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Optimizing Thiadiazole Analogues of Resveratrol vs. Three Chemopreventive Targets

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Abstract

Chemoprevention is an approach to decrease cancer morbidity and mortality through inhibition of carcinogenesis and prevention of disease progression. Although the trans stilbene derivative resveratrol has chemopreventive properties, its action is compromised by weak non-specific effects on many biological targets. Replacement of the stilbene ethylenic bridge of resveratrol with a 1,2,4-thiadiazole heterocycle and modification of the substituents on the two aromatic rings afforded potential chemopreventive agents with enhanced potencies and selectivities when evaluated as inhibitors of aromatase and NF- κ B and inducers of quinone reductase 1 (QR1).

Keywords

Thiadiazole; Cancer Chemoprevention; Aromatase; QR1; NF- κ B

1. Introduction

Drugs that prevent disease from developing offer attractive alternatives to those used in treatment. The use of aspirin to prevent coronary heart diseases is a familiar example.¹ Tamoxifene² and fenasteride³ are examples of drugs that have been employed as breast and prostate cancer chemopreventive agents, respectively.

The concept of cancer chemoprevention refers to delaying and/or preventing cancer development.⁴ Cancer chemoprevention can theoretically be achieved by terminating the effect of carcinogens by inhibiting or down-regulating phase I enzymes such as aromatase and inducible nitric oxide synthase (iNOS) that are capable of generating carcinogenic species.^{5,6} Aromatase, the key enzyme in endogenous estrogen production, is an established target in breast cancer chemotherapy.⁷ Since aromatase transcription is mediated by a known cancer-promoting kinase I κ B kinase β (IKK β), its inhibitors are also considered to be chemopreventive agents.⁸ iNOS is involved in the immune response by catalyzing a high level of NO production.⁹ However, excessive and persistent expression of iNOS and production of NO can contribute to pathological conditions including inflammatory diseases

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as well as cancer. In fact, the overexpression of iNOS has been reported in many cancer types.¹⁰ On the other hand, chemoprevention could also be achieved by activating cytoprotective enzymes such as quinone reductase 1 (QR1).¹¹ An additional target for development of cancer chemopreventive agents is NF- κ B, which regulates the expression of anti-apoptotic genes and is up-regulated in cancer cells due to gene mutation or secretion of NF- κ B activating factors.^{12,13} Therefore, down-regulation of NF- κ B hinders cancer progression and cell differentiation via apoptosis activation. Based on our understanding of these various biological pathways, cancer chemoprevention could be achieved by selectively targeting any of the proteins involved.

Grapes have been reported to reduce the risk of certain types of cancer.¹⁴ Grapes contain resveratrol,¹⁵ a naturally occurring trans stilbene with anticancer¹⁶ and cancer chemopreventive potential.^{17,18} However resveratrol lacks potency and specificity, and is converted rapidly into inactive metabolites.^{8,19} There is therefore interest in design and synthesis of resveratrol analogues with enhanced potencies, selectivities, and metabolic stabilities.

Resveratrol target specificity vs. aromatase and quinone reductase 2 (QR2) has been improved by varying the substituents on the two phenyl rings.²⁰ Replacement of the alkene linker with heterocyclic systems is a complementary strategy that has been used to enhance the antitumor efficacy of the cis stilbene derivative combretastatin.²¹ In this report, both strategies have been combined. First, the trans stilbene ethylenic bridge of the resveratrol scaffold was replaced with five-membered heterocycles. Replacement of the trans stilbene ethylenic bridge with a 1,2-diphenyl heterocyclic system would change the geometric configuration of the two peripheral rings to resemble a cis stilbene (Figure 1). To keep the geometry of these two phenyl rings relatively unchanged and close to that of the trans stilbene template, the phenyl rings were attached on the 1 and 3 positions of a five-membered heterocycle (Figure 1). Based on this design, it was hypothesized that 1,3-diaryl five-membered heterocycles would retain the chemopreventive properties of the trans stilbene nucleus. This hypothesis has been tested, a lead compound was obtained, and then a series of analogues with different substituents were prepared. In order to address the chemopreventive target specificity, all of the compounds have been evaluated against a number of enzymes that are involved in chemopreventive pathways such as aromatase, NF- κ B, iNOS, and QR1. Moreover, the direct antiproliferative effects of the compounds were evaluated in the estrogen receptor-positive breast cancer cell line MCF-7 and their antioxidant capacities were evaluated using a DPPH assay.

trans-Resveratrol was used as a reference compound in this study. In addition, its cis and double bond-reduced analogues were also tested to investigate whether the constrained trans geometry of the two aromatic rings on the alkene are necessary for biological activity in each case.

2. Results and Discussion

2.1. Chemistry

Methyl bromocyanoacetate (**1**), prepared by bromination of commercially available methyl cyanoacetate with *N*-bromosuccinimide (NBS), was allowed to react with a set of thioamides **2** in absolute methanol at room temperature (Scheme 1). Non-commercially available thioamides **2ff**, **2gg**, **2hh**, and **2ii** were prepared from the reactions of their corresponding amides with Lawesson's reagent in dry THF.

The formation of the thiadiazole ring was confirmed by NMR and mass spectral data. For example, the ¹³C NMR spectra of all of the 1,2,4-thiadiazoles **3a-ii** showed two distinct

downfield quaternary carbon signals at about δ 182 and 175 ppm corresponding to the thiadiazole-C3 and -C5 carbons, respectively. The non-equivalence of these two carbons eliminates the corresponding 1,3,4-thiadiazole structure.

The diphenols **3jj** and **3kk** were prepared from their methoxy analogues **3g** and **3ii** by treatment with hydrogen bromide in glacial acetic acid at reflux temperature for 8 h (Scheme 2).

Catalytic hydrogenation of the dinitro derivative **3ff** afforded the corresponding diamino derivative **3ll** (Scheme 3).

2.2. Biological Results

A preliminary study was conducted to test the hypothesis that 1,3-diaryl five-membered heterocycles can retain the chemopreventive properties of the stilbene nucleus. The stilbene analogue **3a** with a thiadiazole system at the position of the stilbene double bond was synthesized and tested for its potential chemopreventive activity. Interestingly, compound **3a** showed moderate to weak activity against aromatase and NF- κ B, and a good induction ratio with QR1. Based on these initial results, it could be concluded that the 3,5-diaryl-1,2,4-thiadiazole scaffold retains some of the chemopreventive activities of resveratrol.

Next, to increase the selectivity towards aromatase, a structure-based drug design approach was conducted using the available enzyme crystal structure.²² The existing SAR data of the stilbene series on aromatase could not be directly applied here because the two peripheral rings of the stilbene and 3,5-disubstituted-1,2,4-thiadiazole systems do not overlap precisely, and the bridge provided by the heterocyclic system is slightly longer than an ethylenic bridge.

Compound **3a** was docked into the active pocket of aromatase (PDB ID 3eqm) using GOLD software.²³ The initial expectation was that the mild activity of the lead compound **3a** could be due to the interaction between the thiazole and the heme iron. However, none of the top ten poses showed such an interaction. Instead, the hypothetical binding mode of the lead compound **3a** with aromatase (Figure 2) indicated that one of the phenyl rings lies over the heme and the other one is surrounded by the Met374 and Val373 residues without any interaction with the heme iron.

