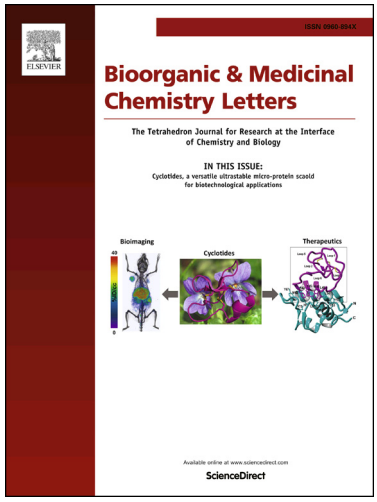

Synthesis and biological evaluation of 4,6-diaryl-2-pyrimidinamine derivatives as anti-breast cancer agents

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Synthesis and biological evaluation of 4,6-diaryl-2-pyrimidinamine derivatives as anti-breast cancer agents

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Same contribution to this paper.

Abstract

Breast cancer is the most frequently diagnosed cancers and the leading causes of cancer death among females worldwide. Estrogen receptor positive has been identified as the predominant internal reasons, involving in more than 70% breast cancer patients and SERMs which competes with estradiol for the binding to ERα in breast tissue are widely used in the treatment of ER+ breast cancer, such as tamoxifen, raloxifene. However, many SERMs may cause negative side effects due to their estrogenic activity in other tissues and approximate 50% of patients with ER-positive tumors either initially do not respond or become resistant to these drugs. Here, a series of designed 4,6-diaryl-2-pyrimidinamine derivatives had been synthesized to treat estrogen receptor positive breast cancer by simultaneously antagonizing ER and inhibiting VEGFR-2. Bioactivity evaluation showed that these compounds could significantly inhibit the proliferation of MCF-7, HUVEC and Ishikawa cells. Further studies identified compound **III-3A** could antagonize against estrogen action and inhibit the phosphorylation of VEGFR-2 as well as inhibit angiogenesis in vivo. The results indicated designed 4,6-diaryl-2-pyrimidinamine derivatives can be used to further study as anti-breast cancer drugs.

Keywords

4,6-diaryl-2-pyrimidinamine derivatives, Anti-breast cancer, SERMs, VEGFR-2

Breast cancer is the most frequently diagnosed cancers and the leading causes of cancer death among females worldwide according to GLOBOCAN 2012.[1] Susceptible population of breast cancer have common characteristics, including advanced age, low parity, delayed age at first delivery, short duration of breastfeeding, overeating, limited exercise and so on. Breast cancer is a heterogeneous disease and multiple subtypes have been defined. The overexpressed of estrogen receptor α (ERα) which is a member of the large superfamily of nuclear receptors has been identified as the predominant internal reasons, involving in more than 70% breast cancer patients, and this type of patients are tagged as ER positive (ER+).[2] In this case, the continuous activation of ERα by estrogens induce the proliferation of tumor cell, so the blocking-up of ER signaling by competitively binding to ER with anti-estrogens or estrogen deprivation is an effective therapeutic strategy [3]. Selective estrogen receptor modulators (SERMs) which compete with estradiol for the binding to ERα in breast tissue are widely used in the treatment of ER+ breast cancer, such as tamoxifen, raloxifene.[4] However, many SERMs may cause negative side effects due to their estrogenic activity in other tissues, such as endometrial cancer.[5] Moreover, up to approximate 50% of patients with ER-positive tumors either initially do not respond or become resistant to these drugs within 5 years of treatment.[6] Thus contribute to a significant obstacle towards ER+ breast

cancer treatment. Hence, there is a great need for the development of new drugs to improve therapeutical effect in the breast cancer treatment.

