular weight brown pigmented species, known as melanin. Tyrosinase inhibitors normally either render the copper within the active site inactive by chelation, obviating the substrate–enzyme interaction, or inhibit oxidation via an electrochemical process.[27,28] Since, it has been observed that flavonoids, stilbenes, and tropolones manifest tyrosinase inhibitory activities, we have honed our research goals both to evaluate other phenolic skeletons for their tyrosinase inhibition properties and also to elucidate their kinetic modes.

The 3,5-diaryl-4,5-dihydropyrazoles has been reported as potent and selective inhibitors of Kinesin spindle protein (KSP).[29] Dihydropyrazoles are potent, cell active KSP that induces apoptosis and generates aberrant mitotic spindles in human ovarian carcinoma cells. Based on literature search it has been found that pyrazole derivatives could have potential biological activity, and therefore, we had decided to work on the pyrazole derivatives, their synthesis, characterization and evaluation for their biological activity.[30,31]

2. Results and discussion

2.1. Chemistry

Natural products have played an important role in drug discovery and are source of scaffolds for the development of new entities. Rohitukine is one of such compound isolated, characterized and synthesized at Hoechst Research Centre, Bombay India. Rohitukine is a novel 4*H*-1-benzopyrane-4-one with combined anti-inflammatory and immunomodulatory properties in acute and chronic model of inflammation. Further from Rohitukine a synthetic compound was synthesized and tasted for anticancer activity was Flavopiridol. Flavopiridol is a novel semi synthetic flavone. Flavopiridol is known to inhibit potently the activity of multiple cyclin-dependent kinases (IC_{50} 40 nm).

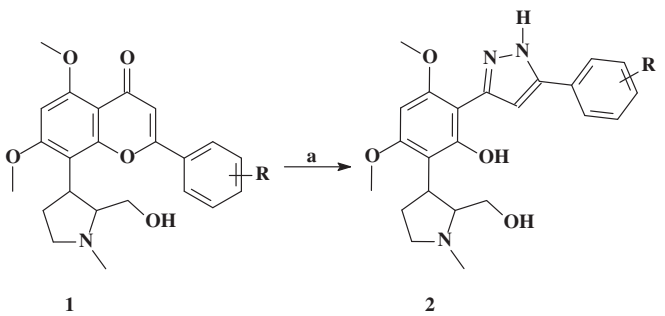
While synthesizing Rohitukine, five membered ring contracted 2-hydroxymethyl pyrrolidine moiety was observed.[32]. Which was used to synthesize flavones having anticancer activity, we have decided to derivatize these flavones to pyrazoles.

In the present investigation 2-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-6-(5-phenyl-1*H*-pyrazol-3-yl)-phenol (**2**) has been synthesized from 8-(2-(hydroxymethyl)-1-methylpyrrolidin-3-yl)-5,7-dimethoxy-2-phenyl-4*H*-chromen-4-one (**1**) and hydrazine hydrate in absolute ethyl alcohol under the refluxed conditions for appropriate time (Scheme 1). Substituted 8-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-5,7-dimethoxy-2-phenyl-chromen-4-one (**1**) were prepared according to known literature method.[32] The residue was purified on column chromatography (silica gel with 1% MeOH and 1% aq ammonia in $CHCl_3$). The compound's structure was confirmed by spectral data (IR, ^1H NMR and MS). The chemical profile of the compounds is as shown in Table 1.

2.2. Biological evaluation

In vitro assays are increasingly being used in drug metabolism studies to screen novel chemicals. Their advantages are twofold: first, they allow testing early in the drug discovery phase, providing important information on chemical characteristics; second, human cells or cell constituents can be utilized, increasing the relevance to man.[1]

Anticancer activity of the synthesized compounds was evaluated by using cell line inhibition viz. MCF-7 (breast cancer cell line), PC-3 (prostate cancer cell line), HL-60 (promyelocytic leukemia cell line), H-460 (lung cancer cell line) and HCT-116 (colon cancer cell line) (Table 2). The compound **2a** and **2d** revealed promising anticancer activity (61–73%), followed by **2i**, **2n**, **2b**, **2c** and **2o** compounds (51–67%). While compounds **2e**, **2f**, **2g**, **2h**, **2k**, **2l** and **2m** (11–29%) revealed slight to moderate activity. Structure–activity relationship (SAR) study observed that only halo substituents (Cl and Br) are involved in improving the anticancer activity except fluoro substituent. However, the substituent CH_3



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Scheme 1. Reagents and conditions: (a) hydrazine hydrate, ethanol, reflux, 6–8 h.

