Enhanced Tumor Selectivity of 5-Fluorouracil Using a Reactive Oxygen Species-Activated Prodrug Approach

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Supporting Information

ABSTRACT: We report the design, synthesis, and evaluation of novel 5-fluorouracil (5FU) prodrugs **1a**, **1b** that are efficiently activated by the high level of reactive oxygen species (ROS) in cancer cells. Prodrugs **1a**, **1b** selectively kill cancer cells over normal cells and are well-tolerated in mice. The strategy described herein can extend application of chemotherapeutic drugs.

KEYWORDS: 5-Fluorouracil, prodrugs, reactive oxygen species, anticancer

The FDA-approved chemotherapy drug 5-fluorouracil (5FU, Figure 1) was first introduced by Heidelberger et

Figure 1. Chemical structures of 5FU and clinically used 5FU-prodrugs.

al. in 1957 as an antineoplastic antimetabolite of the uracil anabolic pathway.¹ In the human body, 5FU interferes with DNA synthesis by blocking the activity of the nucleotide synthesis enzyme thymidylate synthase (TS), which catalyzes the conversion of deoxyuridylic acid to thymidylic acid.² As a single agent or in combination with other chemotherapeutics, 5FU has been widely prescribed for a variety of solid tumors including breast cancer, colorectal cancer, stomach cancer, pancreatic cancer, and cervical cancer by injection and skin

cancer as a cream. While remarkably benefiting cancer patients, 5FU also possesses major side effects such as myelosuppression, central neurotoxicity, and gastrointestinal toxicity.³ Moreover, 5FU is metabolically unstable with the majority of the dose converted to 3-fluoroalanine by the pyrimidine degrading enzyme dihydropyrimidine dehydrogenase (DPD).⁴

To overcome the limitation of 5FU, prodrug strategies have been actively pursued. Among numerous small molecule 5FU-prodrugs described in the literature,⁵ four have been successfully used in the clinic, including tegafur, doxifluridine, carmofur, and capecitabine (Figure 1). These compounds are orally available and activated in the human body via different mechanisms. Activation of tegafur involves either a 5'-hydroxylation catalyzed by the cytochrome P450 enzyme CYP2A6⁶ or an enzymatic cleavage of the N1–C2' bond of the molecule.⁷ Doxifluridine is a second-generation of nucleotide-based prodrug of 5FU that has been approved in several Asian countries.⁸ Its activation in tumors is achieved by either thymidine phosphorylase⁹ or pyrimidine-nucleoside phosphorylase.¹⁰ The lipophilic 5FU analogue carmofur overcomes the problem of 5FU degradation by DPD.¹¹ The carbamoyl group of carmofur is enzymatically removed in the human body to release 5FU. Finally, capecitabine is another carbamate-based 5FU-prodrug that is activated in the liver and cancer cells

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Figure 2. ROS-activated SFU prodrugs **1a**, **1b**.

through sequential reactions catalyzed by carboxylesterase and cytidine deaminase.¹²

Reactive oxygen species (ROS), the metabolic byproducts of oxygen metabolism, play an important role in maintaining cellular redox homeostasis. Unlike the normal cell environment where the ROS level is controlled by balancing its production and elimination, cancer cells exhibit enhanced levels of ROS such as superoxide ($\text{O}_2^{\bullet-}$),¹³ hydrogen peroxide (H_2O_2),¹⁴ and hydroxyl radical ($\text{HO}^\bullet$)¹⁵ due to increased metabolic activities and mitochondrial malfunction. It has been shown that high levels of ROS in cancer cells are also associated with DNA mutations that lead to tumor angiogenesis¹⁶ and metastasis¹⁷ and drug resistance.¹⁸ At the same time, the growth and transformation of cancer cells are promoted with the condition of a modest rise of intracellular ROS.¹⁹

While ROS themselves have been exploited for the development of tumor-selective therapeutics by amplifying oxidative stress,^{20–22} ROS-activated prodrugs have also been developed to obtain tumor selective agents.^{23,24} As commonly used bioprecursors, arylboronic acids and esters have been widely employed as specific ROS-activated groups for various drugs including SAHA,²⁵ aminopterin and methotrexate,²⁶ SN-38,²⁷ nitrogen mustards,^{28,29} nitric oxide donors,^{30,31} aminoferrocene,^{32–34} and matrix metalloproteinases (MMP) inhibitors.³⁵ To the best of our knowledge, a similar strategy has not been described for SFU.