The significance of the 2-pyrimidinamine scaffold in medicinal chemistry is widely known. Many of these compounds are serving as anticancer agent (Figure 1). Here, we noticed that 4,6-diaryl-2-pyrimidinamine derivatives shows good activity in pharmacological studies.[7-10]. These compounds have similar toluylene likely to tamoxifen (Figure 2), and the distance of two 4-C in phenyl is approximate equal to tamoxifen (9.8Å vs 9.3Å, simulation by Chem3D). Moreover, 2-pyrimidinamine scaffold is the key skeleton of pazopanib and JNJ-17029259[11] which are the vascular endothelial growth factor receptor-2 (VEGFR-2) inhibitors. It's known that VEGFR-2 plays key role in the angiogenesis pathway which participates in the proliferation, metastasis, and invasion of breast cancer cells.[12] And studies identified that VEGFR-2 involve in tamoxifen resistance via the Ras/MAPK pathway.[13,14] The combination of tamoxifen and a low dose of brivanib alaninate, a VEGFR-2 inhibitor, was reported not only to maximize therapeutic efficacy but also to retard SERM resistant tumour growth.[15] Thus urge us to design new molecules which have potential to anti-breast cancer via the inhibition of ER and VEGFR-2 based on 4,6-diaryl-2-pyrimidinamine scaffold (Figure 2). Studies found that the phenolic groups which exist in 4-hydroxytamoxifen and raloxifene, interact to the binding domain of ERα, mimicking to estradiol A-ring.[4] Meanwhile, the side chains, 4-[2-(dimethylamino)ethoxy]phenyl of tamoxifen or 4-[2-(1-piperidinyl)ethoxy]phenyl of raloxifene are critical for anti-estrogenic activity, and this side chain also exist in JNJ-17029259 (the Blue Oval, Figure 2). So according to the combination principle of medicinal chemistry, it's easy to design new structure based on the 4,6-diaryl-2-pyrimidinamine scaffold (Figure 2).

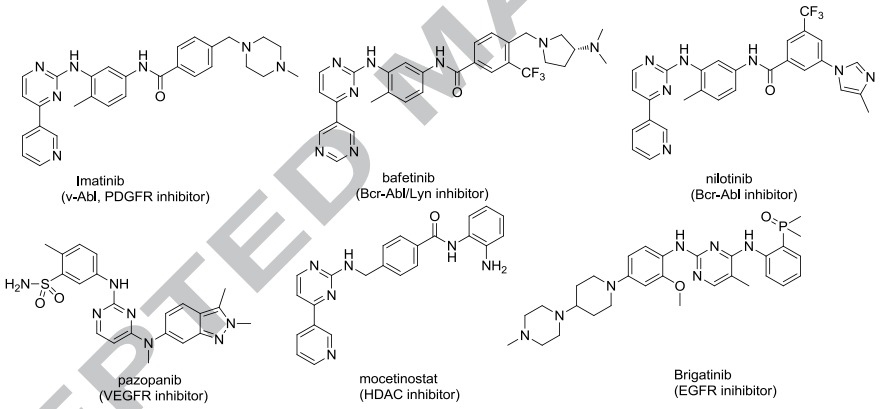


Figure 1 .Anti-cancer drugs with the 2-pyrimidinamine scaffold

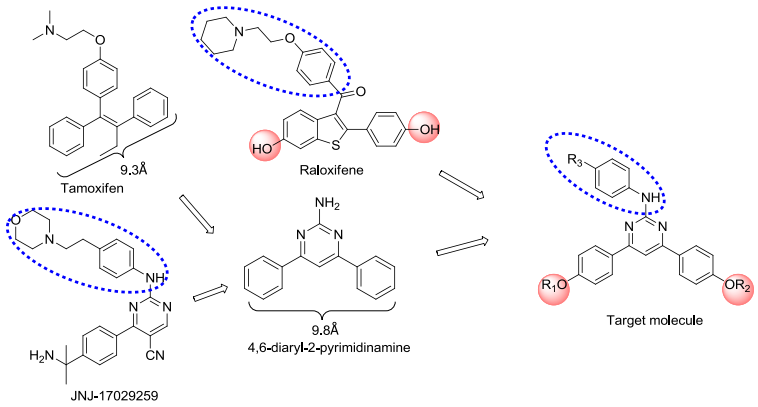
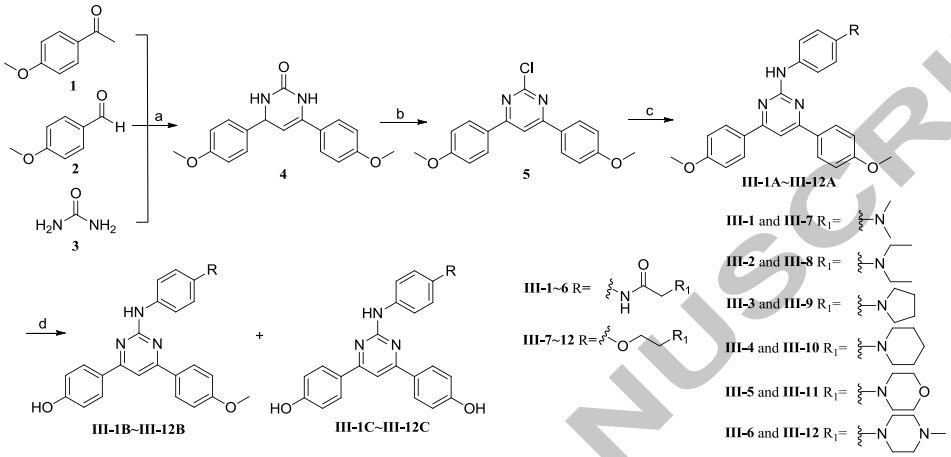