Table 1
Preparation of 3,5-diaryl pyrazole derivatives

Compound	Substituents (R)	Molecular formula	Molecular weight	Yield (%)	Reaction time (h)
2a	2-Cl	$C_{23}H_{26}ClN_3O_4$	443	52	6
2b	2-Br	$C_{23}H_{26}BrN_3O_4$	488	51	6
2c	2,4-Cl	$C_{23}H_{25}Cl_2N_3O_4$	478	46	6
2d	2-Cl - 4-NO_2	$C_{23}H_{25}ClN_4O_6$	488	48	6
2e	2-F	$C_{23}H_{26}FN_3O_4$	427	41	6
2f	2- CH_3	$C_{24}H_{29}N_3O_4$	423	57	6
2g	2-OCH_3	$C_{24}H_{29}N_3O_5$	439	55	6
2h	2,4-OCH_3	$C_{25}H_{31}N_3O_6$	469	59	6.5
2i	3-Cl	$C_{23}H_{26}ClN_3O_4$	443	54	6
2j	3-Br	$C_{23}H_{26}BrN_3O_4$	488	63	6
2k	4-CH_3	$C_{24}H_{29}N_3O_4$	423	59	6
2l	4-F	$C_{23}H_{26}FN_3O_4$	427	46	6
2m	4-OCH_3	$C_{24}H_{29}N_3O_5$	439	63	6
2n	4-Cl	$C_{23}H_{26}ClN_3O_4$	443	46	6
2o	4-Br	$C_{23}H_{26}BrN_3O_4$	488	50	6

Table 2
Anticancer activity of 3,5-diaryl pyrazole derivatives

Compound	Anticancer activity at 10 μM concn				
	MCF-7	PC-3	HL-60	H-460	HCT-116
2a	63	69	67	69	73
2b	55	51	62	53	61
2c	58	63	59	61	59
2d	61	65	63	65	67
2e	15	21	19	23	19
2f	11	17	14	18	16
2g	13	29	17	15	21
2h	21	16	24	19	17
2i	59	67	61	55	63
2j	65	58	52	61	59
2k	17	11	21	13	19
2l	19	15	26	16	21
2m	15	22	19	13	16
2n	61	65	63	58	55
2o	63	59	57	61	49
Flavopiridol 0.5 μM	74	77	72	78	87

MCF-7—breast cancer cell line.
PC-3—prostate cancer cell line.
HL-60—promyelocytic leukemia cell line.
H-460—lung cancer cell line.
HCT-116—colon cancer cell line.

Table 3
Anti-inflammatory activity of 3,5-diaryl pyrazole derivatives

Compound	% Inhibition at 10 μM		Toxicity
	TNF-α	IL-6	
2a	24	47	0
2b	19	39	0
2c	18	41	0
2d	21	42	23
2e	0	11	7
2f	0	13	18
2g	0	10	0
2h	0	11	0
2i	11	35	0
2j	13	43	0
2k	0	10	14
2l	11	28	11
2m	0	12	0
2n	16	35	0
2o	17	31	0
Dexamethasone (1 μM)	71	85	0

and OCH_3 imparted low activity. In general all the cells lines MCF-7, PC-3, HL-60, H-460 are equally inhibited by the synthesized compounds except HCT-116.

Ant-inflammatory activity of the compound was determined for TNF-α and IL-6 inhibition (Table 3). The compounds showed good to moderate cytokine inhibition in the range of 10–47% for IL-6 and only 11–21% for TNF-α. Compounds **2a**, **2c**, **2d** and **2i** give IL-6 inhibitory activity while **2a** and **2d** inhibit TNF-α actively. However rest of the compounds has shown slight to moderate TNF-α inhibition.

The rule of three, relating to activity–exposure–toxicity, presents the single most difficult challenge in the design and advancement of drug candidates to the development stage. Absorption, distribution, metabolism and excretion (ADME) studies are widely used in drug discovery to optimize this balance of properties necessary to convert lead compounds into drugs that are both safe and effective for human patients.[33] Therefore, in vitro bioavailability of the synthesized compounds was determined using CCK-8 cells. The compounds were found to be nontoxic except **2d, 2f, 2k** and **2l** but at an acceptable range. Therefore, the compounds are biologically safe and used as the therapeutic agent for future drug discovery study.