The model provided a rationale for synthesis of pyridyl analogues in which one of the pyridine rings might coordinate with the heme iron of aromatase. The aromatase active site is known to be large and quite flexible,²⁴ so it was very difficult to predict the best nitrogen positions from our simple computational model. Therefore, all of the possible 2-, 3- and 4-pyridyl derivatives were taken into consideration. Both 3-pyridyl and 4-pyridyl derivatives **3b** and **3c** were synthesized successfully; however, the 2-pyridyl analogue could not be synthesized and it has never been reported. The 3-pyridyl analogue **3b** was found to be greater than 250 times more potent than the lead compound **3a** as an aromatase inhibitor and it displayed an IC_{50} of 0.2 μ M (Table 1). One of the pyridine nitrogens is calculated to coordinate with the heme iron and the other nitrogen is calculated to hydrogen bond with the NH of Met374 (Figure 3). Moving the nitrogen atom to position-4 (compound **3c**) partially suppressed the aromatase inhibitory activity (IC_{50} 0.8 μ M, Table 1). Compounds **3a** and **3b** displayed a high degree of structural specificity, being the only compounds in the series that were aromatase inhibitors with IC_{50} values less than 50 μ M except of the diamino compound **3ll**, which had an IC_{50} of 26.5 μ M. Both **3a** and **3b** had a high degree of selectivity for aromatase vs. the other targets investigated.

Based on these results, it seems that 3,5-dipyridyl-1,2,4-thiadiazoles could serve as a new class of non-steroidal aromatase inhibitors. Non-steroidal aromatase inhibitors in general have superior therapeutic benefits compared with their steroidal counterparts.²⁵

As discussed earlier, the unsubstituted thiadiazole **3a** exhibited some NF- κ B inhibitory activity (IC_{50} 47 μ M) plus a high QR1 induction ratio (IR 8.6). Although the crystal structure of NF- κ B is available,²⁶ a structure-based design approach to optimize **3a** is not possible because the mechanism of inhibition is not known. Similarly, the mechanism of QR1 induction by **3a** is yet to be determined. Therefore, a large set of mono- and disubstituted thiadiazoles was prepared and both QR1 and NF- κ B assays were conducted in order to eventually elucidate more extensive structure-activity-relationships (SAR). The results are summarized in Table 2. In addition, the same set of compounds was tested in a number of other indicative chemoprevention assays including DPPH free radical quenching, inhibition of NO production, and antiproliferative activity in the MCF-7 breast cancer cell line to test for activity and selectivity. The results are listed in Table 3.

QR1 plays an important role in carcinogen deactivation.²⁷ The thiadiazoles reported here have proven to be very effective QR1 inducers, with 23 of the tested compounds increasing the QR1 activity by a factor of two or more (Table 2). The top thiadiazole QR1 inducers include the unsubstituted thiadiazole **3a** and the 2-halogen-substituted thiadiazoles such as **3h** and **3i**. The most potent QR1 inducer is the 2-fluoro derivative **3o** (Table 2), which displayed a QR1 induction ratio of 10.5. The more bulky ortho-substituted derivatives such as the iodo and trifluoro derivatives **3t** and **3cc** had lower activity. The size and the electronic properties of the meta substituents also have a large impact on the QR1 induction ratio. The smaller and the more electronegative the substituent, the higher the IR, as could be observed for the 3-fluoro derivative **3n** (IR = 8.4). As the size of the meta substituents became larger and the electronegativity decreased, the IR decreased too, as seen in the case of the 3-bromo **3p** and 3-iodo **3m** analogues with IR values of 7.3 and 4.4, respectively. Thiadiazoles with more polar and larger meta substituents such as the 3-nitrile analogue **3hh** had much lower induction ratios. Replacement of the halide in the meta position with methyl or methoxy led to lower IR values as seen in the 3-tolyl and 3-methoxy analogues **3s** and **3ii**. The para-substituted diphenyl thiadiazoles had weak or moderate IR values regardless the nature of the substituents as seen in the cases of **3d-g**, **3j-l**, **3z**, **3ff** and **3gg**. Furthermore, adding a para substituent to the ortho-substituted active thiadiazoles such as **3h** and **3i** afforded **3dd** and **3ee** and the IR values dropped from 7.0 and 7.4 to 1.5 and 4.5, respectively. In terms of selectivity, the most potent QR1 inducer **3o** has high QR1 selectivity since it has very low activities when tested vs. the other chemopreventive targets.

Among the substituted **3a** derivatives, the hydrophobic thiadiazoles with meta-substituted phenyl rings exhibited potent NF- κ B inhibitory activity in the sub-micromolar range (Table 2). The 3-methylphenyl thiadiazole derivative **3s** (IC_{50} 0.8 μ M) was found to be slightly more potent than *trans*-resveratrol, while its methoxy analogues **3ii** (IC_{50} 0.4 μ M) is a more potent NF- κ B inhibitor. Both **3s** and **3ii** retained some QR1 induction activity (IRs 4.8 and 6.3, respectively). The more polar 3-hydroxy derivative **3kk** (IC_{50} 38 μ M) is less potent than its methoxy analogue **3ii**; however, it is still more active than the unsubstituted analogue **3a**. Unlike the meta-methyl or -methoxy derivatives, their para analogues **3g** and **3x** are weakly active. Finally, the para amino and hydroxyl derivatives showed moderate NF- κ B IC_{50} values, i.e. **3jj** (IC_{50} 19 μ M) and **3ll** (IC_{50} 7.6 μ M).

Free radicals can cause oxidative damage to DNA, proteins, carbohydrates and lipids, with high chemical reactivity, and consequently lead to various diseases including cancer. To evaluate the antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging was performed according to the method of Lee et al. as described in the

experimental section. In general, all of the compounds had weak or no antioxidant properties. The only compound that showed some antioxidant activity was the 4-amino derivative **3ii** (Table 3).

In normal physiology, a large amount of NO is produced by iNOS as an inflammatory mediator in host the immune system. However, NO, as one of reactive nitrogen species, can damage DNA and form nitrative (8-nitroguanine) or oxidative DNA (8-oxodG) species that eventually result in carcinogenesis.²⁸ In view of this, the inhibitory activities of the compounds on NO production were evaluated using the RAW 264.7 cell line-based assay to determine anti-inflammatory and cancer chemopreventive capacity. As a result, **3jj** and **3ii**, which have hydroxyl or amino groups in the para-position, showed better inhibition than the reference compound (*trans*-resveratrol) with IC₅₀ values of 13.5 and 23.3 μ M, respectively. It is noteworthy that these are the only compounds that have hydrogen-bond donor moieties in the para position, suggesting that a hydrogen bond in this position might play an important role in inhibition of NO production.

Lastly, the antiproliferative potentials of the newly synthesized thiadiazoles and the reference compounds were tested with cultured MCF-7 breast cancer cells and the results are summarized in Table 3. Except for the moderately cytotoxic 4-hydroxy derivative **3jj** (IC₅₀ 4.7 μ M), all of the compounds had weak antiproliferative activities.

3. Conclusion

The attempted optimization of the relatively weak activities of resveratrol against its many biological targets led to the 3,5-diaryl-1,2,4-thiadiazole scaffold, which provided a means to increase both potency and selectivity in aromatase and NF- κ B inhibition assays, as well as in a QR1 induction assay. The pyridyl derivatives **3b** and **3c** had 30-125 times higher aromatase inhibitory activity (IC₅₀ values of 0.2 and 0.8 μ M, respectively) than *trans*-resveratrol, in addition to the advantage of the higher degree of target selectivity. Ortho-halo substituted derivatives **3h**, **3i**, and **3o** had very high QR1 induction ratios (IR values 7.0, 7.4, and 10.5, respectively) with a very high degree of selectivity. It is noteworthy to mention that these IR values ranked among or even above that of the best known QR1 inducers. Finally, the meta-methyl or methoxy substituted derivatives **3s** and **3ii** showed equivalent or one fold more NF- κ B inhibitory activity (IC₅₀ values 0.8 and 0.4 μ M, respectively) than the *trans*-resveratrol. However, both **3s** and **3ii** are more selective than *trans*-resveratrol towards NF- κ B, they still have some QR1 induction properties.