Figure 2 The design of target molecules based on 4,6-diaryl-2-pyrimidinamine scaffold

The synthetic route of target 4,6-diaryl-2-pyrimidinamine derivatives is shown in Scheme 1. The key intermediate, 4,6-bis(4-methoxyphenyl)-3,4-dihydropyrimidin-2(1H)-one (**4**), was prepared from the commercially available urea,

1-(4-methoxyphenyl)-ethanone, and 4-methoxybenzaldehyde according to Biginelli reaction in high yields. Then, intermediate **4** was dehydrogenize and the hydroxy was replaced by chloro under the reflux of phosphorus oxychloride to give 2-chloro-4,6-diaryl-2-pyrimidine (**5**). This pyrimidine reacted a nucleophilic replacement with substitutional aniline to give new compounds **III-1A**~**III-12A**. At last, under the catalysis of BBr_3, the takeoff of one or two methoxy group of compounds **III-1A**~**III-12**A obtained the end-products **III-1B**~**III-12B** and **III-1C**~**III-12C**. Finally, we got three series of novel small compounds. All of the synthesized compounds have been confirmed by NMR and mass spectrometry.



Scheme 1. Reagents and conditions: a) acetonitrile, $FeCl_3$•$6H_2O$, TMSCl, 80°C, 10h; b) $POCl_3$, 110°C, 5h, c) dry dioxane, $Pd(OAc)_2$, Cs_2CO_3, BINAP, 100°C, 8h. d) dry dichloromethane, BBr_3, 0°C /rt, 4h.

To evaluate the anticancer activity, all synthesized compounds were screened against human breast cancer cell line MCF-7, human umbilical vein endothelial cell (HUVEC) as well as human endometrial cancer cell line Ishikawa (Table 1). Human Breast cancer cell line MCF-7 belongs to ER+ cancer cell, and is good target cell for the screen of ER antagonist. Meanwhile, human umbilical vein endothelial cell (HUVEC) have played a major role as a model system for the study of the regulation of endothelial cell function and are suitable for the evaluation of antiangiogenesis effect by antiproliferative experiment. Here, most compounds show low micromole IC_{50} to MCF-7, and are better than tamoxifen in our antiproliferative experiment. There are no significant difference between the acylamino side chain and ethyoxyl side chain except in certain amino groups. Certainly, diethylaminoethoxyl and piperdinoethoxyl are better than the acylamino side chain (**III-8** vs **III-2**, **III-10** vs **III-4**) no matter whether the methyl of 4,6-phenol in pyrimidine exist or not, but morpholinyl acetamide is better than the another (**III-5** vs **III-11**). When we removed two methyl, the inhibitory activity of dimethylaminyl or pyrrolidinyl derivatives significant decreased (**III-1C**, **III-3C**, **III-7C** and **III-9C**), while other compounds have no difference. Furthermore, studies identified these compounds have good inhibitory activity to HUVEC and most of them are comparable to sunitinib. However, several double demethylation derivatives (**III-1C**, **III-5C** and **III-11C**) showed low inhibitory activity. From the results, we can simply summary the structure-activity relationship. The linker of side chain including the acetamido and ethyoxyl are not the key factor of activity. When the terminal amino group is diethylamine (**III-2**), the inhibitory activity significant decreased, this indicated the terminal may be suitable for the small amino group. Moreover, although many SERMs contain the bisphenol, the demethylation can't improve the activity of synthesized compounds in cell level, and several compounds' activity even declined perhaps because of physicochemical property.

Table 1 Cancer cells antiproliferative activity (IC_{50}, μM)

Compound	MCF-7 IC_{50}/μM	HUVEC IC_{50}/μM	Ishikawa IC_{50}/μM	Compound	MCF-7 IC_{50}/μM	HUVEC IC_{50}/μM	Ishikawa IC_{50}/μM
III-1A	2.5	2.8	3.6	III-7A	3.6	4.6	5.1
III-1B	3.4	3.8	6.3	III-7B	3.6	4.7	5.2