In a preliminary screening, using mushroom tyrosinase as a representative enzyme, we observed that 3,5-diaryl pyrazole derivatives showed significant inhibition of tyrosinase L-DOPA oxidation. More detailed bioassay for some compounds was subsequently conducted. Data shown in Table 4, all 3,5-diaryl pyrazole derivatives examined for tyrosinase inhibition, apart from all 3,5-diaryl pyrazole derivatives showed dose dependent inhibition against both monophenolase and diphenolase activity. As the concentration of inhibitors raised, the residual enzyme activity drastically decreased (Fig. 1). All inhibitors manifested a similar relationship between the enzyme activity and enzyme concentration. From the progress curve obtained, compounds (**2a**, **2d**, **2h** and **2l**) showing solid lines below the line of enzyme activity has indicative of enzyme inhibition and vice versa.

Taken as an ensemble, the following general features of SAR can be deduced from compounds (**2a**–**2o**). The presence of the methoxy groups at 2,4-position is important for inhibition (**2h**, IC_{50} = 1.75). While, compounds lacking this substitution showed 10 fold higher IC_{50} values from other inhibitors (**2a**, IC_{50} = 22.8, **2d**, IC_{50} = 17.8 and **2l**, IC_{50} = 15.5). This effect of great magnitude obtained in IC_{50} values was due to the presence of halo substituents (–Cl, –Br and –F).

The kinetic behavior of the L-tyrosine on L-DOPA oxidation catalyzed by the mushroom tyrosinase at different concentrations of compounds (**2h**, **2l**, **2d** and **2a**) were studied. In this experiment, the initial velocity of the enzyme was monitored via dopachrome formation at 475 nm. As shown in Figure 2, the Lineweaver–Burk plots of $1/v$ versus 1/[S] result in a family of straight lines with the same y-axis intercept, as illustrated, respectively for the four tyrosinase inhibitors. In Figure 2 the reciprocal of concentration of L-tyrosine is the abscissa 1/[L-DOPA] and the ordinate $1/v$ is the reciprocal

Table 4
Inhibitory effects of 3,5-diaryl pyrazole derivatives on mushroom tyrosinase activities

Entry	L-Tyrosine		L-DOPA	
	IC_{50} (μM)	Type of inhibition (K_i, μM)	IC_{50} (μM)	Type of inhibition (K_i, μM)
2a	22.8	Competitive (10.6)	29.4	Competitive (11.8)
2d	17.8	Competitive (9.85)	21.5	Competitive (14.6)
2h	1.75	Competitive (0.84)	2.84	Competitive (0.96)
2l	15.5	Competitive (11.2)	19.6	Competitive (12.6)

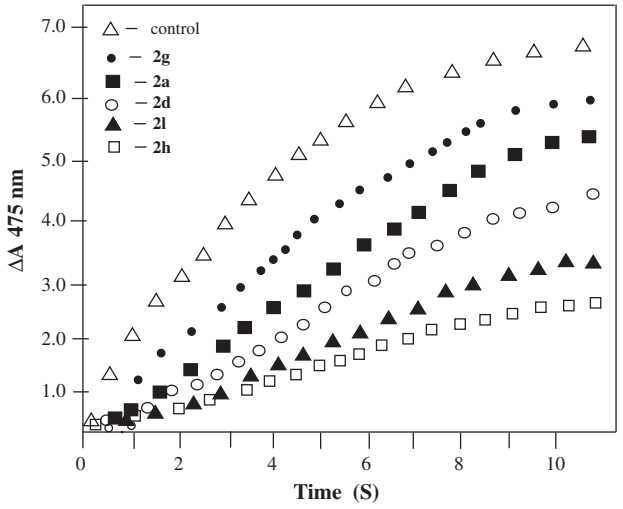

Figure 1. Progress curve for oxidation of L-DOPA catalyzed by mushroom tyrosinase in the presence of of 3,5-diaryl pyrazole derivatives (**2h > 2l > 2d > 2a > 2g**). Data shown in the figure is mean of three different experiments.