4. Experimental Section

4.1. General

All biologically tested compounds had purity $\geq$ 95% as indicated by HPLC. ¹H NMR spectra were run at 300 MHz and ¹³C NMR spectra were run at 75.46 MHz in deuterated chloroform (CDCl₃) or dimethyl sulfoxide (DMSO-*d*₆). Chemical shifts are given in parts per million (ppm) on the delta (δ) scale. Chemical shifts are related to that of the solvent. Mass spectra were recorded at 70 eV. Melting points were determined using capillary tubes with a Mel-Temp apparatus and are uncorrected. HPLC analyses were performed using a 5 μ M C-18 reverse phase column; the default setting was used (λ 254).

4.2. Preparation of Thioamides 2. General Procedure

Amides (1 mmol) and Lawesson's reagent (490 mg, 1.2 mmol) were added to dry THF (15 mL). The reaction mixture was stirred at room temperature for 1 h, or heated under reflux for 5 h in the case of the 4-nitro derivative. The solvent was evaporated under reduced pressure and the residue was partitioned between aq NaHCO₃ (25 mL) and ethyl acetate (25

mL). The organic solvent was separated and dried over anhydrous MgSO_4 . The crude product was further purified by silica gel flash chromatography, using hexane-ethyl acetate (4:1), to yield the corresponding thioamides as yellow solids (42-60%). 4-Nitrothioamide (**2ff**),²⁹ 4-cyanothiobenzamide (**2gg**)³⁰ and 3-cyanothiobenzamide (**2hh**),³¹ 3-methoxythiobenzamide (**2ii**)³² have been previously reported.

4.3. Preparation of 1,2,4-Thiadiazole Derivatives 3a-ii

Thioamide (1 mmol) was added to a solution of methyl bromocyanoacetate (**1**, 215 mg, 1.2 mmol) in absolute methanol (5 mL) at room temperature. The reaction mixture was stirred for 15 sec. to 1 min. The precipitate was collected by filtration, washed with methanol-water (5 mL), and dried. In the case of oily compound **3z**, it was purified by silica gel chromatography using hexane-ethyl acetate (9:1). Compounds **3a**,^{33,34} **3b**,^{35,36} **3e**,³⁷ **3f**,³⁸ **3h**,³⁹ **3d**,⁴⁰ **3s**,⁴¹ **3c**,⁴² **3ff**,⁴³ **3jj**,⁴⁴ **3kk**⁴⁵ have previously been reported.

4.3.1. 3,5-Bis(2-bromophenyl)-1,2,4-thiadiazole (3h)—White solid (99%): mp 98 °C. ^1H NMR (CDCl_3) δ 8.51 (d, J = 7.8 Hz, 1 H), 7.96 (t, J = 5.1 Hz, 2 H), 7.85 (d, J = 7.5 Hz, 1 H), 7.56 (m, 4 H); ^{13}C NMR (CDCl_3) δ 187.01, 172.78, 138.14, 136.55, 131.12, 129.60, 128.95, 128.66; ESIMS (m/z , rel intensity) 399/397/395 (MH^+ , 12/60/100); HRMS (ESI), m/z MH^+ 394.8858, calcd for $\text{C}_{14}\text{H}_8\text{Br}_2\text{N}_2\text{S}$ 394.8853; HPLC purity 99.61% (MeOH- H_2O , 95:5).

4.3.2. 3,5-Bis(4-(trifluoromethyl)phenyl)-1,2,4-thiadiazole (3j)—White solid (99%): mp 81 °C. ^1H NMR (CDCl_3) δ 8.49 (d, J = 5.1 Hz, 2 H), 8.16 (d, J = 5.1 Hz, 2 H), 7.80 (d, J = 5.1 Hz, 2 H), 7.76 (d, J = 5.1 Hz, 2 H); ^{13}C NMR (CDCl_3) δ 186.85, 172.54, 135.42, 133.30, 132.80, 128.55, 127.72, 126.30, 125.65, 125.63; CIMS (m/z , rel intensity) 374 (MH^+), 355 ($\text{MH}^+ - \text{HF}$, 100); HRMS (ESI), m/z MH^+ 374.0316, calcd for $\text{C}_{10}\text{H}_8\text{F}_6\text{N}_2\text{S}$ 374.0312; HPLC purity 98.30% (MeOH- H_2O , 95:5).

4.3.3. 3,5-Bis(4-(tert-butyl)phenyl)-1,2,4-thiadiazole (3k)—White solid (99%): mp 91-92 °C. ^1H NMR (CDCl_3) δ 8.35 (d, J = 5.1 Hz, 2 H), 7.98 (d, J = 5.1 Hz, 2 H), 7.53 (m, 4 H), 1.38 (s, 9 H); ^{13}C NMR (CDCl_3) δ 187.73, 174.15, 155.37, 153.57, 130.68, 128.00, 127.20, 126.10, 125.40, 36.01, 31.15; ESIMS (m/z , rel intensity) 351 (MH^+ , 100); HRMS (ESI), m/z MH^+ 351.1892, calcd for $\text{C}_{22}\text{H}_{26}\text{N}_2\text{S}$ 351.1895; HPLC purity 96.26% (MeOH- H_2O , 95:5).

4.3.4. 3,5-Bis(4-iodophenyl)-1,2,4-thiadiazole (3l)—Pale yellow solid (100%): mp 224-226 °C. ^1H NMR (CDCl_3) δ 8.10 (d, J = 5.4 Hz, 2 H), 7.88 (d, J = 5.2 Hz, 2 H), 7.86 (d, J = 5.4 Hz, 2 H), 7.76 (d, J = 5.2 Hz, 2 H); ^{13}C NMR (CDCl_3) δ 187.30, 173.04, 138.50, 137.89, 136.75, 132.08, 129.89, 128.76, 98.77, 97.21; CIMS m/z (rel intensity) 491 (MH^+ , 52); HRMS (ESI), m/z 489.8489 MH^+ , calcd for $\text{C}_{14}\text{H}_8\text{I}_2\text{N}_2\text{S}$ 489.4898; HPLC purity 96.8% (MeOH- H_2O , 95:5).

4.3.5. 3,5-Bis(3-iodophenyl)-1,2,4-thiadiazole (3m)—White solid (100%): mp 102-103 °C. ^1H NMR (CDCl_3) δ 8.74 (dd, J = 1.6, 1.8 Hz, 1 H), 8.41 (dd, J = 1.6, 1.6 Hz, 1 H), 8.36 (dd, J = 7.8, 1.6 Hz, 1 H), 7.98 (dd, J = 7.8, 2.1 Hz, 1 H), 7.90 (dd, J = 7.8, 1.2 Hz, 1 H), 7.83 (dd, J = 7.8, 1.2 Hz, 1 H), 7.26 (m, 2 H); ^{13}C NMR (CDCl_3) δ 186.59, 172.14, 140.81, 139.33, 137.16, 135.94, 134.38, 132.18, 130.84, 130.38, 127.43, 126.71, 94.81, 94.37; CIMS m/z (rel intensity) 491 (MH^+ , 52); HRMS (ESI), m/z 489.8501 MH^+ , calcd for $\text{C}_{14}\text{H}_8\text{I}_2\text{N}_2\text{S}$ 489.4898; HPLC purity 97.20% (MeOH- H_2O , 95:5).

