Development of Novel Anticancer Agents with a Scaffold of Tetrahydropyrido[4,3-*d*]pyrimidine-2,4-dione

Zonghui Ma, Ge Gao, Kunsen Fang, and Haiying Sun*

Jiangsu Key Laboratory of Drug Design and Optimization, Department of Medicinal Chemistry, China Pharmaceutical University, Nanjing 210009, China

Supporting Information

ABSTRACT: ONC201 is a small molecular anticancer agent currently in multiple Phase II clinical trials. Based on the pharmacophore of ONC201, a series of small molecular compounds with a core structure of tetrahydropyrido[4,3-*d*]pyrimidine-2,4-dione were designed and synthesized. Preliminary mechanism studies of these compounds indicated that they can inhibit the phosphorylation of AKT and ERK, induce the dephosphorylation of Foxo3a, and promote the expression of TRAIL and the enhancement of activating transcription factor 4 (ATF4) in PC-3 cells. Structure–activity relationship (SAR) studies indicated that modifications of the substituted groups on the core structure can significantly improve the cellular activities of these compounds. The most potent compounds are over 100 times more potent than ONC201 in inhibition of cell growth in a panel of different types of human cancer cell lines.

KEYWORDS: TRAIL, AKT, ERK, Foxo3a, ATF4

Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a tumor suppressor which induces apoptosis in a wide range of human cancer cell lines by binding to and activating pro-apoptotic death receptors 4 and 5 (DR4 and DR5). TRAIL can potently induce apoptosis in cancer cells while it shows low toxicity to normal cells; therefore, recombinant TRAIL and TRAIL-agonist antibodies targeting DR4 and DR5 have been evaluated in multiple clinical trials. However, these macro-biomolecules did not show sufficient effects in these trials.^{1,2} The reasons for the failures of these trials are not clear, but one hypothesis is that their poor stability and biodistribution may limit their usage as anticancer agents.³ To overcome these drawbacks, Allen et al. initiated a campaign for searching small molecules which can up-regulate the expression of TRAIL gene and enhance the level of endogenous TRAIL by screening the NCI chemical library. Their effort led to the identification of a small molecular compound ONC201 (also known as TIC10, Figure 1) as an inducer of the expression of TRAIL gene.^{4,5} ONC201 can inhibit cell growth and induce robust apoptosis in a broad range of tumor cells, and the apoptosis induced by ONC201 is p53 independent.⁵ Detailed mechanism studies for ONC201 have been performed, and the results indicated that ONC201

exerts its anticancer activity through two pathways, including both TRAIL-dependent and -independent pathways.^{6–8} In the TRAIL-dependent pathway, ONC201 induces dual-inhibition of the phosphorylations of AKT and ERK, which leads to the dephosphorylation of Foxo3a. Dephosphorylated Foxo3a then translocates from the cytoplasm into the nucleus and binds to the promoter of TRAIL gene to up-regulate the transcription of TRAIL gene.⁶ In the TRAIL-independent pathway, ONC201 can induce endoplasmic reticulum stress responses (ER) or integrated stress responses (ISR) which culminate at the enhancement of activating transcription factor 4 (ATF4), that leads to the expression of many ATF4 targeted genes.^{7,8} In solid tumors ONC201 can function through both of the two pathways, but in hematological malignancies it seems to act only through the TRAIL-independent mechanism.^{6–8}

In preclinical studies ONC201 exhibited excellent drug properties, such as robust antiproliferative and pro-apoptotic effects, benign toxicity profile, optimum aqueous solubility, favorable pharmacokinetic properties, and oral bioavailability. It also shows broad synergistic effects with approved and investigational cancer therapies in many solid tumors and hematological malignancies in a preclinical model.^{9–11} Currently ONC201 is being evaluated in multiple Phase II clinical trials.¹²

Because of the promising anticancer activity of ONC201, we are interested in the design of novel anticancer agents with similar mechanism of functions as ONC201. In this paper, we

Figure 1. Design of analogues of ONC201.

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reported our design, synthesis, and preliminary mechanism studies of a class of tetrahydropyrido[4,3-*d*]pyrimidine-2,4-diones using ONC201 as a lead compound.

