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3-(2′-Bromopropionylamino)-benzamides as novel S-phase arrest agents

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Abstract—We report the synthesis, antiproliferative activity, and SAR of novel 3-(2′-bromopropionylamino)-benzamides. Many of the benzamide compounds showed potent cytotoxicities against Molt-3 leukemia cells. Several compounds exihibited cytotoxicities (under 6.5 μM) against five solid tumor cell lines. The mechanism of action of the most potent benzamide **10l** does not involve targeting on tubulin but it causes cell cycle S-phase arrest. This active S-phase arrest agent merits further investigation.

Cell cycle control is crucial for error-free transmission of the entire genetic material to subsequent generations. Defective regulatory function results in genetic modifications that contribute to tumorigenesis. The loss of cell cycle control leading to deregulated cell proliferation is a hallmark of cancer. Many chemotherapeutic agents in clinical use kill rapidly dividing cancer cells by inducing cell cycle arrest at various phases and subsequently lead to apoptosis. For example, antitubulin agents, taxanes, and vinca alkaloids target tubulin dynamics which induce G_2/M phase arrest; on the other hand, DNA topoisomerase I inhibitors, camptothecin analogues, cause the S-phase arrest of cancer cells.[1,2] However, multidrug resistance (MDR) and undesired side effects have limited their clinical use.[3] Thus, it is very important to develop effective anticancer agents with both improved safety profiles and novel mechanism of actions.

We have recently reported a small molecule compound 3-(2′-bromopropionylamino)-benzoylurea(JIMB01) (Chart 1, **1**) which has strong antiproliferative activities in a panel of thirteen human tumor cell lines including MDR B-cell lymphoma.[4] JIMB01 interferes with the assembly of microtubules, arrests the cell cycle at G_2/M phase, and induces apoptotic cell death in a variety of cancer types. Moreover, JIMB01 has shown promising in vivo efficacy in a hepatocarcinoma (Bel-7402) tumor model.[4] With the goal to elucidate the SAR and further enhance the anticancer activity of JIMB01, we synthesized a series of novel 3-(2′-bromopropionylamino)-benzamides, which display more potent antiproliferative activity and a different mechanism of action is shown for **10l**.

The SAR studies of 3-haloacetamido benzoylurea compounds (Chart 1, **2**–**5**) have shown that the 3-haloacetamido group and the acylurea unit must be in a meta-relationship for potent cytotoxicity and antitubulin activity.[5] In the original work, replacement of the acylurea group with an ethyl ester group resulted in 3-haloacetamido benzoyl ethyl ester compounds (Chart 1, **6**–**9**).[6,7] The 3-iodo or bromoacetamido benzoyl ethyl esters displayed improved solubility and maintained the inhibitory effect on microtubule assembly. However, this modification induced different effects on the tumor cell cycle: 3-iodoacetamido benzoyl ethyl ester blocked at the G1–S transition in addition to its activity in G_2/M phase. A related simple benzamide, 3-aminobenzamide, a poly (ADP-ribose) polymerase (PARP) inhibitor, was

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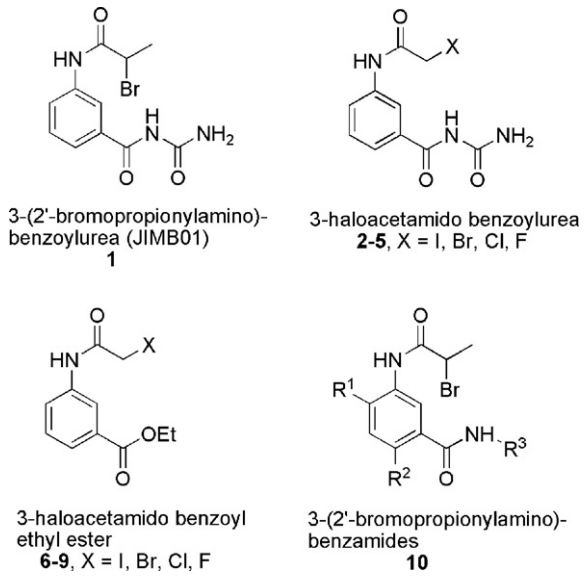
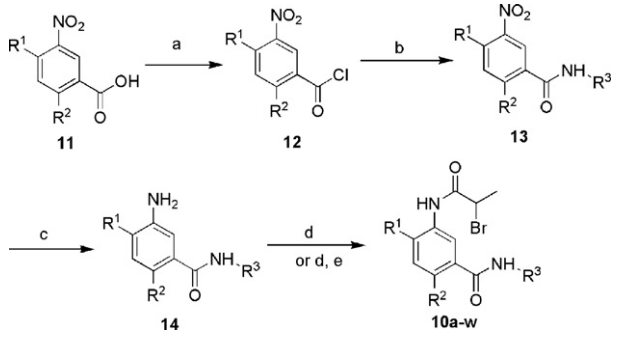