of the change of velocity, which is reciprocal of tyrosinase activity. The pattern of lines and their arrangement in presence of each inhibitor for each graph indicated that they are all competitive inhibitors [**2a**, K_i = 10.6 μM, **2d**, K_i = 9.85 μM, **2h**, K_i = 0.84 μM and **2l**, K_i = 11.2 μM] with their inhibitory activity toward tyrosinase decreasing with the increasing concentrations of the substrate. Most competitive inhibitors of tyrosinase share some structural commonality with that of product. As reported from earlier studies, for successful diphenolase inhibition, a 3,4-dihydroxy group in the B-ring of required in order to make the structure of the inhibitor molecule resemble L-DOPA. This leads to the competitive displacement of L-DOPA from the active site of the cofactor in a lock-and key model. This is also seen in molecules such as quercetin and chalcones.[34,35] Tepper et al. have reported that halides ions such as I^-, F^-, Cl^-, and Br^- directly interacted with the copper ions in the active site of tyrosinase from *Streptomyces antibioticus* and this resulted in the inhibition of L-DOPA conversion.[36] These results implied that different several manners of inhibition mechanism by halide ions on tyrosinase from various sources could exist. Likewise, this is consistent to the general recognition that mushroom tyrosinase activity was inhibited by those 3,5-diaryl pyrazole derivatives which poses either nucleophilic methoxy group or halides ions such as F^-, Cl^-, and Br^- along with the phenolic skeleton.

3. Conclusion

In an attempt to generate broad spectrum 3,5-diaryl pyrazole derivatives, we have synthesized total **15** compounds with significant anticancer, anti-inflammatory and Tyrosinase inhibitory activity. Compounds **2a** and **2d** were identified as the potent anticancer and inflammatory agent against all selected cell lines and IL-6 (an anti-inflammatory target), respectively. The 3,5-diaryl pyrazole derivatives were assayed for mushroom tyrosinase inhibition. Five of these compounds, emerged to be the potent inhibitors of Mushroom tyrosinase. We progressed to clearly demonstrate that all of these derivatives were competitive inhibitors. This study explored novel compounds that can fight against tyrosinase born illness as well as food depreciation.

4. Experimental

4.1. General

Melting points were recorded in open capillaries with an electrical melting point apparatus and were uncorrected. An IR spectrum (KBr disks) was recorded using a Perkin–Elmer 237 spectrophotometer. ^{1}H NMR spectra was recorded on a Bruker Avance (300 MHz) spectrometer in $CDCl_3$ solutions, with TMS as an internal reference. A mass spectrum was recorded on a Shimadzu GCMS QP 1000 EX. All the reagents and solvents used were of analytical grade, and were used as supplied, unless otherwise stated. TLC was performed on aluminum plates coated with silica gel (Merck) for monitoring the reactions.

4.2. Synthesis of 3,5-diaryl pyrazole derivatives

To a solution of 8-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-5,7-dimethoxy-2-phenyl-chromen-4-one **(1)** (1.16 mmol) in absolute ethanol (20 mL) was added hydrazine hydrate (87 μl, 1.74 mmol) and refluxed for 6 h (Scheme 1). Reaction mixture was cooled to 25 °C diluted with chloroform adsorbed on silica and passed through a silica gel column eluting with 1% MeOH/$CHCl_3$ and 1% aq ammonia to get required pyrazole derivatives.

The physical and spectral data of pyrazole derivatives are given below.

4.2.1. 2-[5-(2-Chloro-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2a)

Mp: 210–212 °C; IR cm^{-1}: 3422, 1648, 1598; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (m, 1H), 7.4 (m, 1H), 7.32 (m, 2H), 7.31 (s, 1H), 6.1 (s, 1H), 4.06 (m, 1H), 3.98 (s, 3H), 3.94 (s, 3H), 3.69 (m, 1H), 3.49 (m, 1H), 3.26 (m, 1H), 2.92 (m, 1H), 2.5 (s, 3H), 2.43 (m, 1H), 2.08 (m, 2H); MS: *m/z* 444 (M^+).

4.2.2. 2-[5-(2-Bromo-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2b)

Mp: 188–190 °C; IR cm^{-1}: 3423, 1649, 1596; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.71 (m, 1H), 7.46 (m, 1H), 7.34 (m, 2H), 7.30(s, 1H), 6.1 (s, 1H), 4.02 (m, 1H), 3.97 (s, 3H), 3.95 (s, 3H), 3.68 (m, 1H), 3.48 (m, 1H), 3.26 (m, 1H), 2.92 (m, 1H), 2.51 (s, 3H), 2.42 (m, 1H), 2.08 (m, 2H); MS: *m/z* 488 (M^+).