ONC201 has a unique tricyclic structure. Analyzing its structure, we think that N₁ in the five membered ring can function as a hydrogen bond acceptor, while the ethylenyl group in the five membered ring can provide hydrophobic interaction. Based upon this analysis, we designed a class of analogues of ONC201 with a core structure of tetrahydropyrido[4,3-*d*]pyrimidine-2,4-dione (**1**, Figure 1), in which N₁ in ONC201 (Figure 1) is replaced with an oxygen atom to maintain the ability as a hydrogen bond acceptor, and a small hydrophobic substitute group R¹ is introduced to N₁ in **1** to mimic the hydrophobic effect of the ethylenyl group in the five membered ring in ONC201. R² and R³ are the groups mimicking the benzyl and 2-methylbenzyl groups, respectively, in ONC201 (Figure 1).

We at first designed and synthesized compounds **2–6** by introducing different substituted groups to N₁ of general structure **1**. In these five compounds the R² and R³ groups in **1** were kept as benzyl groups. In our initial screen for the anticancer cellular activities of ONC201, we have found that ONC201 has modest anticancer cellular activity in the MDA-MB-231 cell line; therefore, **2–6** were tested together with ONC201 in this cell line for their abilities in inhibiting cell growth, and the results are shown in Table 1. ONC201 shows

Table 1. Cell Growth Inhibition of Compounds **2–6 and ONC201 in Human Breast Cancer MDA-MB-231 Cell**

Compound	R	MDA-MB-231 IC ₅₀ (μM)
ONC201		14.6 ± 3.2
2	H	32.0 ± 5.6
3	Me	1.9 ± 0.8
4	Et	2.0 ± 0.7
5	iPr	52.3 ± 11.7
6	cyclopentyl	67.1 ± 13.2

an IC₅₀ of 14.6 μM in this cell line, while compound **2** which does not have a substituted group on N₁ shows an IC₅₀ of 32.0 μM and is about 2-times less potent than ONC201. Compounds **3** and **4** which contain a methyl or an ethyl group on N₁ show significantly improved cellular activity with IC₅₀s of 1.9 and 2.0 μM, respectively, and are about 7-times more potent than ONC201, suggesting that a small hydrophobic group at this position is critical to the cellular activity. However, compounds **5** and **6** which have an isopropyl or a cyclopentyl group on N₁ respectively are 4- to 5-times less potent than ONC201, indicating that the size of the substituted group on N₁ has critical influence to the cellular activity. To reach potent cellular activity, R¹ can only be a methyl or an ethyl group; a larger hydrophobic group on N₁ is detrimental.

In order to find whether these compounds have similar structure–activity relationship (SAR) in their anticancer cellular activities in other cancer cell lines, we selected a more potent compound **4** and a less potent compound **5** and screened their anticancer cellular activities together with ONC201 in a panel of different types of human cancer cell

lines, and the results are reported in Table 2. ONC201 shows IC₅₀ values between 3 and 15 μM to most of the cancer cell

Table 2. Cell Growth Inhibition of Compounds **4, **5**, and ONC201 in a Panel of Human Cancer Cell Lines**

Cell lines	ONC201 IC ₅₀ (μM)	4 IC ₅₀ (μM)	5 IC ₅₀ (μM)
Colo205	15.1 ± 3.3	2.9 ± 1.1	48.1 ± 16.3
KYSE140	58.1 ± 15.9	13.8 ± 4.3	65.9 ± 21.6
PC3	8.9 ± 3.2	1.7 ± 0.3	22.9 ± 8.3
MDA-MB-468	8.9 ± 1.6	1.8 ± 0.3	27.6 ± 6.9
H3122	8.7 ± 2.3	1.6 ± 0.2	21.8 ± 4.4
THP1	14.4 ± 4.5	4.5 ± 1.4	18.6 ± 3.5
RS4;11	31.4 ± 14.8	3.9 ± 1.8	38.8 ± 9.6
MV4;11	3.0 ± 1.3	0.4 ± 0.1	6.7 ± 1.8
Molm13	7.5 ± 1.9	0.9 ± 0.2	28.9 ± 7.4
Molm14	6.3 ± 2.2	0.6 ± 0.2	15.3 ± 3.9

lines we have tested, but it is less sensitive to KYSE140 and RS4;11 cell lines with IC₅₀ values of 58.1 and 31.4 μM, respectively. It has been reported that ONC201 inhibits cell growth in most of sensitive human cancer cell lines with IC₅₀s between 1 and 10 μM; thus, our results are consistent with the reported results. Similar to their activities to MDA-MB-231 cells, compound **5** is a few fold less potent than ONC201 to all of these cell lines, while compound **4** is 5–10 times more potent than ONC201. It is of note that the cells sensitive to ONC201 are also sensitive to compounds **4** and **5**, and the cells not sensitive to ONC201 are also not sensitive to compounds **4** and **5**, suggesting compounds **4** and **5** have similar mechanism of functions as ONC201. Among all of the cell lines we have screened, PC3, MDA-MB-468, and H3122 are the most sensitive solid tumor cell lines, while MV4;11 is the most sensitive hematological tumor cell line to these compounds.