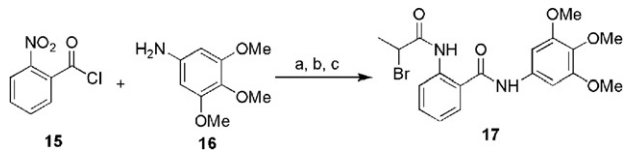
Chart 1. Small molecule antiproliferative agents: benzoylurea, ethyl ester and benzamides.

shown to affect DNA synthesis and to inhibit DNA repair.[8,9] Additionally, several other classes of benzamides or pyridinyl amides have been reported such as tubulin, protein tyrosine kinase, or NF-κB inhibitors.[10–13] Thus, we explored the replacement of the acylurea group with the amide group in the lead compound JIMB01. Here, we report the synthesis, cytotoxicity, SAR analysis, and preliminary mode of action information for this series of novel benzamide compounds.

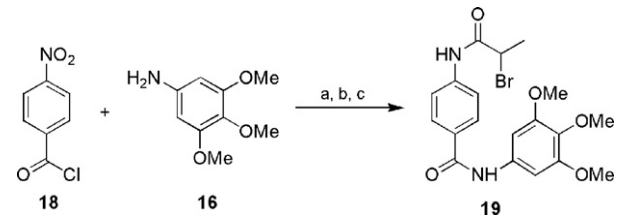
The synthesis of 3-(2′-bromopropionylamino) benzamides is shown in Schemes 1–4. The benzoyl chlorides (either commercially available or directly prepared from the corresponding benzoic acid) were allowed to react with alkyl or arylamines in the presence of triethylamine in DMF to yield the nitrobenzamides. Then reduction of the nitro group by $SnCl_2{\cdot}2H_2O$ in ethanol at reflux yielded the corresponding amino compounds.[14] Finally, acylation with 2-bromopropionyl bromide afforded the desired benzamides.[5] The corresponding sulfonamide compound **24** was prepared from 3-nitrobenzenesulfonyl chloride **23** by reaction with 3,4,5-trimethoxyaniline **16**, followed by our general reduction and acylation procedure, as shown in Scheme 5. Condensation of 3-nitrobenzoylisocyanate **25** with aniline **16** following reported procedures gave the benzoylurea compound and ultimately the final compound **26** (see Scheme 6).[15]



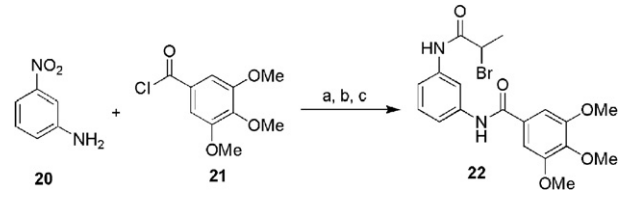
Scheme 1. Reagents and conditions: (a) SO_2Cl, EtOAc, reflux; (b) R^3-NH_2, NEt_3, DMF; (c) $SnCl_2$, EtOH, reflux; (d) 2-bromopropionyl bromide, DMF; (e) HCl (gas), EtOAc.



Scheme 2. Reagents and condition: (a) NEt_3, DMF; (b) $SnCl_2$, EtOH, reflux; (c) 2-bromopropionyl bromide, DMF.