4.2.3. 2-[5-(2,4-Dichloro-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2c)

Mp: 128–130 °C; IR cm^{-1}: 3418, 1644, 1596; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (d, 1H, J = 8.5), 7.58 (d, 1H, J = 8.6),

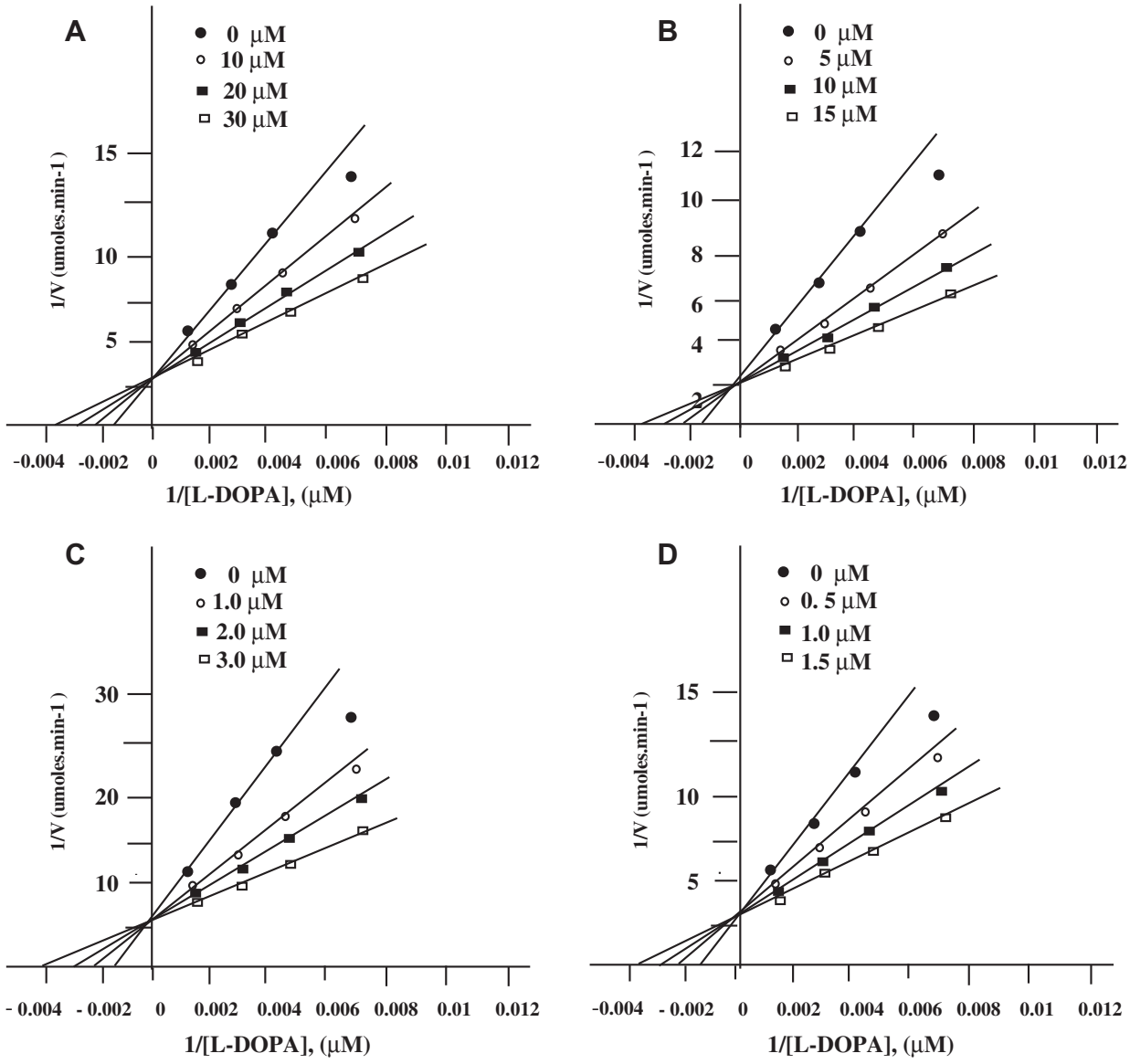

Figure 2. Lineweaver–Burk plots for the inhibition of diphenolase activity of Mushroom tyrosinase by 3,5-diaryl pyrazole derivatives (**2a**, **2d**, **2h** and **2l**). (A) Concentrations of derivative **2a** for curves from top to bottom were 0, 10, 20 and 30 μM. (B) Derivative **2d** (0, 5, 10 and 15 μM). (C) Derivative **2h** (0, 1.0, 2.0 and 3.0 μM) and derivative **2l** (0, 0.5, 1.0 and 1.5 μM), respectively.