4.3.6. 3,5-Bis(3-fluorophenyl)-1,2,4-thiadiazole (3n)—White solid (100%): mp 112-113 °C. ^1H NMR (CDCl_3) δ 8.15 (d, J = 7.8 Hz, 1 H), 8.08 (dd, J = 9, 0.9 Hz, 1 H),

7.77 (s, 1 H), 7.76 (d, $J = 1.8$ Hz, 1 H), 7.50-7.45 (m, 2 H), 7.26-7.17 (m, 2 H); ^{13}C NMR (CDCl_3) δ 186.87, 172.52, 163.92, 161.95, 134.55, 132.26, 130.97, 130.26, 123.90, 123.27, 118.95, 117.42, 115.29, 114.22; CIMS m/z (rel intensity) 275 (MH^+ , 49); HRMS (ESI), m/z 274.0374 M^+ , calcd for $\text{C}_{14}\text{H}_8\text{F}_2\text{N}_2\text{S}$ 274.0376; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.7. 3,5-Bis(2-fluorophenyl)-1,2,4-thiadiazole (3o)—White solid (100%): mp 96-97 °C. ^1H NMR (CDCl_3) δ 8.47 (dt, $J = 0.5, 7.5$, 1 Hz, 1 H), 8.33 (dt, $J = 0.5, 7.5$, 1 Hz, 1 H), 7.56 (dt, $J = 1.5, 8, 5.5$ Hz, 1 H), 7.49 (dt, $J = 1.5, 8, 5.5$ Hz, 1 H), 7.37 (t, $J = 8$ Hz, 1 H), 7.31 (m, 3 H); ^{13}C NMR (CDCl_3) δ 182.03, 172.89, 163.92, 133.21, 133.14, 131.82, 131.74, 128.73, 124.97, 124.14, 116.87, 116.70, 116.02, 115.85; CIMS m/z (rel intensity) 275 (MH^+ , 45); HRMS (ESI), m/z 274.0378 M^+ , calcd for $\text{C}_{14}\text{H}_8\text{F}_2\text{N}_2\text{S}$ 274.0376; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.8. 3,5-Bis(3-bromophenyl)-1,2,4-thiadiazole (3p)—White solid (100%): mp 114-115 °C. ^1H NMR (CDCl_3) δ 8.53 (t, $J = 1.5$ Hz, 1 H), 8.29 (d, $J = 7.8$ Hz, 1 H), 8.21 (t, $J = 1.8$ Hz, 1 H), 7.93 (dt, $J = 8.7, 1.6$ Hz, 1 H), 7.68 (dt, $J = 7.8, 1.8$, 1 Hz, 1 H), 7.60 (dt, $J = 8.7, 1.8$, 1 Hz, 1 H), 7.40 (d, $J = 7.5$ Hz, 1 H), 7.35 (d, $J = 7.8$ Hz, 1 H); ^{13}C NMR (CDCl_3) δ 186.26, 172.28, 134.88, 134.34, 133.40, 132.13, 131.33, 130.78, 130.26, 130.14, 126.81, 126.10, 123.38, 122.81; CIMS m/z (rel intensity) 399/ 397/ 395 (MH^+ , 45/100/49); HRMS (ESI), m/z 393.8775 MH^+ , calcd for $\text{C}_{14}\text{H}_8\text{Br}_2\text{N}_2\text{S}$ 393.8775; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.9. 3,5-Bis(2,3-dichlorophenyl)-1,2,4-thiadiazole (3q)—White solid (100%): mp 165-166 °C. ^1H NMR (CDCl_3) δ 8.53 (dd, $J = 8.1, 1.2$ Hz, 1 H), 7.86 (dd, $J = 7.6, 1.3$ Hz, 1 H), 7.66 (dd, $J = 8.1, 1.3$ Hz, 1 H), 7.60 (dd, $J = 7.8, 1.3$ Hz, 1 H), 7.43 (t, $J = 8.1$ Hz, 1 H), 7.34 (t, $J = 7.5$ Hz, 1 H); ^{13}C NMR (CDCl_3) δ 182.92, 169.34, 134.42, 134.29, 134.09, 132.80, 132.05, 131.70, 131.42, 130.32, 128.90, 127.99, 127.20; CIMS m/z (rel intensity) 379/377/375 (MH^+ , 43/100/78); HRMS (EI), m/z 373.9009 M^+ , calcd for $\text{C}_{14}\text{H}_6\text{Cl}_4\text{N}_2\text{S}$ 373.9006; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.10. 3,5-Bis(4-chloro-2-methylphenyl)-1,2,4-thiadiazole (3r)—White solid (100%): mp 73-74 °C. ^1H NMR (CDCl_3) δ 8.12 (d, $J = 8.4$ Hz, 1 H), 8.00 (d, $J = 8.4, 1.3$ Hz, 1 H), 7.36-7.26 (m, 4 H), 2.71 (s, 3 H), 2.65 (s, 3 H); ^{13}C NMR (CDCl_3) δ 185.28, 172.58, 139.81, 138.72, 136.97, 135.44, 132.25, 131.59, 131.27, 130.92, 130.42, 128.43, 126.69, 126.00, 22.11, 21.84; CIMS m/z (rel intensity) 337/ 335 (MH^+ , 16/51); HRMS (EI), m/z 334.0092 M^+ , calcd for $\text{C}_{16}\text{H}_{12}\text{Cl}_2\text{N}_2\text{S}$ 334.0098; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.11. 3,5-Bis[2-(trifluoromethyl)phenyl]-1,2,4-thiadiazole (3t)—White solid (10%): mp > 260 °C. ^1H NMR (CDCl_3) δ 7.94-7.79 (m, 4 H), 7.73-7.59 (m, 4 H); ^{13}C NMR (CDCl_3) δ 184.88, 171.18, 132.07, 131.94, 131.65, 130.92, 129.85, 129.39, 129.21, 128.96, 128.44, 126.91, 126.85, 126.77, 125.49, 121.87; CIMS m/z (rel intensity) 375 (MH^+ , 13); HRMS (EI), m/z 374.0307 M^+ , calcd for $\text{C}_{16}\text{H}_8\text{F}_6\text{N}_2\text{S}$ 374.0312; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.12. 3,5-Bis(2,5-dichlorophenyl)-1,2,4-thiadiazole (3u)—Off-white solid (100%): mp 116-117 °C. ^1H NMR (CDCl_3) δ 8.61 (d, $J = 2.7$ Hz, 1 H), 8.08 (d, $J = 2.7$ Hz, 1 H), 7.54-7.37 (m, 4 H); ^{13}C NMR (CDCl_3) δ 182.04, 168.53, 133.80, 132.74, 132.11, 132.03, 131.94, 131.53, 130.84, 130.54, 130.06; APCIMS m/z (rel intensity) 379/377/375 (MH^+ , 52/100/64); HRMS (ESI), m/z 374.9082 MH^+ , calcd for $\text{C}_{14}\text{H}_7\text{Cl}_2\text{N}_2\text{S}$ 374.9079; HPLC purity 96.8% (MeOH- H_2O , 95:5).

4.3.13. 3,5-Bis(3,4-dichlorophenyl)-1,2,4-thiadiazole (3v)—Off-white solid (100%): mp 127–128 °C. ^1H NMR (CDCl_3) δ 8.44 (d, J = 1.8 Hz, 1 H), 8.18 (dd, J = 1.8, 8.1 Hz, 1 H), 8.14 (d, J = 2 Hz, 1 H), 7.82 (dd, J = 2, 8.1 Hz, 1 H), 7.56 (dd, J = 1.7, 8.1 Hz, 1 H); ^{13}C NMR (CDCl_3) δ 186.01, 171.68, 136.44, 134.82, 133.90, 133.07, 132.19, 131.36, 130.78, 130.19, 129.97, 128.97, 127.34, 126.47; CIMS m/z (rel intensity) 379/377/375 (MH^+ , 58/100/75); HRMS (CI), m/z 373.8999 M^+ , calcd for $\text{C}_{14}\text{H}_6\text{Cl}_4\text{N}_2\text{S}$ 373.9006; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.14. 3,5-Bis(3-chloro-2-methylphenyl)-1,2,4-thiadiazole (3w)—Off-white solid (100%): mp 82–83 °C. ^1H NMR (CDCl_3) δ 7.90 (d, J = 6 Hz, 1 H), 7.88 (d, J = 6 Hz, 1 H), 7.54 (d, J = 6 Hz, 1 H), 7.48 (d, J = 6 Hz, 1 H), 7.28 (q, J = 6, 8.7 Hz, 2 H), 2.697 (s, 3 H), 2.691 (s, 3 H); ^{13}C NMR (CDCl_3) δ 186.53, 171.91, 136.41, 135.77, 134.36, 131.88, 130.74, 129.50, 128.65, 127.07, 126.55, 18.01, 17.87; CIMS m/z (rel intensity) 337/335 (MH^+ , 71/100); HRMS (EI), m/z 334.0092 M^+ , calcd for $\text{C}_{16}\text{H}_{12}\text{Cl}_2\text{N}_2\text{S}$ 334.0098; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.15. 3,5-Di-*p*-tolyl-1,2,4-thiadiazole (3x)—White solid (100%): mp 124–125 °C. ^1H NMR (CDCl_3) δ 8.30 (d, J = 8.4 Hz, 2 H), 7.92 (d, J = 8.1 Hz, 2 H), 7.31 (d, J = 8.4 Hz, 2 H), 7.29 (d, J = 8.1 Hz, 2 H), 2.43 (s, 3 H), 2.41 (s, 3 H); ^{13}C NMR (CDCl_3) δ 187.89, 173.73, 142.39, 140.41, 130.34, 129.85, 129.36, 128.27, 128.06, 127.36, 21.62, 21.52; ESIMS m/z (rel intensity) 267 (MH^+ , 100); HRMS (ESI), m/z 267.0960 MH^+ , calcd for $\text{C}_{16}\text{H}_{15}\text{N}_2\text{S}$ 267.2950; HPLC purity 99.62% (MeOH- H_2O , 95:5).