III-1C	39.5	60.6	8.0	III-7C	19.4	4.7	1.6
III-2B	49	22.0	16.4	III-8A	5.0	2.2	4.3
III-2C	27	19.0	17.3	III-8B	5.6	3.5	6.4
III-3A	2.2	2.5	1.9	III-8C	5.1	2.5	4.8
III-3B	4.5	2.3	5.5	III-9A	2.6	2.1	3.9
III-3C	19.7	7.0	8.3	III-9B	4.6	3.1	5.9
III-4B	>100	10.6	1.4	III-9C	57	4.7	4.5
III-4C	26.4	7.4	13.5	III-10A	32.4	5.4	1.9
III-5A	33.1	-	-	III-10B	17.1	4.5	4.8
III-5B	19.2	10.2	42.2	III-10C	6.1	4.6	5.0
III-5C	15.6	47	60.4	III-11B	95.1	7.7	31.9
III-6A	1.8	5.0	5.7	III-11C	-	74.9	-
III-6B	4.1	4.7	4.2	III-12A	4.2	3.0	5.7
III-6C	5.2	6.8	4.9	III-12B	4.8	2.4	5.3
Tamoxifen	19.5			III-12C	5.2	2.5	5.4
				Sunitinib		3.0	

Furthermore, the cell line Ishikawa bears estrogen and progesterone receptors, the cells have been used in numerous basic research areas. It is known that tamoxifen will stimulate endometrial cell growth and induce endometrial cancer after long term treatment.[16] Here, we evaluated the synthesized compounds to the effect of uterus by the Ishikawa antiproliferative experiment (**Table 1**). And the simulation of tamoxifen to Ishikawa had been confirmed. Tamoxifen simulated the cell line Ishikawa's proliferation under the concentration of 10μM and weakly inhibited Ishikawa's proliferation above 40μM in our experiment. Fortunately, the compounds which we synthesized could significant inhibit the proliferation of Ishikawa cells and the IC_{50} of most molecules are under the 10μM. This indicated synthesized compounds will not induce endometrial cancer.

In order to identify the function of synthesized compounds to ER and VEGFR-2, we choose **III-3A** for further study. Because progesterone receptor (PgR) is the target gene of estrogen, the expression level of PgR is commonly used to assess estrogenic or antiestrogenic activity. Here we used real-time polymerase chain reaction (RT-PCR) in the ER positive MCF-7 cells to evaluate the modulation of progesterone receptor. As shown in Figure 3A, presence of 10 nM estradiol (E2) was able to remarkably elevate the mRNA expression of PgR gene compared to the vehicle control. While both tamoxifen and **III-3A** can't simulate the mRNA expression of PgR gene without E2. When combination with 10nM E2, tamoxifen under the concentration of 1μM could significant reduce the expression of PgR mRNA compared with the E2 group. Compound **III-3A** also exhibited stronger antagonism against the expression of PgR mRNA, although weaker than tamoxifen. This result identified that **III-3A** could competitively bind to ER against E2. Moreover, we explored if **III-3A** could block VEGF signalling pathway by inhibiting the VEGFR-2 tyrosine kinase phosphorylation. A Western blotting assay for total and phosphorylated (active) VEGFR-2 was performed (Figure 3B). The positive control group sunitinib could significantly inhibit the phosphorylation of VEGFR-2 in HUVEC. Despite weaker than sunitinib, Compound **III-3A** could make p-VEGFR-2 fall to approximate 50% compared to control group at 1μM. These results identified compound **III-3A** could antagonize against estrogen and inhibit the phosphorylation of VEGFR-2.

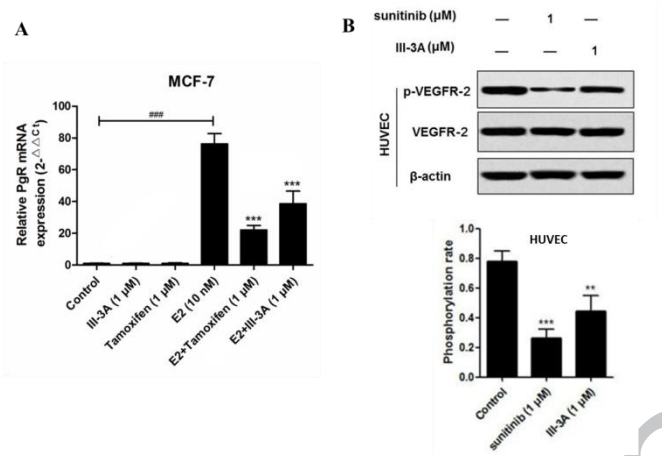


Figure 3. The effect of **III-3A** to PR and VEGFR-2. **A**). The mRNA expression of PR was examined by Real-time PCR. The data illustrated the increased mRNA expression of PR induced by E2 was reversed by **III-3A** in MCF-7 cells. **B). III-3A** inhibits the phosphorylation of VEGFR-2 in HUVEC cells. Expression of p-VEGFR-2 and VEGFR-2 in HUVEC cells were examined by western blots. Densitometric analysis was performed to determine the phosphorylation rate of VEGFR-2. From the chart, the phosphorylation of VEGFR-2 in HUVEC were inhibited by sunitinib and compound **III-3A**. Values are mean ±SD (n=3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. E2 group in A or Control group in B. ### $P < 0.001$ vs Control group.