In order to explore the mechanism of function of our designed compounds, we performed preliminary mechanism studies for compounds **4** and **5** in comparison with ONC201. We at first evaluated these compounds for their ability in promoting TRAIL expression and inducing the production of SubG1 DNA in PC-3 and MDA-MB-468 cell lines, and the results indicated that all of these three compounds can dose dependently up-regulate TRAIL expression and enhance the production of SubG1 DNA (Figure 3). Consistent to their cellular activities, compound **4** at 2.5 μM is more potent than 10 μM of ONC201 in promoting TRAIL expression and

Figure 3. Compounds **4**, **5**, and ONC201 promote the expression of TRAIL and the production of SubG1 DNA.

increasing the production of SubG1 DNA, while compound **5** is less potent than ONC201.

In the TRAIL dependent pathway, ONC201 can inhibit the phosphorylation of AKT and ERK, which leads to the dephosphorylation of Foxo3a; therefore, we treated PC3 cells with different concentrations of compounds **4**, **5**, and ONC201 and performed Western blot analysis for these biomarkers (Figure 4). The results indicated that all of these three

Figure 4. Western blot analysis for compounds **4** and **5** in comparison with ONC201 using human prostate cancer cell PC3. Cell was treated for 24 h.

compounds can dose dependently inhibit the phosphorylation of ERK and AKT and reduce the phosphorylation of Foxo3a, and compound **4** is more potent than ONC201 while **5** is less potent than ONC201; therefore, the ability of these compounds in inducing the changes of these biomarkers is also consistent with their cellular activity.

It has been reported that in the TRAIL independent pathway, ONC201 can induce ER and ISR which culminate at the up-regulation of ATF4; therefore, we also performed Western blot analysis for ATF4, and the results indicated that all of these three compounds can dose dependently induce the up-regulation of ATF4, and again, compound **4** is more potent than ONC201, while compound **5** is less potent than ONC201 (Figure 4). Our biological studies thus suggest that our designed compounds have similar mechanism of functions as ONC201.

After confirming the mechanism of functions of our designed compounds, we then designed and synthesized compounds **7–25** (Table 3) to explore the substituted group on N₃ of the tetrahydropyrido[4,3-*d*]pyrimidine-2,4-dione core structure for its influence to the cellular activity using compound **4** as the lead compound. In these compounds the substituted groups on N₁ and N₆ were kept as ethyl and benzyl groups, respectively. These compounds have been evaluated for their anticancer cellular activities in two sensitive cell lines H3122 and MV4;11, and the results are reported in Table 3. In these two cell lines, **4** shows IC₅₀ values of 1.6 and 0.4 μM, respectively. Compounds **7** and **8**, in which a methyl or an ethyl group was used to replace the benzyl group on the N₃ of compound **4**, respectively, are 45 times less potent than **4** in inhibiting the cell growth of the H3122 cell line and 25 times

Table 3. Cell Growth Inhibition of Compounds **7–25** in H3122 and MV4;11 Cell Lines

Compounds	R	H3122 IC ₅₀ (μM)	MV4;11 IC ₅₀ (μM)
ONC201		8.7 ± 2.3	3.0 ± 1.3
4	Bn	1.6 ± 0.2	0.4 ± 0.1
7	methyl	78.8 ± 20.9	10.1 ± 2.9
8	ethyl	72.9 ± 18.7	9.8 ± 2.3
9		69.2 ± 15.4	8.9 ± 3.1
10		51.9 ± 16.2	5.9 ± 2.2
11		14.8 ± 3.3	2.7 ± 0.9
12		4.7 ± 1.6	0.9 ± 0.2
13		3.9 ± 1.9	0.6 ± 0.2
14		0.8 ± 0.2	0.2 ± 0.1
15		3.9 ± 1.3	0.6 ± 0.2
16		2.6 ± 0.9	0.3 ± 0.1
17		0.4 ± 0.1	0.07 ± 0.02
18		2.7 ± 0.8	0.6 ± 0.2
19		1.6 ± 0.4	0.2 ± 0.1
20		0.6 ± 0.2	0.08 ± 0.02
21		1.9 ± 0.5	0.5 ± 0.1
22		0.7 ± 0.2	0.09 ± 0.03
23		1.9 ± 0.3	0.4 ± 0.1
24		0.9 ± 0.3	0.2 ± 0.1
25		0.6 ± 0.2	0.09 ± 0.03