4.3.16. 3,5-Di(naphthalen-2-yl)-1,2,4-thiadiazole (3y)—White solid (100%): mp 120–121 °C. ^1H NMR (CDCl_3) δ 8.98 (s, 1 H), 8.61 (s, 1 H), 8.50 (dd, J = 1.5, 7.5 Hz, 1 H), 8.12 (dd, J = 1.5, 7.5 Hz, 1 H), 8.02–7.89 (m, 6 H), 7.61–7.54 (m, 4 H); ^{13}C NMR (CDCl_3) δ 188.15, 173.90, 134.96, 134.30, 133.24, 133.06, 130.23, 129.18, 128.97, 128.61, 128.43, 127.94, 127.78, 127.65, 127.10, 126.44, 125.18, 124.19; CIMS m/z (rel intensity) 339 (MH^+ , 100); HRMS (CI), m/z 338.0876 M^+ , calcd for $\text{C}_{22}\text{H}_{14}\text{N}_2\text{S}$ 338.0878; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.17. 3,5-Bis(4-butylphenyl)-1,2,4-thiadiazole (3z)—Colorless viscous oil (96%). ^1H NMR (CDCl_3) δ 8.29 (d, J = 8.0 Hz, 2 H), 7.95 (d, J = 8.0 Hz, 2 H), 7.32 (d, J = 8.0 Hz, 2 H), 7.31 (d, J = 8.0 Hz, 2 H), 2.69 (t, J = 7.5 Hz, 4 H), 1.66 (q, J = 7.5 Hz, 4 H), 1.63 (q, J = 7.5 Hz, 4 H), 0.96 (t, J = 7.5 Hz, 6 H); ^{13}C NMR (CDCl_3) δ 187.91, 173.78, 147.38, 145.40, 130.49, 129.23, 128.70, 128.24, 127.40, 35.65, 35.58, 33.40, 33.29, 29.66, 22.28, 13.90; ESIMS m/z (rel intensity) 373 (MNa^+ , 100), 351 (MH^+ , 98); HRMS (ESI), m/z 351.1901 MH^+ , calcd for $\text{C}_{12}\text{H}_{27}\text{N}_2\text{S}$ 351.1889; HPLC purity 96.59% (MeOH- H_2O , 95:5).

4.3.18. 3,5-Bis(5-chloro-2-methylphenyl)-1,2,4-thiadiazole (3aa)—Off-white solid (100%): mp 80–81 °C. ^1H NMR (CDCl_3) δ 8.18 (d, J = 0.9 Hz, 1 H), 8.10 (d, J = 0.9 Hz, 1 H), 7.38 (dd, J = 2.1, 7.8 Hz, 1 H), 7.29 (dd, J = 0.9, 7.8 Hz, 1 H), 7.26 (dd, J = 0.9, 7.5 Hz, 1 H), 2.69 (s, 3 H), 2.64 (s, 3 H); ^{13}C NMR (CDCl_3) δ 185.08, 172.31, 136.42, 135.38, 133.27, 133.06, 132.83, 132.30, 131.53, 131.33, 130.94, 130.69, 129.70, 129.23, 21.68, 21.52; CIMS m/z (rel intensity) 337/335 (MH^+ , 66/100); HRMS (CI), m/z 334.0096 M^+ , calcd for $\text{C}_{16}\text{H}_{12}\text{Cl}_2\text{N}_2\text{S}$ 334.0098; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.19. 3,5-Bis(4-bromo-2-methylphenyl)-1,2,4-thiadiazole (3bb)—Off-white solid (100%): mp 74 °C. ^1H NMR (CDCl_3) δ 8.17 (d, J = 2.1 Hz, 1 H), 8.10 (d, J = 2.1 Hz, 1 H), 7.40 (dd, J = 2.1, 7.5 Hz, 1 H), 7.33–7.25 (m, 4 H), 2.69 (s, 3 H), 2.65 (s, 3 H); ^{13}C NMR (CDCl_3) δ 185.07, 172.29, 136.36, 135.34, 133.24, 133.01, 132.78, 132.25, 131.48, 131.29, 130.90, 130.64, 129.66, 129.20, 21.60, 21.46; CIMS m/z (rel intensity) 427/425/423 (MH^+ ,

18/100/53); HRMS (CI), m/z 421.9090 M^+ , calcd for $C_{16}H_{12}Br_2N_2S$ 421.9088; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.20. 3,5-Bis(2-iodophenyl)-1,2,4-thiadiazole (3cc)—Yellowish-white solid (100%): mp 112 °C. 1H NMR ($CDCl_3$) δ 8.34 (d, J = 6.9 Hz, 1 H), 8.18 (d, J = 7.8 Hz, 1 H), 8.08 (d, J = 7.8 Hz, 1 H), 7.84 (d, J = 6.9 Hz, 1 H), 7.63 (t, J = 7.8 Hz, 1 H), 7.56 (d, J = 7.2 Hz, 1 H), 7.34 (dt, J = 2.4, 7.5 Hz, 1 H), 7.28 (dt, J = 2.4, 7.8 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 186.69, 172.17, 141.17, 140.74, 137.57, 135.78, 132.07, 131.79, 131.51, 130.92, 128.55, 128.10, 97.19, 95.59; CIMS m/z (rel intensity) 491 (MH^+ , 100); HRMS (CI), m/z 489.8501 M^+ , calcd for $C_{14}H_8I_2N_2S$ 489.8498; HPLC purity 100% (MeOH- H_2O , 95:5).

4.3.21. 3,5-Bis(2-bromo-4-methylphenyl)-1,2,4-thiadiazole (3dd)—Off-white solid (100%): mp 73 °C. 1H NMR ($CDCl_3$) δ 8.49 (d, J = 8.4 Hz, 1 H), 7.86 (d, J = 8.4 Hz, 1 H), 7.58 (s, 2 H), 7.29 (d, J = 8.4 Hz, 1 H), 7.25 (d, J = 8.5 Hz, 1 H), 2.41 (s, 3 H), 2.40 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 184.33, 170.50, 143.14, 141.35, 134.55, 134.27, 131.97, 131.14, 128.99, 128.84, 128.15, 123.25, 121.77, 21.05, 20.96; CIMS m/z (rel intensity) 427/425/423 (MH^+ , 18/100/51); HRMS (CI), m/z 421.9090 M^+ , calcd for $C_{16}H_{12}Br_2N_2S$ 421.9087; HPLC purity 95.01% (MeOH- H_2O , 95:5).