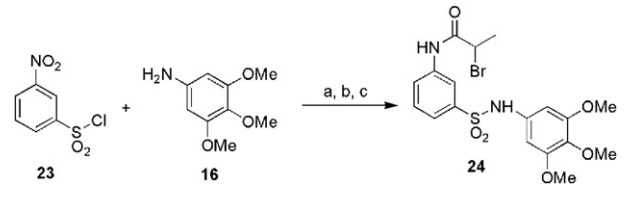


Scheme 3. Reagents and condition: (a) NEt_3, DMF; (b) $SnCl_2$, EtOH, reflux; (c) 2-bromopropionyl bromide, DMF.

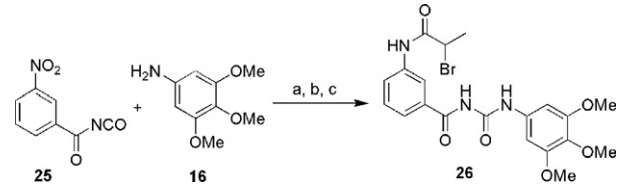


Scheme 4. Reagents and condition: (a) NEt_3, DMF; (b) $SnCl_2$, EtOH, reflux; (c) 2-bromopropionyl bromide, DMF.

The novel series of 3-(2′-bromopropionylamino) benzamides and their analogues were evaluated for their antiproliferative activity against the Molt-3 leukemia cell line and were compared with the lead compound JIMB01, as shown in Table 1.[16] Fourteen of the 28

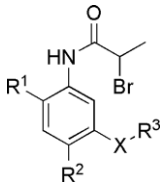


Scheme 5. Reagents and condition: (a) NEt_3, DMF; (b) $SnCl_2$, EtOH, reflux; (c) 2-bromopropionyl bromide, DMF.



Scheme 6. Reagents and condition: (a) 1,4-dioxane; (b) $SnCl_2$, EtOH, reflux; (c) 2-bromopropionyl bromide, DMF.

Table 1. Antiproliferative activity of new compounds in Molt-3 T-cell leukemia cells



Compound	R^1	R^2	X	R^3	IC_{50} (μM)[a]
JIMB01	H	H	–CONHCONH$_2$	—	1.60
10a	H	H	–CONH–	–$CH_2CH_2CH_3$	8.94
10b	H	H	–CONH–	–$CH_2CH_2CH_2CH_3$	>10
10c	H	H	–CONH–	–$CH_2CH_2NMe_2$·HCl	>10
10d	H	H	–CO–	1-MePiperizine-4-yl·HCl	>10
10e	H	H	–CONH–	3,4,5-OMe$_3$Ph	0.67
10f	OMe	H	–CONH–	3,4,5-OMe$_3$Ph	4.24
10g	F	H	–CONH–	3,4,5-OMe$_3$Ph	2.20
10h	Cl	H	–CONH–	3,4,5-OMe$_3$Ph	2.25
10i	H	OH	–CONH–	3,4,5-OMe$_3$Ph	1.08
10j	H	F	–CONH–	3,4,5-OMe$_3$Ph	0.42
10k	H	H	–CONH–	4-MeOPh	0.80
10l	H	H	–CONH–	3,4-OMe$_2$Ph	0.12
10m	H	H	–CONH–	4-OH-3-OMePh	0.51
10n	H	H	–CONH–	3,4-O–CH_2–O–Ph	0.62
10o	H	H	–CONH–	3,5-OMe$_2$Ph	0.61
10p	H	H	–CONH–	4-FPh	2.00
10q	H	F	–CONH–	4-OMePh	0.25
10r	H	F	–CONH–	4-OEtPh	>10
10s	H	F	–CONH–	3,4-OMe$_2$Ph	0.47
10t	H	F	–CONH–	4-OH-3-OMePh	0.85
10u	H	F	–CONH–	2-OMePy-4-yl	0.50
10v	H	H	–CONH–	6-OMebenzothiazole-2-yl	1.66
10w	H	H	–CONH–	Carbazole-3-yl	1.51
22	H	H	–NHCO–	3,4,5-OMe$_3$Ph	5.66
24	H	H	–SO_2NH–	3,4,5-OMe$_3$Ph	1.27
26	H	H	–CONHCONH–	3,4,5-OMe$_3$Ph	4.22
17					5.72
19					1.79
Podophyllotoxin					0.014
CA-4					0.09

[a] Values were determined in duplicate as described in Ref. 16.

compounds prepared were more active than lead compound JIMB01. Eleven compounds showed IC_{50} values under 1 μM.

