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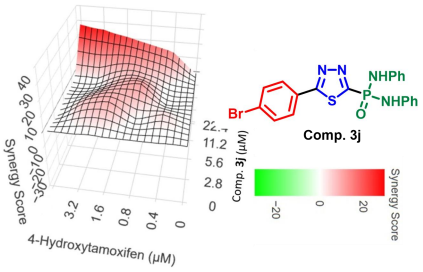
Discovery of diarylphosphoryl and diaminophosphoryl substituted 1,3,4-thiadiazoles for combination therapy of breast cancer

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Compound (4-bromophenyl)-1,3,4-thiadiazol-*N,N'*-diphenylphosphonic diamide 3j exhibited the promising antiproliferative activity against MCF-7 breast cancer cells, with an IC_{50} of 26 μM. Combination studies revealed that compound 3j acts synergistically with several clinically relevant anticancer agents, including an AKT inhibitor, everolimus, docetaxel, doxorubicin, and 4-hydroxytamoxifen, as evidenced by high synergy scores in MCF-7 cells.

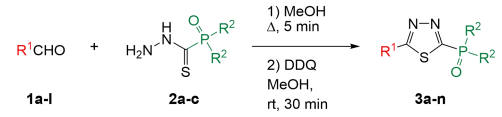


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The development of anticancer agents with improved selectivity and reduced adverse effects remains a major focus of medicinal chemistry research.[1] Amongst others, 1,3,4-thiadiazoles represent an important class of compounds in modern anticancer drug discovery, as the mesoionic nature of the thiadiazole ring facilitates membrane permeability and promotes strong interactions with biological targets.[2-3] Besides, thiadiazole-based compounds and their combinations have progressed into clinical evaluation. Examples include NSC 4728, an inosine monophosphate dehydrogenase inhibitor, evaluated in phase I trials for solid tumors; carbonic anhydrase inhibitors such as acetazolamide in combination with platinum-based chemotherapy, etoposide, and radiotherapy for small cell lung cancer; and acetazolamide combined with temozolomide for malignant glioma (phase I).[4] Glutaminase inhibitors, such as CB-839, are being evaluated both as monotherapy and in combination with multiple agents (e.g., paclitaxel, panitumumab, nivolumab, , capecitabine, *etc.*).[5]

Recently, in our continuing search[6-9] we have identified 2-(2'-P(O)(NH^{t}Bu)$_2$-1',3',4'-thiadiazole)-$\Delta^{1,3,5(10)}$-estratrien-17-one as a promising anticancer agent.[10] Here we report synthesis and first-time biological evaluation of anticancer activity of 2-phosphoryl-5-aryl-substituted 1,2,3-thiadiazoles. The development of these derivatives was guided by a scaffold simplification strategy to evaluate the necessity of the complex steroidal core present in phosphorus(V)-substituted 1,3,4-thiadiazole anticancer agents. By replacing the rigid steroidal framework with a versatile aryl moiety, we aimed to assess the retention of antiproliferative activity and delineate the structure-activity relationships governing the altered cytotoxicity profiles of phosphorus(V)-substituted 1,3,4-thiadiazoles. *In vitro* growth-inhibitory effects against breast carcinoma (MCF-7) was assessed for fourteen synthesized compounds. The remarkable antiproliferative activity of (4-bromophenyl)-1,3,4-thiadiazol phosphonic diamide **3j** led us to investigate its anticancer efficacy on the MCF-7 cell line in combination with antiproliferative agents, namely AKT Inhibitor X, everolimus, docetaxel, doxorubicin, and 4-hydroxytamoxifen.

A series of phosphine oxide and phosphoramide-substituted 1,3,4-thiadiazoles **3** was obtained from arylaldehydes **1** by reaction with phosphorylthioformic acid hydrazides **2** (Scheme 1), as described by us previously.[11] The following standard one-pot protocol was used: 1) a mixture of aldehyde **1** and hydrazide **2** in methanol was refluxed for 5 min; 2) the resulting mixture was then stirred at room temperature for 30 min in the presence of DDQ. The oxidizing agent DDQ was added to the reaction mixture after the completion of the dehydrocondensation step resulting in the formation of 1,3,4-thiadiazoline intermediate.