less potent than **4** in inhibiting the cell growth of the MV4;11 cell line, suggesting that a larger hydrophobic group on N₃ is critical for the cellular activity. Replacement of the phenyl ring of the benzyl group with an aliphatic ring (compounds **9–11**) significantly decreases the cellular activity. In these three compounds, the anticancer cellular activity increases with the enlargement of the ring, but the most potent compound **11** in which a cyclohexyl group is used to replace the phenyl ring is still 7- to 9-times less potent than **4** in these two cell lines, indicating that the phenyl ring is important to the cellular activity.

Introduction of a halo atom to the ortho or meta position of this phenyl ring does not have significant influence on the

cellular activity. For example, compounds **12** and **13**, which have a fluoro atom on the ortho or meta position of the phenyl ring respectively, show comparable cellular activities in both of the two cell lines and are only 2-times less potent than compound **4**. Compounds **18** and **19**, which have a bromo atom on the ortho or meta position respectively, are slightly more potent than **12** and **13**, suggesting that a larger halo atom on these two position is more favorable to the cellular activity. Different from the ortho and meta substitutions, introduction of a halo atom to the para position of this phenyl ring can improve the cellular activity by 2–5-fold (compounds **14**, **17**, and **20** vs **4**). Compound **17** which has a chloro atom on the para position of this phenyl ring shows IC₅₀ values of 0.4 and 0.07 μ M to H3122 and MV4;11 cell lines, respectively, and is the most potent compound in this series. We have also tried introduction of other hydrophobic groups, such as methoxyl, methyl, and trifluoromethyl groups (**21**–**25**) to different positions of the phenyl ring and found the same trend. Interestingly, it seems that the position for introducing the substituted group but not the substituted group itself has more significant influence on the cellular activity. If these groups were introduced to the para position, the cellular activity can be improved, but if the groups were introduced to the ortho or meta position, the cellular activity was not dramatically influenced.

We then explored the substituted group on N₆ for its influence on the anticancer cellular activity by designing and synthesizing compounds **26**–**40**. In these compounds the substituted groups on N₁ and N₃ were kept as ethyl and benzyl groups, respectively, while various hydrophobic groups were used to replace the benzyl group on N₆ of compound **4**. These compounds were also evaluated for their abilities in inhibiting cell growth in H3122 and MV4;11 cell lines, and the results are reported in Table 4. Similar to the modifications of the substituted group on N₃, replacement of the benzyl group on N₆ of compound **4** with small hydrophobic groups, such as methyl, ethyl, or cyclopropylmethyl significantly decreases the cellular activities (compounds **26**–**28** vs **4**) by more than 30-fold. Replacement of the benzyl group with a phenylethyl group (compound **29**) also decreases the cellular activities by more than 10-fold, indicating that this benzyl group is also critical for the cellular activity. We then tried introduction of a halo atom to different positions of the phenyl ring. For this phenyl ring, introduction of a F, Cl, or Br atom to the ortho position dramatically decreases the cellular activity by 4–20-fold (**30**, **33**, and **36** vs **4**), indicating that a substituted group on the ortho position is detrimental to the cellular activity. Introduction of a halo atom to the para position does not have significant influence on the cellular activity (**32**, **35**, and **38** vs **4**), but compounds **31**, **34**, and **37** which have a F, Cl, and Br at the meta position of the phenyl ring have 3–20 times more potent cellular activities in these two cell lines than **4**. Compound **34** shows IC₅₀ values of 0.3 and 0.02 μ M in inhibiting the cell growth in these two cell lines and is the most potent compound in this series. We have also tried introduction of a methoxy group to the meta and para positions of the phenyl ring and found meta substituted compound **39** is slightly more potent than **4** while para substituted compound **40** is about 8 times less potent than **4**. These results also suggested that the meta substituted compound has more potent cellular activity, but **39** is 4 and 10 times less potent than **34** in inhibiting the cell growth of

Table 4. Cell Growth Inhibition of Compounds **26**–**40** in H3122 and MV4;11 Cell Lines