We first evaluated the cytotoxic effect of *N*-alkyl substitution of benzamide. The synthesized compounds **10a** and **10b** showed decreased potency by more than five-fold compared to lead compound JIMB01. In order to improve water solubility, we also introduced dimethylaminoethyl and *N*-methylpiperizine groups and evaluated the cytotoxicies of their hydrochloride salt. While the compounds **10c** and **10d** were more water-soluble, unfortunately, they showed lower activity. These results demonstrated that *N*-alkyl group and cation containing side chains were not suitable for potent antiproliferative activity.

Replacement of the *N*-alkyl group with *N*-aryl group yielded novel *N*-aryl benzamides. Because the 3,4,5-trimethoxyphenyl group is found in many antitubulin compounds, such as colchicine, podophyllotoxin, and combretastatin A-4 (CA-4),[17] we first evaluated the influence of 3,4,5-trimethoxyphenyl group on this series of 3-(2′-bromopropionylamino) benzamide compounds. The 3,4,5-trimethoxyphenyl compound **10e** (IC_{50} value 0.67 μM) is 2.3 times more potent than JIMB01.

The effect of various substitutions on the benzene ring in the benzamides was also examined. Substitution of R^1 of **10** with methoxy, fluoro, or chloro (**10f**–**10h**) reduced cytotoxicity by 3–7 times. However, when R^2 was substituted with hydroxy (**10i**), inhibitory activity comparable to compound **10e** was observed. Substitution of R^2 with a fluorine atom (**10j**) led to a modest increase in antiproliferative activity.

We further evaluated the influence of various substitutions on the *N*-phenyl ring of the benzamide. A single

methoxy substitution of the phenyl ring (**10k**) led to a slight loss of potency. However, 3,4-dimethoxy substitution of the phenyl ring (**10l**) led to a 5.5-fold increase in activity relative to **10e**. 4-Hydroxy-3-methoxy, 3,4-O–CH_2–O, and 3,5-dimethoxy phenyl substituted benzamides (**10m**–**10o**) showed similar cytotoxicities to that of the 3,4,5-trimethoxyphenyl compound **10e**, all showed greater activity than the lead compound JIMB01. These results suggest that mono, di, or trimethoxy substitutions on the 3, 4, or 5 position of phenyl ring are all well tolerated and increase the activity. The 3,4-dimethoxy substitution seems to be the most effective. However, changing 4-methoxy to a 4-fluoro (**10p**) yielded a three-fold loss of activity.

Although introduction of a fluorine *ortho* to the acetamide (R^1) did not improve potency, we synthesized compounds **10q**–**10u** with an *ortho*-fluorine to the benzamide (R^2). The results were different compared to the corresponding non-*ortho*-fluorine compounds. *ortho*-Fluorine substitution improved activity for 3,4,5-trimethoxyphenyl and 4-methoxyphenyl compounds and reduced it for 3,4-dimethoxy and 4-hydroxy-3-methoxy substituted compounds. It is also surprising to note that replacement of 4-methoxyphenyl by 4-ethoxyphenyl led to a dramatic loss of activity. The 2-methoxypyridine-4-yl substituted *ortho*-fluorine compound **10u** displayed similar activity to that of the 3,4-dimethoxyphenyl compound **10s**. The 4-methoxyphenyl compound **10q** exihibited the most potent activity in this series of *ortho*-fluorine substituted benzamides.

Replacement of the *N*-phenyl ring with 2-(6-methoxy)-benzothiazolyl (**10v**) and 3-carbazolyl groups (**10w**) gave 2- to 2.5-fold loss in activities as compared to compound **10e**; however, these two compounds are still as potent as the lead compound JIMB01. In order to understand the importance of the amide linkage, we further synthesized the benzamide **22**, the phenylsulfonamide **24**, and the benzoylurea **26**. All of these compounds displayed reduced cytotoxicities. Moving the amide group from *meta* to *para* or *ortho* position (**17**, **20**) also resulted in loss of inhibitory activity. These results revealed that the amide linkage and the position on the phenyl ring are important for antiproliferative activity.