3a, R^1, R^2 = C_6H_5, 71%
3b, R^1 = 4-MeC_6H_4, R^2 = C_6H_5, 68%
3c, R^1 = 4-F-3-PhOC_6H_3, R^2 = C_6H_5, 83%
3d, R^1 = 4-PhCH$_2$OC_6H_4, R^2 = C_6H_5, 63%
3e, R^1 = C_6H_5, R^2 = ^{t}BuNH, 95%
3f, R^1 = 4-MeC_6H_4, R^2 = ^{t}BuNH, 80%
3g, R^1 = 4-EtOC_6H_4, R^2 = PhNH, 72%
3h, R^1 = 4-AcO-3MeOC_6H_3, R^2 = PhNH, 75%
3i, R^1 = 2,3,4-MeOC_6H_2, R^2 = PhNH, 70%
3j, R^1 = 4-BrC_6H_4, R^2 = PhNH, 86%
3k, R^1 = antracene-9-yl, R^2 = PhNH, 67%
3l, R^1 = 5-methylthiophen-2-yl, R^2 = PhNH, 68%
3m, R^1 = pyridine-3-yl, R^2 = PhNH, 86%
3n, R^1 = 1-phenylethyl, R^2 = PhNH, 64%

Scheme 1 *Reaction conditions: 1) aldehyde **1** (0.17 mmol, 1.1 equiv for **2a,c** (R^2 = Ph, ^{t}BuNH); 0.78 mmol, 5.2 equiv for **2b**(R^2 = PhNH)) phosphorylthioformic acid hydrazides **2** (0.15 mmol, 1.0 equiv), methanol (7 mL), reflux, 5 min; 2) DDQ (37 mg, 0.17 mmol, 1.1 equiv), 25 °C, 30 min.*

Benzaldehydes functionalized with electron-donating (OH, OMe, OEt, OAc, OPh, OCH$_2$Ph) and electron-withdrawing (F, Cl, Br) substituents reacted smoothly to form 5-aryl-1,3,4-

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thiadiazoles **3a-g** in 63-95% yields. In addition, anthracenyl-substituted product **3k** was isolated in 67% yield. 5-Methylthiophene-2-carbaldehyde and 3-pyridinecarbaldehyde were reacted with thiohydrazide **2c** to form products **3l,m** in 68% and 86% yields, respectively. Phenylethyl-substituted product **3n** was obtained in 64% yield from 2-phenylpropanal. It should be noted that synthesis of the compounds **3c-e,g-n** was described by us previously,[11] while compounds **3a,b,f** are new.

All synthesized compounds **3a-n** were screened for antiproliferative activity (Table 1). The viability of the *in vitro* cultured breast cancer cell line MCF-7 was evaluated using the MTT assay. According to modern requirements for the design of antiproliferative agents, the compounds were considered to be active if their IC_{50} values were lower than 50 μM. Cisplatin was included as a reference anticancer agent under the same experimental conditions. Treatment with cisplatin resulted in a 50% reduction in MCF-7 cell viability at a concentration of approximately 6.5 μM. As shown in Table 1, nine compounds were considered inactive under the applied experimental conditions, whereas five compounds demonstrated measurable antiproliferative activity, with IC_{50} values below 50 μM. Among the tested derivatives, the highest activity toward MCF-7 cells was observed for the (4-bromophenyl)-1,3,4-thiadiazol phosphonic diamide **3j**. Compound **3j** exhibited an IC_{50} value of 26 μM, corresponding to approximately fourfold lower potency than that of the reference drug cisplatin. Although its activity was lower than that of cisplatin, compound **3j** was therefore identified as the most promising antiproliferative derivative within the synthesized series and was selected for subsequent combination studies.

Table 1 Antiproliferative activity of compounds **3**; the results of the MTT test after 72 hours incubation with compounds are presented.