4.3.22. 3,5-Bis(2,4-dichlorophenyl)-1,2,4-thiadiazole (3ee)—White solid (100%): mp 80–81 °C. 1H NMR ($CDCl_3$) δ 8.56 (d, J = 5.7 Hz, 1 H), 8.03 (d, J = 8.4 Hz, 1 H), 7.61 (d, J = 2.1 Hz, 1 H), 7.58 (d, J = 2.1 Hz, 1 H), 7.46 (dd, J = 2.1, 8.4 Hz, 1 H), 7.39 (dd, J = 2.1, 8.4 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 185.47, 168.82, 137.91, 136.27, 134.33, 134.05, 133.07, 131.36, 130.77, 130.20, 128.06, 127.17; APCIMS m/z (rel intensity) 381/379/377/375 (MH^+ , 12/41/100/66); HRMS (CI), m/z 374.9082 MH^+ , calcd for $C_{14}H_7Cl_4N_2S$ 374.9084; HPLC purity 98.28% (MeOH- H_2O , 95:5).

4.3.23. 4,4'-(1,2,4-Thiadiazole-3,5-diyl)dibenzonitrile (3gg)—White solid (100%): mp > 260 °C. IR (KBr) 3096, 2923, 2230, 1607, 1466 cm^{-1} ; 1H NMR ($CDCl_3$) δ 8.50 (d, J = 8.7 Hz, 2 H), 8.17 (d, J = 8.5 Hz, 2 H), 7.86 (d, J = 8.5 Hz, 2 H), 7.82 (d, J = 8.7 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 186.61, 172.27, 136.01, 133.82, 133.15, 132.61, 128.84, 127.97, 118.41, 117.78, 115.56, 114.05; CIMS m/z (rel intensity) 289 (MH^+ , 100); HRMS (CI), m/z 288.0474 M^+ , calcd for $C_{16}H_8N_4S$ 288.0470; HPLC purity 98.44% (MeOH- H_2O , 95:5).

4.3.24. 3,3'-(1,2,4-Thiadiazole-3,5-diyl)dibenzonitrile (3hh)—White solid (100%): mp 176–177 °C. 1H NMR ($CDCl_3$) δ 8.70 (s, 1 H), 8.62 (d, J = 8.4 Hz, 1 H), 8.37 (s, 1 H), 8.26 (d, J = 8.1 Hz, 1 H), 7.82 (d, J = 8.4 Hz, 1 H), 7.78 (d, J = 8.1 Hz, 1 H), 7.69–7.64 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 186.32, 171.78, 135.11, 133.76, 133.38, 132.30, 132.01, 131.44, 130.77, 130.37, 129.71, 118.27, 117.54, 114.00, 113.15; CIMS m/z (rel intensity) 289 (MH^+ , 100); HRMS (CI), m/z 288.0476 M^+ , calcd for $C_{16}H_8N_4S$ 288.0470; HPLC purity 95.01% (MeOH- H_2O , 95:5).

4.3.25. 3,5-Bis(3-methoxyphenyl)-1,2,4-thiadiazole (3ii)—Pale yellow solid (148 mg, 100%): mp 71 °C. 1H NMR ($CDCl_3$) δ 7.99 (d, J = 7.8 Hz, 1 H), 7.92 (t, J = 2.1, 3.3 Hz, 1 H), 7.55 (m, 2 H), 7.38 (m, 2 H), 7.02 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 187.86, 173.44, 160.04, 159.79, 134.04, 131.69, 130.26, 129.69, 120.90, 120.00, 117.84, 116.65, 112.96, 112.05, 55.42, 55.34; CIMS m/z (rel intensity) 299 (MH^+ , 100); HRMS (ESI), m/z 298.0781 M^+ , calcd for $C_{16}H_{14}N_2O_2S$ 298.0776; HPLC purity 97.91% (MeOH- H_2O , 95:5).

4.3.26. 3,5-Bis(4-aminophenyl)-1,2,4-thiadiazole (3ll)—Orange-yellow solid (40%): mp 208–209 °C. 1H NMR (CD_3COCD_3) δ 8.07 (d, J = 8.7 Hz, 2 H), 7.79 (d, J = 8.7 Hz, 2 H), 6.78 (d, J = 8.7 Hz, 2 H), 6.74 (d, J = 8.7 Hz, 2 H), 5.52 (brs, 2 H), 5.15 (brs, 2 H); ^{13}C

NMR (CD₃COCD₃) δ 188.17, 174.30, 153.12, 151.28, 130.15, 129.57, 122.75, 119.72, 114.55, 114.33; CIMS m/z (rel intensity) 269 (MH⁺, 100); HRMS (CI), m/z 268.0789 M⁺, calcd for C₁₄H₁₂N₄S 268.0783; HPLC purity 98.99% (MeOH-H₂O, 80:20).

4.4. Molecular Modeling

Compounds of interest were built with Sybyl 7.1 software and minimized to 0.01 kcal/mol by the Powell method, using Gasteiger-Hückel charges and the Tripos force field. The energy-optimized compounds were docked into the androgen binding pocket of aromatase after removal of the structure of the natural ligand. The parameters were set as the default values for GOLD. The maximum distance between hydrogen bond donors and acceptors for hydrogen bonding was set to 3.5 Å. After docking, the first pose conformations of compounds of interest were merged into the ligand-free protein. The new ligand-protein complex was subsequently subjected to energy minimization using the Amber force field with Amber charges. During the energy minimization, the structure of the compounds of interest and a surrounding 10 Å sphere of the protein were allowed to move. The structure of remaining protein was kept frozen. The energy minimization was performed using the Powell method with a 0.05 kcal/(mol Å) energy gradient convergence criterion and a distance dependent dielectric function.

4.5. Aromatase Assay

Aromatase activity was assayed as previously reported, with the necessary modifications to assay in a 384-well plate.⁴⁶ Briefly, the test compound (3.5 μ L) was preincubated with 30 μ L of NADPH-regenerating system (2.6 mM NADP⁺, 7.6 mM glucose 6-phosphate, 0.8 U/mL glucose-6-phosphate dehydrogenase, 13.9 mM MgCl₂, and 1 mg/mL albumin in 50 mM potassium phosphate buffer, pH 7.4) for 10 min at 37 °C. The enzyme and substrate mixture (33 μ L of 1 μ M CYP19 enzyme, BD Biosciences, 0.4 μ M dibenzylfluorescein, 4 mg/mL albumin in 50 mM potassium phosphate, pH 7.4) was added, and the plate was incubated for 30 min at 37 °C before quenching with 25 μ L of 2 N NaOH. After termination of the reaction and shaking for 5 min, the plate was further incubated for 2 h at 37 °C. This enhances the ratio of signal to background. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission). IC₅₀ values were based on three independent experiments performed in duplicate using five concentrations of test substance. Naringenin (IC₅₀ = 0.23 μ M) was used as a positive control.

Kinetic aromatase assays were carried out in order to determine inhibitor mechanism. Test compound was preincubated with 30 μ L of NADPH regenerating system (2.6 mM NADP⁺, 7.6 mM glucose 6-phosphate, 0.8 U/mL glucose 6-phosphate dehydrogenase, 13.9 mM MgCl₂, and 1 mg/mL albumin in 50 mM potassium phosphate buffer, pH 7.4) for 10 min at 37 °C. The IC₅₀ was used as the final concentration for each inhibitor. Substrate was added at five concentrations: 16, 8, 4, 2, and 1 μ M. CYP19 (1 μ M) was added and fluorescence was measured at 485 nm (excitation) and 530 nm (emission) every 10 s for at least 5 min. Error values represent three independent experiments for each compound.

4.6. Quinone Reductase 1 (QR1) Assay

Hepa 1c1c7 (mouse hepatoma) cells were used in the assay. Cells were incubated in a 96-well plate with test compounds at a maximum concentration of 50 M, digitonin was used to permeabilize cell membranes, and enzyme activity was measured by the reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Production was measured by absorption at 595 nm. A total protein assay using crystal violet staining was run in parallel. 4'-Bromoflavone (CD = 0.01 M) was used as a positive control.