We further evaluated the antiproliferative activities of several potent compounds **10e**, **10j**, **10l**, **10m**, **10q**, **10u** against a panel of five human solid tumor cell lines in vitro. Table 2 contains these results along with comparative data for CA-4 and podophyllotoxin.[16] Although these compounds' activities against solid tumor cell lines are less potent than against Molt-3 leukemia cell line ($IC_{50} < 0.67$ μM), the IC_{50} values of these most potent *N*-aryl benzamides were between 0.86 and 6.48 μM. The 3,4-dimethoxy substituted benzamide compound **10l** is slightly more active than the 3,4,5-trimethoxyphenyl substituted compound **10e** against these human solid tumor cell lines. However, the 4-hydroxy-3-methoxy compound **10m** showed different sensitivities compared to the 3,4-dimethoxy and 3,4,5-trimethoxy substitutions inhibiting these solid tumor cells. The compound **10j** with *ortho*-fluorine substitution showed similar activities with non-fluorine compound **10e**. The 4-methoxyphenyl and 2-methoxy pyridine-4-yl compounds **10q**, **10u** with *ortho*-fluorine substitution also displayed comparable activities with 3,4,5-trimethoxy substituted compound **10e**. These results suggest that the substitutions with various methoxy groups and *ortho*-fluorine are of little influence on the cytotoxicities against these five solid tumor cell lines, which is different from that of the Molt-3 leukemia cell line.

The lead compound JIMB01 has shown complete inhibition of free tubulin assembly at a concentration of 4 μM.[4] The mechanism of action of JIMB01 involves targeting on tubulin which blocks the cell cycle at the G_2/M phase. To investigate whether the antiproliferative activities of the novel *N*-aryl benzamide compounds involved interaction with tubulin, the most active compound **10l** was evaluated for inhibition of polymerization of purified tubulin in a cell-free system. The results are shown in Figure 1. Vincristine was used as a positive control. Inhibition of tubulin assembly was examined by compound **10l** at concentrations of 0.5–10 μM. Surprisingly, the results showed that the compound **10l** does not significantly interfere with the assembly of microtubules. Then, we further examined the influence of **10l** on the cancer cell cycle using flow cytometric analysis with T-cell leukemia cells (Fig. 2). Treatment with **10l** at a concentration of 6.14 μM for 6 h induced a major shift from G_0/G_1 to the S-phase (52.9%) compared to control (32.2%). The S-phase peak increased continuously from 6 to 12 h (62.7%) and maintained a high level at 24 h (61.9%). However, the G_2/M phase proportion of cells was little changed under treatment with **10l**. These results further confirmed that the mechanism of action of the benzamide compound **10l** was different from the lead compound JIMB01 and that it is a novel S-phase arrest agent. As mentioned previously, replacement of the acylurea group with an ethyl ester group resulted in 3-iodoacetamido benzoyl ethyl

Table 2. Antiproliferative activities of **10e**, **10j**, **10l**, **10m**, **10q**, **10u**, CA-4, and podophyllotoxin in human cancer cell lines

Cell line	Human tumor	IC_{50} (μM)[a]							
		10e	**10j**	**10l**	**10m**	**10q**	**10u**	CA-4	Podophyllotoxin
Molt-3	T-cell leukemia	0.67	0.42	0.12	0.51	0.25	0.50	0.009	0.014
MCF-7	Breast cancer	1.47	2.11	1.23	0.86	1.39	2.20	0.012	0.015
Bel-7402	Hepatoma	2.64	2.05	2.33	5.26	3.37	2.70	0.010	0.009
DU-145	Prostate cancer	1.93	2.03	1.87	6.48	6.45	2.35	0.009	0.052
PC-3	Prostate cancer	2.46	2.18	2.36	3.13	2.43	2.42	0.003	0.010
DND-1	Melanoma	2.26	4.16	1.40	1.22	2.56	2.57	0.003	0.012

[a] Values were determined in triplicate as described in Ref. 16.