7.46 (s, 1H), 7.3 (s, 1H), 5.59 (s, 1H), 4.02 (m, 1H), 3.96 (s, 3H), 3.94 (s, 3H), 3.67 (m, 1H), 3.47 (m, 1H), 3.25 (m, 1H), 2.91 (m, 1H), 2.5 (s, 3H), 2.44 (m, 1H), 2.06 (m, 2H); MS: *m/z* 479 (M^+).

4.2.4. 2-[5-(2-Chloro-4-nitro-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2d)

Mp: 197–199 °C; IR cm^{-1}: 3426, 1646, 1592; ^1H NMR (300 MHz, $CDCl_3$): δ 8.6 (s, 1H), 8.2 (d, 1H, J = 8.6), 7.82 (d, 1H, J = 8.4), 7.4 (s, 1H), 6.1 (s, 1H), 4.01 (m, 1H), 3.94 (s, 3H), 3.90 (s, 3H), 3.6 (m, 1H), 3.4 (m, 1H), 3.24 (m, 1H), 2.91 (m, 1H), 2.48 (s, 3H), 2.42 (m, 1H), 2.07 (m, 2H). MS: *m/z* 489 (M^+).

4.2.5. 2-[5-(2-Fluoro-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxy-methyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2e)

Mp: 115–121 °C; IR cm^{-1}: 3421, 1646, 1592; ^1H NMR (300 MHz, $CDCl_3$): δ 7.78 (m, 1H), 7.4 (m, 1H), 7.36 (m, 2H), 7.3 (s, 1H), 5.58 (s, 1H), 4.04 (m, 1H), 3.97 (s, 3H), 3.95 (s, 3H), 3.67 (m, 1H), 3.47 (m, 1H), 3.24 (m, 1H), 2.91 (m, 1H), 2.48 (s, 3H), 2.4 (m, 1H), 2.04 (m, 2H). MS: *m/z* 428 (M^+).

4.2.6. 2-(2-Hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-6-(5-*o*-tolyl-1*H*-pyrazol-3-yl)-phenol (2f)

Mp: 152–156 °C; IR cm^{-1}: 3426, 1653, 1596; ^1H NMR (300 MHz, $CDCl_3$): δ 7.6–7.32 (m, 4H), 7.3 (s, 1H), 6.0 (s, 1H), 4.04 (m, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.67 (m, 1H), 3.44 (m, 1H), 3.21 (m, 1H), 2.9 (m, 1H), 2.48 (s, 3H), 2.43 (m, 1H), 2.32 (s, 3H), 2.08 (m, 2H); MS: *m/z* 424 (M^+).

4.2.7. 2-(2-Hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-6-[5-(2-methoxy-phenyl)-1*H*-pyrazol-3-yl]-phenol (2g)

Mp: 137–141 °C; IR cm^{-1}: 3424, 1650, 1598; ^1H NMR (300 MHz, $CDCl_3$): δ 7.64–7-4 (m, 4H), 7.31 (s, 1H), 5.59 (s, 1H), 4.06 (m, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.86 (s, 3H), 3.69 (m, 1H), 3.46 (m, 1H), 3.24 (m, 1H), 2.91 (m, 1H), 2.5 (s, 3H), 2.41 (m, 1H), 2.06 (m, 2H); MS: *m/z* 440 (M^+).

4.2.8. 2-[5-(2,4-Dimethoxy-phenyl)-1H-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2h)

Mp: 88–90 °C; IR cm^{-1}: 3428, 1656, 1600; ^1H NMR (300 MHz, $CDCl_3$): δ 7.7 (d, 1H, J = 8.7), 7.62 (d, 1H, J = 8.4), 7.42 (s, 1H), 7.30 (s, 1H), 6.1 (s, 1H), 4.04 (m, 1H), 3.95 (s, 3H), 3.94 (s, 3H), 3.9 (s, 3H), 3.86 (s, 3H), 3.66 (m, 1H), 3.46 (m, 1H), 3.24 (m, 1H), 2.90 (m, 1H), 2.5 (s, 3H), 2.46 (m, 1H), 2.04 (m, 2H); MS: m/z 470 (M^+).

4.2.9. 2-[5-(3-Chloro-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2i)

Mp: 143–148 °C; IR cm^{-1}: 3420, 1646, 1597; ^1H NMR (300 MHz, $CDCl_3$): δ 7.78 (s, 1H), 7.66 (d, 1H, J = 7.8), 7.44 (d, 1H, J = 7.8), 7.32 (s, 1H), 6.0 (s, 1H), 4.06 (m, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.67 (m, 1H), 3.47 (m, 1H), 3.24 (m, 1H), 2.91 (m, 1H), 2.48 (s, 3H), 2.41 (m, 1H), 2.07 (m, 2H); MS: m/z 444 (M^+).