4.7. NF- κ B Luciferase Assay

Studies were performed with NF- κ B reporter stably-transfected human embryonic kidney cells 293 from Panomics (Fremont, CA). This cell line contains chromosomal integration of a luciferase reporter construct regulated by NF- κ B response element. The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light, which is detected with a luminometer. Data were expressed as % inhibition at 50 μ M or IC₅₀ values (i.e., concentration of test sample required to inhibit TNF- α activated NF- κ B activity by 50%). After incubating treated cells, they were lysed in Reporter Lysis buffer. The luciferase assay was performed using the Luc assay system from Promega, following the manufacturer's instructions. In this assay, *N* α -tosyl-L-phenylalanine chloromethyl ketone (TPCK) was used as a positive control; IC₅₀ = 5.09 μ M.

4.8. Nitrite Assay

RAW 264.7 mouse macrophage cells were incubated in a 96-well culture plate for 24 h. The cells were pretreated with various concentrations of compounds dissolved in phenol red-free DMEM for 30 min followed by 1 μ g/mL of LPS treatment for 240 h. The level of nitrite, a stable end product of NO, in the cultured media was measured using a colorimetric reaction with Griess reagent. The optical density (OD) was measured at 540 nm and the level of nitrite was estimated using a standard curve with known concentrations of sodium nitrite. The positive control in this assay was N^G-L-monomethyl arginine (L-NMMA); IC₅₀ = 19.7 μ M. In parallel, the cytotoxic effects of compounds were evaluated by SRB assay

4.9. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Assay

To evaluate antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging was performed according to the method described by Lee *et al.* In this assay,⁴⁷ antioxidants convert the stable free radical DPPH to an inactive hydrazine form, accompanied by color fading from purple to pale yellow. Briefly, 95 μ L of DPPH radical solution in ethanol (316 μ M) was added in a 96-well plate containing 5 μ L of each compound dissolved in 100% DMSO, and the mixture incubated for 30 min at 37 °C. The OD of each well was measured at 515 nm using a microplate reader. The DPPH radical scavenging activity of each sample was evaluated by calculating % inhibition as follows: % inhibition = $(1 - \text{OD}_{\text{sample}} / \text{OD}_{\text{control}}) \times 100$.

4.10. SRB Assay⁴⁸

Serially diluted test compounds in DMSO were transferred to 96-well plates and incubated for 72 h at 37 °C in a CO₂ incubator. The incubation was stopped by adding trichloroacetic acid (10%), which fixes cells. The cells were washed, air-dried, stained with 0.4% SRB solution, and optical densities were determined at 515 nm using a microplate reader. In each case, a zero-day control was performed by adding an equivalent number of cells (1×10^4 cells per well), incubating at 37 °C for 30 min, and processing as described above. Percent of cell survival was calculated using the formula: $(\text{OD}_{\text{tested compound}} - \text{OD}_{\text{zero-day}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{zero-day}}) \times 100$.

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Abbreviations

ARE antioxidant response element

BBO	multinuclear broadband observe
CD	concentration to double QR1 activity
DMEM	Dulbecco's Modified Eagle Medium
DRE	dioxin response element
IC₅₀	sample concentration which causes 50% inhibition
DPPH	2,2-diphenyl-1-picrylhydrazyl
iNOS	inducible nitric oxide synthase
IR	QR1 induction ratio
LPS	lipopolysaccharide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	nuclear factor erythroid 2-related factor 2
QNP	quattro nucleus probe
QR1	quinone reductase 1
TSO	<i>trans</i> -stilbene oxide
SRB	sulforhodamine B

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Figure 1.
Design of 1,3-diaryl five- membered heterocycles from the stilbene template.

Figure 2.

Hypothetical interaction between lead compound **3a**, and Met374, Arg115, Val370 and heme in the human aromatase active site. The heme backbone is colored orange. Many amino acid residues are deleted for the sake of clarity (PDB ID 3eqm). The stereoview is programmed for wall-eyed viewing.

Figure 3. Hypothetical interaction between lead compound **3b** and Met374, Arg115 and heme in the human aromatase active site. Hydrogen bonds are represented by yellow dashed line. Many amino acid residues are deleted for the sake of clarity (PDB ID 3eqm). The stereoview is programmed for wall-eyed viewing.

Figure 4.
Summary of the chemopreventive activities of 3,5-diaryl-1,2,4-thiadiazoles.

***trans*-resveratrol** ***cis*-resveratrol** **dihydroresveratrol**

*Reagents and conditions: (a) methanol, 23 °C, 1 min., 10-100%.

Scheme 1^a

Scheme 2^a

^aReagents and conditions: (a) H_2/Pd , methanol, 23 °C, 24 h, 40%.

Scheme 3^a

Table 1

Inhibition of the Human Aromatase by Thiadiazole **3** Derivatives.

Compound	Aromatase Assay		Compound	Aromatase Assay	
	% Max Inhib ^a	IC ₅₀ ^b (μM)		% Max Inhib ^a	IC ₅₀ ^b (μM)
3a	60.2 ± 2.8	> 50	3v	23.0 ± 0.8	> 50
3b	97.2 ± 1.4	0.2 ± 0.04	3w	19.8 ± 1.2	> 50
3c	98.0 ± 2.2	0.8 ± 0.02	3x	21.3 ± 0.4	> 50
3d	9.7 ± 0.9	> 50	3y	15.8 ± 0.9	> 50
3e	45.2 ± 3.7	> 50	3z	35.5 ± 0.7	> 50
3f	37.4 ± 2.3	> 50	3aa	22.0 ± 1.1	> 50
3g	11.1 ± 1.1	> 50	3bb	47.8 ± 1.6	> 50
3h	44.8 ± 0.8	> 50	3cc	39.4 ± 1.0	> 50
3i	49.7 ± 1.9	> 50	3dd	48.3 ± 0.9	> 50
3j	41.0 ± 1.2	> 50	3ee	40.3 ± 0.4	> 50
3k	43.5 ± 2.1	> 50	3ff	29.4 ± 0.8	> 50
3l	36.7 ± 0.6	> 50	3gg	38.5 ± 1.4	> 50
3m	30.6 ± 1.4	> 50	3hh	22.1 ± 0.6	> 50
3n	41.8 ± 0.7	> 50	3ii	26.6 ± 0.7	> 50
3o	53.2 ± 1.8	> 50	3jj	50.4 ± 1.6	> 50
3p	48.4 ± 1.3	> 50	3kk	24.4 ± 0.8	> 50
3q	45.9 ± 0.5	> 50	3ll	70.4 ± 1.9	26.5 ± 1.4
3r	35.3 ± 0.6	> 50	<i>trans</i> -resveratrol	67.8 ± 2.0	25.0 ± 0.8
3s	32.9 ± 1.1	> 50	<i>cis</i> -resveratrol	44.6 ± 1.7	> 50
3t	21.6 ± 0.7	> 50	dihydroresveratrol	37.8 ± 0.8	> 50
3u	16.6 ± 0.3	> 50			

^aThe inhibition percentage was tested at a concentration of 50 μM of each compound.^bThe IC₅₀ values (μM) are the concentrations that cause 50% inhibition of aromatase.