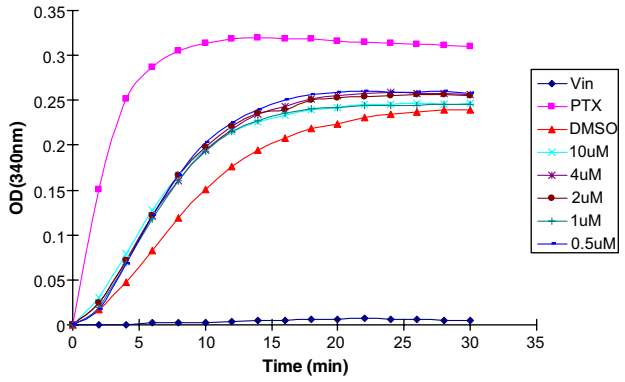


Figure 1. Effect of **10l** on tubulin assembly.[16] Free purified β-tubulin from bovine brain (1 mg/mL) in reaction buffer was incubated with GTP and Mg^{2+} at 37 °C for assembly in the absence or presence of **10l** (0.5–10 μM), vincristine (20 μM) or paclitaxel (20 μM). Tubulin assembly was determined every 2 min by OD at 340 nm. Each point represents the mean of two independent experiments.

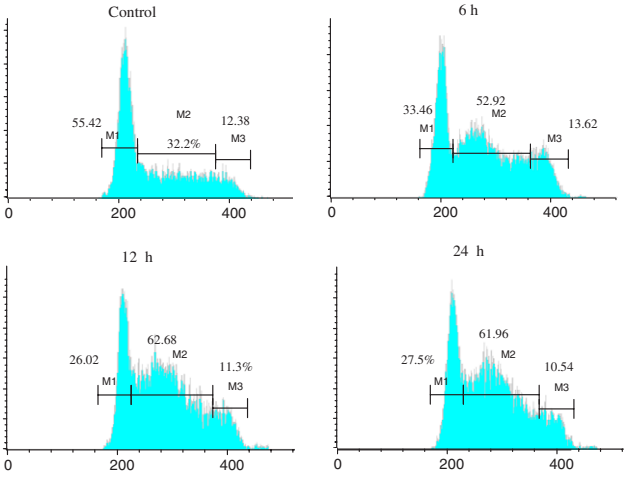


Figure 2. Cell cycle block at the S-phase in T-cell leukemia cells treated with **10l**. CEM cells treated with **10l** (6.14 μM) were analyzed for cell cycle distribution using flow cytometry. The major block at S-phase appeared between 6 and 24 h post-treatment.

ester which blocked the G_1–S transition in addition to its activity in the G_2/M phase. However, the ethyl ester still inhibited microtubule polymerization at a concentration of 7.5 μM.[7] It is unclear whether the mechanism of action has an intrinsic linkage between benzoyl ethyl esters and benzamides or not.

Compounds which have been clearly shown to cause arrest in the S-phase are uncommon. Camptothecin analogues, several of clinical significance, are perhaps the best known small molecules to cause S-phase arrest.[2] Others include noscapine analogues,[18] resveratrol,[19] 7-hydroxystaurosporine,[20] β-lapachone,[21] beicalein,[22] and deguelin.[23] The mechanism of action of this series of benzamides as novel S-phase arrest agents merits further investigation.

In conclusion, a series of novel 3-(2′-bromopropionylamino)-benzamides were synthesized by replacing the acylurea with an amide group in the lead antitubulin compound JIMB01. Many new benzamide compounds showed more potent antiproliferative activities against Molt-3 leukemia cells than the lead compound JIMB01. Several compounds showed effective activities against five solid tumor cell lines. Preliminary mechanism of action studies demonstrated that the most potent benzamide compound **10l** does not inhibit tubulin polymerization and arrests the cancer cell cycle at S-phase, which is different from the lead compound JIMB01. This series of benzamide compounds merits further studies as novel S-phase arrest agents.

Acknowledgments

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