4.2.10. 2-[5-(3-Bromo-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2j)

Mp: 157–163 °C; IR cm^{-1}: 3421, 1646, 1598; ^1H NMR (300 MHz, $CDCl_3$): δ 7.79 (s, 1H), 7.6 (d, 1H, J = 7.6), 7.48 (d, 1H, J = 7.8), 7.30 (s, 1H), 6.0 (s, 1H), 4.04 (m, 1H), 3.96 (s, 3H), 3.92 (s, 3H), 3.66 (m, 1H), 3.42 (m, 1H), 3.24 (m, 1H), 2.90 (m, 1H), 2.48 (s, 3H), 2.41 (m, 1H), 2.06 (m, 2H); MS: m/z 488/490 (M^+).

4.2.11. 2-(2-Hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-6-(5-*p*-tolyl-1*H*-pyrazol-3-yl)-phenol (2k)

Mp: 89–94 °C; IR cm^{-1}: 3420, 1646, 1592; ^1H NMR (300 MHz, $CDCl_3$): δ 7.58 (d, 2H, J = 8), 7.24 (d, 2H, J = 7.8), 7.3 (s, 1H), 6.04 (s, 1H), 4.06 (m, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 3.65 (m, 1H), 3.47 (m, 1H), 3.24 (m, 1H), 2.91 (m, 1H), 2.42 (s, 3H), 2.43 (m, 1H), 2.3 (s, 3H), 2.08 (m, 2H); MS: m/z 424 (M^+).

4.2.12. 2-[5-(4-Fluoro-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxy–methyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2l)

Mp: 146–150 °C; IR cm^{-1}: 3423, 1649, 1596; ^1H NMR (300 MHz, $CDCl_3$): δ 7.68 (t, 2H, J = 6.6), 7.42 (t, 2H, J = 8.7), 7.3 (s, 1H), 6.06 (s, 1H), 4.04 (m, 1H), 3.96 (s, 3H), 3.94 (s, 3H), 3.66 (m, 1H), 3.48 (m, 1H), 3.25 (m, 1H), 2.91 (m, 1H), 2.49 (s, 3H), 2.42 (m, 1H), 2.06 (m, 2H). MS: m/z 428 (M^+).

4.2.13. 2-(2-Hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-6-[5-(4-methoxy-phenyl)-1*H*-pyrazol-3-yl]-phenol (2m)

Mp: 108–114 °C; IR cm^{-1}: 3421, 1651, 1594; ^1H NMR (300 MHz, $CDCl_3$): δ 7.61 (d, 2H, J = 8.2), 7.30 (d, 2H, J = 8.2), 7.30 (s, 1H), 6 (s, 1H), 4.06 (m, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.86 (s, 3H), 3.66 (m, 1H), 3.48 (m, 1H), 3.25 (m, 1H), 2.92 (m, 1H), 2.46 (s, 3H), 2.41 (m, 1H), 2.04 (m, 2H); MS: m/z 440 (M^+).

4.2.14. 2-[5-(4-Chloro-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2n)

Mp: 112–117 °C; IR cm^{-1}: 3420, 1646, 1595; ^1H NMR (300 MHz, $CDCl_3$): δ 7.78 (d, 2H), 7.64 (d, 2H), 7.32(s, 1H), 6.1 (s, 1H), 4.08 (m, 1H), 3.96 (s, 3H), 3.94 (s, 3H), 3.66 (m, 1H), 3.48 (m, 1H), 3.24 (m, 1H), 2.91 (m, 1H), 2.48 (s, 3H), 2.41 (m, 1H), 2.06 (m, 2H); MS: m/z 444 (M^+).

4.2.15. 2-[5-(4-Bromo-phenyl)-1*H*-pyrazol-3-yl]-6-(2-hydroxymethyl-1-methyl-pyrrolidin-3-yl)-3,5-dimethoxy-phenol (2o)

Mp: 165–171 °C; IR cm^{-1}: 3424, 1651, 1596; ^1H NMR (300 MHz, $CDCl_3$): δ 7.8 (d, 2H), 7.66 (d, 2H), 7.31 (s, 1H), 6.1 (s, 1H), 4.06 (m, 1H), 3.95 (s, 3H), 3.92 (s, 3H), 3.68 (m, 1H), 3.46 (m, 1H), 3.26 (m, 1H), 2.90 (m, 1H), 2.47 (s, 3H), 2.4 (m, 1H), 2.07 (m, 2H); MS: m/z 488/490 (M^+).