Table 2Induction of QR1 and Inhibition of NF- κ B

Compd	QR1 Assay		NF- κ B-luciferase	
	IR ^{a,b}	CD ^{c,d} (μ M)	Lucif. act. % inhib ^{b,e}	IC ₅₀ ^f (μ M)
3a	8.63 $\pm$ 0.21	2.1 $\pm$ 0.24	82.4 $\pm$ 5.8	47.4
3b	2.70 $\pm$ 0.09	53.1 $\pm$ 1.3	30.9 $\pm$ 8.6	
3c	1.00 $\pm$ 0.13	> 50	7.2 $\pm$ 7.0	
3d	3.60 $\pm$ 0.18	4.0 $\pm$ 0.36	0.00	
3e	3.00 $\pm$ 0.07	2.07 $\pm$ 0.17	0.00	
3f	2.20 $\pm$ 0.05	16.0 $\pm$ 0.89	0.00	
3g	1.20 $\pm$ 0.20	> 50	21.2 $\pm$ 4.3	
3h	7.00 $\pm$ 0.14	1.73 $\pm$ 0.24	0.00	
3i	7.40 $\pm$ 0.03	1.72 $\pm$ 0.09	25.3 $\pm$ 9.6	
3j	2.00 $\pm$ 0.12	53.3 $\pm$ 1.4	0.00	
3k	3.00 $\pm$ 0.22	27.7 $\pm$ 1.8	0.00	
3l	1.30 $\pm$ 0.08	> 50	0.00	
3m	4.40 $\pm$ 0.04	3.10 $\pm$ 0.42	0.00	
3n	8.40 $\pm$ 0.16	4.90 $\pm$ 0.22	15.4 $\pm$ 12.0	
3o	10.50 $\pm$ 0.25	1.80 $\pm$ 0.36	28.3 $\pm$ 4.1	
3p	7.30 $\pm$ 0.23	0.059 $\pm$ 0.002	0.00	
3q	5.10 $\pm$ 0.18	2.00 $\pm$ 0.14	0.00	
3r	4.90 $\pm$ 0.07	0.44 $\pm$ 0.08	0.00	
3s	4.80 $\pm$ 0.24	4.00 $\pm$ 0.16	84.4 $\pm$ 2.9	0.8
3t	0.70 $\pm$ 0.06	2.10 $\pm$ 0.11	0.00	
3u	4.20 $\pm$ 0.10	18.20 $\pm$ 0.74	40.3 $\pm$ 2.8	
3v	1.50 $\pm$ 0.13	> 50	38.3 $\pm$ 5.3	
3w	2.10 $\pm$ 0.09	4.00 $\pm$ 0.25	20.3 $\pm$ 12.1	
3x	0.80 $\pm$ 0.06	> 50	35.4 $\pm$ 6.1	
3y	1.00 $\pm$ 0.12	16.00 $\pm$ 0.41	48.5 $\pm$ 5.9	
3z	0.80 $\pm$ 0.14	> 50	39.4 $\pm$ 11.2	
3aa	3.80 $\pm$ 0.16	0.470 $\pm$ 0.04	29.7 $\pm$ 5.5	
3bb	2.30 $\pm$ 0.24	0.570 $\pm$ 0.06	6.7 $\pm$ 8.3	
3cc	2.30 $\pm$ 0.17	0.540 $\pm$ 0.03	10.9 $\pm$ 8.3	
3dd	1.50 $\pm$ 0.15	> 50	0.00	
3ee	4.50 $\pm$ 0.21	5.23 $\pm$ 0.22	45.8 $\pm$ 6.6	
3ff	1.30 $\pm$ 0.11	> 50	15.9 $\pm$ 2.8	
3gg	1.80 $\pm$ 0.19	> 50	18.2 $\pm$ 6.9	
3hh	1.60 $\pm$ 0.07	> 50	46.5 $\pm$ 15.3	7.4
3ii	6.30 $\pm$ 0.36	4.03 $\pm$ 0.31	93.9 $\pm$ 1.8	0.4 $\pm$ 0.2
3jj	0.70 $\pm$ 0.13	> 50	78.9 $\pm$ 2.8	19.3 $\pm$ 4.4
3kk	0.1	0.98 $\pm$ 0.07	60.4 $\pm$ 9.3	38.1 $\pm$ 6.7

Compd	QR1 Assay		NF-κB-luciferase	
	IR ^{a,b}	CD ^{c,d} (μM)	Lucif. act. % inhib ^{b,e}	IC ₅₀ ^f (μM)
3II	0.70 ± 0.04	> 50	90.6 ± 8.0	7.6±2.1
trans-resveratrol	2.40 ± 0.12	21.00 ± 0.84	79.0 ± 6.3	0.98±0.2
cis-resveratrol	1.90 ± 0.14	> 50	38.0 ± 8.3	
dihydroresveratrol	1.80 ± 0.21	> 50	16.7± 2.4	

^aIR, induction ratio.

^bTesting concentration, 50 μM.

^cCD is the concentration that doubles the activity.

^dCD values were determined for compounds with induction ratios > 2.

^eNF-κB IC₅₀ calculated when inhibition > 60%.

^fIC₅₀, median inhibitory concentration.

Table 3
Evaluation of antioxidant, antiproliferative, and anti-inflammatory potentials of compounds.

Compounds	DPPH assay		SRB assay (MCF-7)		Nitrite assay	
	% inhibition ^a	IC ₅₀ ^b (μM)	IC ₅₀ ^b (μM)	% inhibition ^c	IC ₅₀ ^b (μM)	
3a	8.26±3.48		24.4±3.3	55.7±6.5		
3b	-16.1±1.1			1.3±2.6		
3c	-35.6±0.4			0.9±3.3		
3d	2.7±6.3			5.1±1.3		
3e	6.9±4.4		18.8±1.7	22.2±2.5		
3f	0.8±4.9			17.1±1.7		
3g	3.1±5.5		17.4±2.1	1.7±4.4		
3h	1.8±7.3		35.0±2.5	9.1±2.2		
3i	2.8±5.3		35.8±4.3	-5.9±5.0		
3j	2.5±5.5			8.5±0.7		
3k	2.9±4.6			0.5±4.4		
3l	16.2±1.4			20.3±3.3		
3m	14.3±0.4		37.1±3.6	15.6±4.7		
3n	1.3±1.4		34.5±2.4	3.2±2.3		
3o	3.8±3.0		31.4±3.0	24.6±1.9		
3p	1.0±0.5		31.0±6.0	22.8±2.3		
3q	11.1±3.1		10.2±2.5	21.4±4.5		
3r	3.7±1.1		39.5±0.07	10.1±3.0		
3s	1.9±1.1		22.1±7.1	0.9±2.2		
3t	2.9±2.9			6.7±2.4		
3u	1.0±1.7		17.8±5.6	17.9±4.2		
3v	9.6±1.4		23.5±3.2	21.5±5.0		
3w	2.3±0.5		30.3±5.7	24.5±0.8		
3x	7.2±2.6			9.6±2.9		
3y	1.1±0.7			0.5±3.7		
3z	2.1±0.2			-3.5±5.6		
3aa	1.3±2.3		29.0±2.8	25.9±5.6		

Compounds	DPPH assay		SRB assay (MCF-7)		Nitrite assay	
	% inhibition ^a	IC ₅₀ ^b (μM)	IC ₅₀ ^b (μM)	% inhibition ^c	IC ₅₀ ^b (μM)	
3bb	2.2±0.1		39.8±0.2	23.3±6.8		
3cc	3.4±1.0			29.0±2.7	30.0±0.1	
3dd	3.4±0.4			25.0±3.3		
3ee	2.9±0.2			25.0±2.5		
3ff	7.3±3.3			1.6±2.9		
3gg	6.0±2.3		31.3±4.1	-0.4±7.2		
3hh	1.4±1.3			26.4±3.5		
3ii	-2.1±3.1		24.2±1.6	17.6±3.1		
3jj	3.4±5.9		4.7±0.7	93.7±0.5	13.50±2.22	
3kk	NT ^d			47.4±13.2		
3ll	33.1±2.4		30.8±3.3	69.6±3.4	23.27±2.42	
<i>trans</i> -resveratrol	65.3±2.3	251.1±7.6		87.6±0.7	30.71±1.96	
<i>cis</i> -resveratrol	33.9±1.8			31.5±3.1		
dihydroresveratrol	14.7±1.3		30.3±3.5	36.2±4.4		

^aThe inhibition percentage was tested at a concentration of 400 μM of each compound.

^bThe IC₅₀ values are the concentrations corresponding to 50% inhibition.

^cThe inhibition percentage was tested at a concentration of 40 μM of each compound.

^dNot tested.