Compounds	R	H3122 IC ₅₀ (μ M)	MV4;11 IC ₅₀ (μ M)
ONC201		8.7 $\pm$ 2.3	3.0 $\pm$ 1.3
4	Bn	1.6 $\pm$ 0.2	0.4 $\pm$ 0.1
26	methyl	84.3 $\pm$ 27.9	19.4 $\pm$ 4.6
27	ethyl	79.6 $\pm$ 24.6	18.8 $\pm$ 3.9
28		54.7 $\pm$ 18.8	13.2 $\pm$ 3.3
29		22.5 $\pm$ 5.9	3.8 $\pm$ 0.9
30		17.3 $\pm$ 4.6	1.6 $\pm$ 0.3
31		0.4 $\pm$ 0.1	0.04 $\pm$ 0.01
32		3.1 $\pm$ 0.8	0.5 $\pm$ 0.2
33		21.9 $\pm$ 2.7	3.3 $\pm$ 0.9
34		0.3 $\pm$ 0.1	0.02 $\pm$ 0.01
35		4.1 $\pm$ 0.9	0.6 $\pm$ 0.2
36		32.5 $\pm$ 11.7	3.9 $\pm$ 1.1
37		0.6 $\pm$ 0.1	0.07 $\pm$ 0.02
38		3.0 $\pm$ 0.9	0.3 $\pm$ 0.1
39		1.2 $\pm$ 0.3	0.2 $\pm$ 0.1
40		11.9 $\pm$ 4.7	1.6 $\pm$ 0.3

H3122 and MV4;11 cell lines, respectively, indicating that a larger group at the meta position is not favorable.

Combining the SAR information obtained from optimizations of the substituted groups on the two phenyl rings, we designed compounds **41**–**49** in which two halo atoms were introduced to the meta position of the phenyl ring in the benzyl group on N₆ and the para position of the phenyl ring in the benzyl group on N₃, respectively. These compounds have also been evaluated for their anticancer cellular activities in H3122 and MV4;11 cell lines, and the results are reported in Table 5. All of these compounds show more potent or comparable cellular activities as the corresponding compounds in which only one of the two benzyl groups on N₃ and N₆ contains halo atom substitution at the corresponding position. For example, compounds **41**–**43** which contain a para fluoro substituted benzyl group on N₃ and a meta fluoro, chloro, or

Table 5. Cell Growth Inhibition of Compounds 41–49 in H3122 and MV4;11 Cell Lines

Compounds	R ¹	R ²	H3122 IC ₅₀ (nM)	MV4;11 IC ₅₀ (nM)
41	F	F	134 ± 29	24 ± 6
42	Cl	F	158 ± 35	21 ± 4
43	Br	F	326 ± 49	55 ± 7
44	F	Cl	217 ± 31	33 ± 7
45	Cl	Cl	147 ± 32	23 ± 6
46	Br	Cl	386 ± 53	59 ± 16
47	F	Br	223 ± 59	39 ± 13
48	Cl	Br	139 ± 41	21 ± 5
49	Br	Br	379 ± 39	62 ± 17

bromo substituted benzyl group on N₆, respectively, show IC₅₀ values of 24, 21, and 55 nM in inhibiting the cell growth of the MV4;11 cell line and are 4–8 times more potent than **14**, in which only the benzyl group on N₃ has a para fluoro substitution (Table 3). These three compounds are also slightly more potent than their corresponding compounds **31**, **34**, and **37**, which contain a benzyl group on N₃ and a fluoro, chloro, or bromo substituted benzyl group on N₆, respectively. Compounds **43**, **46**, and **49**, which contain a meta bromo substituted benzyl group on N₆, show slightly less potent cellular activities than other compounds in this series, indicating that a larger substituted group at the meta position of this phenyl ring is not favorable to the cellular activity, consistent with the SAR found from the optimization of the benzyl group on N₃ (Table 4).

In summary, a series of tetrahydropyrido[4,3-*d*]pyrimidine-2,4-diones have been designed and synthesized as analogues of a clinical lead ONC201. Preliminary mechanism studies indicated that these compounds have similar mechanism of functions as ONC201. These compounds potently inhibit cell growth in a panel of human cancer cell lines, and modifications of the substituted groups on the core structure can dramatically improve the cellular activities. Therefore, these compounds represent a class of novel anticancer agents worthy of further studies. More detailed SAR studies and investigations to identify the protein targets of these compounds are proceeding.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00531.

Experimental details for the synthesis of the designed compounds, assays for cell growth inhibition, Western blot analysis of the biomarkers, the detection of TRAIL concentration, the detection of SubG1 DNA (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: haiyings1969@cpu.edu.cn.

ORCID

Haiying Sun: 0000-0001-5380-5329

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Notes

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