Futhermore, compound **III-3A** was selected to perform chicken chorioallantoic membrane (CAM) assay to investigate their inhibition of angiogenesis in vivo. Compound **III-3A** and the positive control sunitinib dissolved in DMSO were placed on sterile methyl cellulose filter papers at 1μM, 10μM and 20μM with phosphate buffered saline (PBS) as the blank control. Results are shown in Figure 4. Compared with blank control group, compound **III-3A** could significantly inhibit angiogenesis and be similar to sunitinib. Moreover, the inhibitory ability was proportional to the concentration. Overall, compound showed potential anti-angiogenesis activities in vivo.

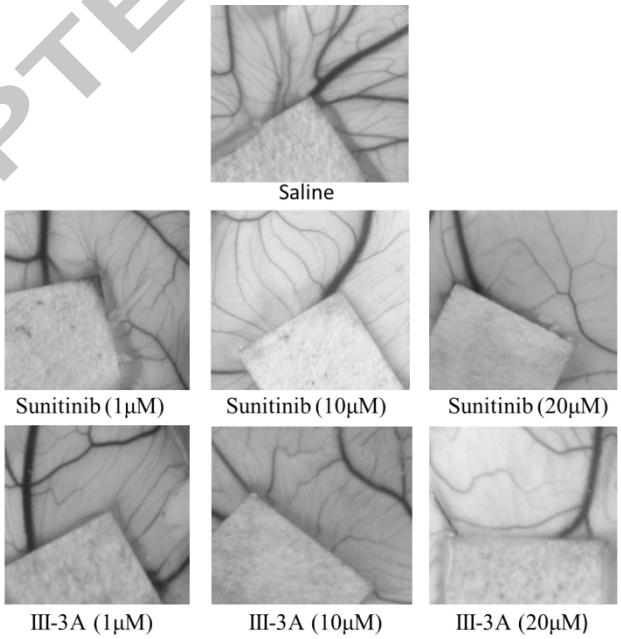


Figure 4 Results of CAM assay.

In conclusion, we have designed and synthesized a series of 4,6-diaryl-2-pyrimidinamine derivatives targeting both ER and VEGFR-2. Biological evaluation showed that most of the synthesized compounds exert stronger inhibitory activity to MCF-7, HUVEC and Ishikawa cells. Further investigation also showed these compounds were expected to inhibit ER positive breast cancer through inhibition of ER and VEGFR-2 simultaneously based on the result of **III-3A** which exhibited stronger antagonism

against the expression of PgR mRNA in MCF-7 and the inhibitory activity to the phosphorylation of VEGFR-2 in HUVEC at 1μM as well as potential anti-angiogenesis effects in vivo. This work may provide a new and potential route to develop effective drugs for breast cancer and will help us for the next research.

Acknowledgments

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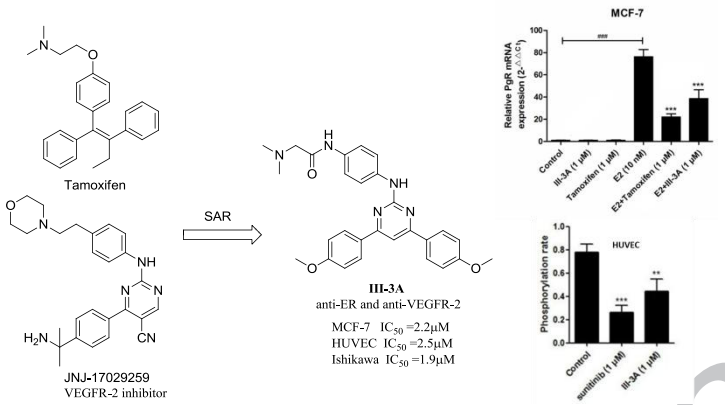

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2. Tamoxifen simulated the cell line Ishikawa's proliferation under the concentration of 10μM in our experiment.
3. A series of designed 4,6-diaryl-2-pyrimidinamine derivatives could simultaneously antagonize ER and inhibit VEGFR-2.