Entry	Comp.	Cell viability, %						IC_{50}, μM
		0	3.1	6.2	12.5	25	50	
1	**3a**	100	91	86	85	79	74	> 50
2	**3b**	100	91	89	81	76	70	> 50
3	**3c**	100	98	87	79	77	72	> 50
4	**3d**	100	88	83	72	67	54	> 50
5	**3e**	100	97	96	89	80	79	> 50
6	**3f**	100	94	86	84	81	79	> 50
7	**3g**	100	93	88	82	77	39	41 ± 3.5
8	**3h**	100	92	86	77	74	56	> 50
9	**3i**	100	83	77	75	58	39	41 ± 4.0
10	**3j**	100	93	90	76	51	25	26 ± 2.2
11	**3k**	100	83	80	77	72	61	> 50
12	**3l**	100	91	84	75	70	48	47 ± 3.6
13	**3m**	100	95	93	94	83	81	> 50
14	**3n**	100	96	95	90	87	42	43 ± 3.9
15	**cisplatin**	100	65	53	35	21	8	6.5 ± 0.7

The investigation of antiproliferative agents in combination represents one of the key strategies in modern oncology, aimed at enhancing therapeutic efficacy while simultaneously reducing systemic toxicity and overcoming drug resistance.[12-16] The rationale for combination therapy is based on the ability of multiple agents to simultaneously target different molecular pathways, resulting in synergistic antiproliferative or pro-apoptotic effects that exceed the activity of individual drugs.[12] In preclinical studies, the human breast adenocarcinoma cell line MCF-7 remains one of the most widely used *in vitro* models for evaluating such strategies, particularly in the context of hormone-dependent breast cancer.[17-18]

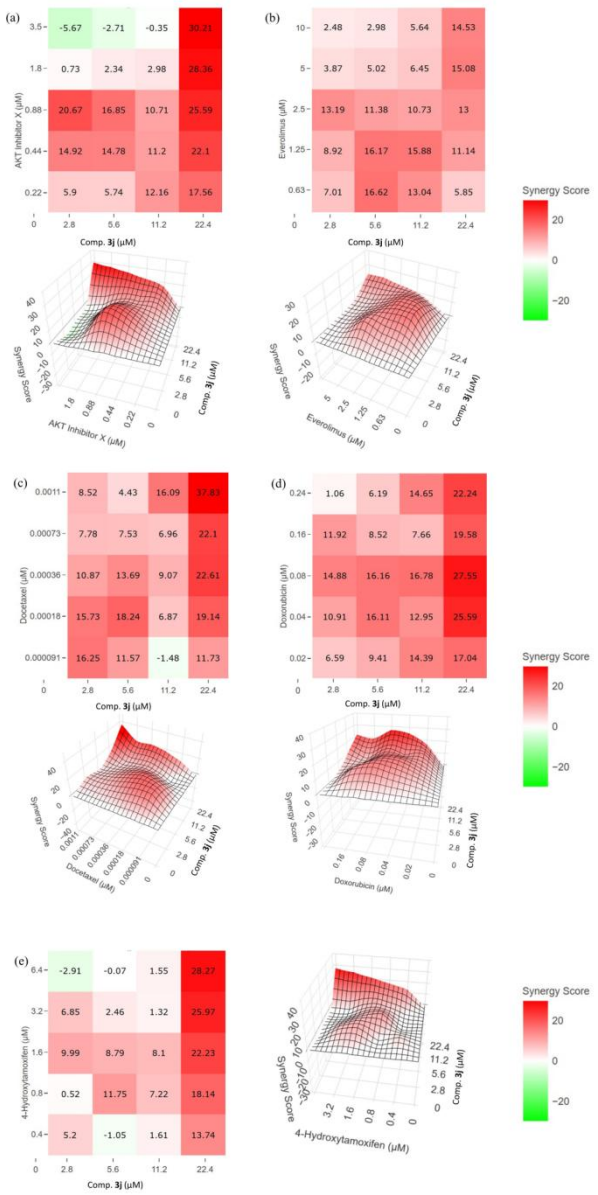


Figure 1 Combined effect of the synthesized thiadiazole derivative **3j** with AKT inhibitor X (**a**), everolimus (**b**), docetaxel (**c**), doxorubicin (**d**), and 4-hydroxytamoxifen (**e**) on the MCF-7 cell line.