4.3. Anticancer activity

Cytotoxic assay is performed on MCF-7 (breast cancer cell line), PC-3 (prostate cancer cell line), HL-60 (promyelocytic leukemia cell line), H-460 (lung cancer cell line) and HCT-116 (colon cancer cell line) cell lines using propidium iodide fluorescence assay.[37] Dyes such as propidium iodide (PI), which bind DNA, provide a rapid and accurate means for quantitating total nuclear DNA. The fluorescence signal intensity of the PI is directly proportional to the amount of DNA in each cell, PI is not able to penetrate an intact membrane, and so cells must first be permeabilized. Seed cells of 3000–7500 cells/well were placed in 2000 μl of tissue culture grade 96 well plates and allowed them to recover for 24 h in humidified 5% CO_2 incubator at 37 °C. After culturing for 24 h compounds (in 0.1% DMSO) were added onto triplicate wells with 10 μM concentrations. 0.1% DMSO alone was used as control. After 48 h in humidified 5% CO_2 incubator at 37 °C condition, the medium was removed and treated with 25 μl of propidium iodide (50 μg/mL in water/medium) per well. The plates were frozen at −80 °C for 24 h then thawed and allowed it to come at room temperature and the plate absorbance was read on a fluorometer (Polar-Star BMG Tech), using 530 nm excitation and 620 nm emission wavelength. Lastly, percent cytotoxicity of the compounds was calculated by using following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Reading of control} - \text{Reading of treated cells}}{\text{Reading of control}} \times 100$$

The results were compared with the standard drug inhibitors flavopiridol (0.5 μM).

4.4. Anti-inflammatory and cytotoxicity assay

Proinflammatory cytokine production by lipopolysaccharide (LPS) in THP-1 cells was measured according to the method described by Hwang et al.[38] During the assay, THP-1 cells were cultured in the RPMI 1640 culture media (Gibco BRL, Pasley, UK) containing 100 U/mL penicillin and 100 mg/mL streptomycin containing 10% fetal bovine serum (FBS, JRH). Cells were differentiated with phorbol myristate acetate (PMA, Sigma). Following cell plating, the test compounds in 0.5% DMSO was added to each well and the plate was incubated for 30 min at 37 °C. Finally, LPS (*Escherichia coli* 0127: B8, Sigma Chemical Co., St. Louis, MO) was added, at a final concentration of 1 μg/mL in each well. Plates were further incubated at 37 °C for 24 h in 5% CO_2. After incubation, supernatants were harvested, and assayed for TNF-α and IL-6 by ELISA as described by the manufacturer (BD Biosciences). The cells were simultaneously evaluated for cytotoxicity using CCK-8 from Dojindo Laboratories. Percent inhibition of cytokine release in comparison to the control was calculated. The 50% inhibitory concentration (IC_{50}) values were calculated by a nonlinear regression method.

4.5. Assay of tyrosinase activity

Mushroom tyrosinase (EC 1.14.18.1) (Sigma Chemical Co.) was used as described previously with some modifications, using either, L-DOPA (diphenolase) or L-tyrosine (monophenolase) as substrate.[39] In spectrophotometric experiments, enzyme activity was monitored by observing dopachrome formation at 475 nm with a UV–vis spectrophotometer (Spectro UV–vis Double beam; Shimadzu, Inc.) at 30 °C. All samples were first dissolved in 0.1% DMSO at 10 μM–50 μM. First, 200 μL of a 2.7 mM L-tyrosine (K_m = 180 μM) or 5.4 mM L-DOPA (K_m = 360 μM) aqueous solution was mixed with 2687 μL of 0.25 M phosphate buffer (pH 6.8). Then, 100 μL of the sample solution and 13 μL of the same

phosphate buffer solution containing mushroom tyrosinase (144 units) were added in this order to the mixture. Each assay was conducted as three separate replicates. The inhibitor concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the Dose response curve.

4.6. Progress curves determination

All reactions were carried out using L-tyrosine as a substrate in 0.25 M phosphate buffer (pH 6.8) at 30 °C. Enzyme activities were measured continuously for 15 min using a UV spectrophotometer. To determine the kinetic parameters associated with time-dependent inhibition of tyrosinase, progress curves with 20 data points (30 s intervals) were obtained at several inhibitor concentrations using fixed substrate concentrations.

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