In subsequent experiments, the antiproliferative activity of compound **3j**, which demonstrated the highest activity among the synthesized derivatives in the MTT assay, was investigated in combination with four clinically relevant or mechanistically distinct antiproliferative agents. Figure 1a presents the combination plots obtained following treatment of MCF-7 cells with compound **3j** and AKT Inhibitor X. The resulting heatmap indicated pronounced synergistic interactions over a substantial part of the tested concentration matrix, with particularly high synergy scores observed at a concentration of 22.4 μM of compound **3j**. These findings suggest that inhibition of AKT signaling may enhance the antiproliferative

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effects of compound **3j**, although further mechanistic studies will be required to establish whether this interaction is directly associated with modulation of the PI3K/AKT signaling pathway.

Everolimus, a targeted inhibitor of mTOR kinase, also demonstrated efficacy in combination with compound **3j**. Synergistic effects were observed across both high and low concentrations of the agents (Figure 1b). The synergy score for 5.6 μM of compound **3j** in combination with 0.63 μM everolimus reached a value of 16. Taxanes, particularly docetaxel, are widely used in combination therapy for breast cancer. For the majority of the tested concentrations of docetaxel and compound **3j**, significant synergistic effects were observed (Figure 1c). At higher concentrations, the synergy score reached up to 37. Similar trends were identified for the combination of compound **3j** with the anthracycline antibiotic doxorubicin (Figure 1d). The maximum synergy score for this drug pair was 27.5.

The gold standard of endocrine therapy for breast cancer is 4-hydroxytamoxifen, a selective modulator of estrogen receptor α activity that has been widely used for over 50 years. At lower concentrations of the tested agents, the synergistic interaction between compound **3j** and 4-hydroxytamoxifen was less pronounced than that observed for combinations of **3j** with docetaxel or doxorubicin (Figure 1e). However, at higher concentrations, significant synergistic effects were detected, with the maximum synergy score for the combination of compound **3j** and 4-hydroxytamoxifen exceeding 28. This observation is particularly relevant in the context of ERα-positive MCF-7 cells and suggests that the activity of **3j** may be compatible with endocrine-based treatment approaches. Nevertheless, additional experiments using multiple ERα-positive breast cancer models, including models with acquired endocrine resistance, would be required to determine the broader applicability of this combination.

Taken together, these data demonstrate that compound **3j**, identified as the most active derivative of the synthesized series, can exhibit synergistic antiproliferative effects when combined with agents targeting distinct cellular pathways. The strongest interactions were observed in combination with docetaxel, doxorubicin, and AKT/mTOR pathway inhibitors, while a pronounced synergistic response was also detected with 4-hydroxytamoxifen at higher concentrations. These findings provide a rationale for further investigation of **3j** in rational combination strategies. Future studies should focus on elucidating the molecular mechanisms underlying these interactions, validating the observed effects in additional breast cancer cell models, and determining whether the synergistic responses are maintained under clinically relevant concentration ranges. Such studies may help to define the therapeutic potential of compound **3j** and establish the most appropriate combination partners for its further preclinical development.

In summary, a representative series of phosphorus(V)-substituted 1,3,4-thiadiazoles was obtained. Among the tested compounds, (4-bromophenyl)-1,3,4-thiadiazol-*N,N'*-diphenylphosphonic diamide **3j** showed remarkable antiproliferative activity against MCF-7 breast cancer cells with IC_{50} = 26 μM. Importantly, **3j** acted synergistically with five clinically relevant anticancer agents - an AKT inhibitor, everolimus, docetaxel, doxorubicin, and 4-hydroxytamoxifen - highlighting phosphorus(V)-substituted 1,3,4-thiadiazole's potential as a scaffold for combination therapy.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.___

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