Herein we report the design and evaluation of novel arylboronate-based prodrugs **1a**, **1b** of SFU (Figure 2). The *p*-boronate-benzyl group was introduced to the N1 position to limit the metabolic degradation by DPD. The arylboronate groups are designed as ROS-sensitive triggers, which react with H_2O_2 to generate the corresponding phenol intermediate. Spontaneous breakdown of this intermediate will release SFU, along with a highly reactive byproduct 4-methylenecyclohexa-2,5-dien-1-one, which is rapidly hydrolyzed in aqueous environment to give the nontoxic 4-(hydroxymethyl)phenol.³⁶

The synthesis of prodrugs **1a**, **1b** and a control compound **1c** is detailed in Scheme 1. The N1-selective benzylation of SFU using either compound **2** or compound **3** in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as the base generated phenylboronate ester **1a** and control **1c** in modest yields. Then compound **1a** was treated with NaIO_4 in the presence of NH_4OAc to yield compound **1b** in modest yields.

We first tested the efficiency of new prodrugs **1a**, **1b** in releasing the parent drug SFU in the presence of the ROS H_2O_2 (Figure 3). Compounds **1a**, **1b** were incubated in a mixture of DMSO in PBS buffer (30%) with the addition of H_2O_2 (5 equiv) at 37 °C. The conversion of prodrugs **1a**, **1b** and the production of SFU were followed using RP-UPLC-MS. Both prodrugs were activated efficiently by H_2O_2 to release SFU. Interestingly, under the assay conditions, the boronic

Scheme 1. Synthesis of Compounds **1a–1c**.^a

^aReagents and conditions: (a) SFU, DBU, DMF, rt, 12 h, 37% for **1a** and 49% for **1c**; (b) NaIO_4 , NH_4OAc , rt, 16 h, 69%.

Figure 3. Release of SFU from compounds **1a**, **1b**. The incubation of each prodrug (100 μM) was carried out in the presence of 5 equiv of H_2O_2 : (A) prodrug **1a** and (B) **1b**. The conversion of prodrugs **1a**, **1b** and release of SFU was determined. The data represent mean $\pm$ SD of triplicates.

ester **1a** hydrolyzed rapidly to generate boronic acid **1b** (Figure 3A).

Next, we studied the anticancer effects of compounds **1a**, **1b** against 60 human cancer cell lines (the Developmental Therapeutics Program of NCI). The cell lines were derived from different cancer types including leukemia, nonsmall-cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer (Supporting Information (SI), Table S1). The assay protocol can be found at: <https://dtp.cancer.gov/default.htm>. Compound **1a** caused over 50% growth inhibition for most of the tested cell lines. In particular, it exhibited the most significant

inhibition (81–96%) against the lung cancer NCI-H522 cells, melanoma MDA-MB-435 cells, ovarian cancer OVCAR-3 cells, and breast cancer MCF7 and MDA-MB-468 cells (Table 1). In

Table 1. Anticancer Effects of Compounds 1a,1b

tumor type	cell line	inhibition (%) ^a	
		1a	1b
lung cancer	NCI-H522	90	44
melanoma	MDA-MB-435	96	65
ovarian cancer	OVCAR-3	95	30
breast cancer	MCF7	81	33
	MDA-MB-468	87	22

^aEach cell line was treated with either compound 1a or compound 1b at the concentration of 50 μ M for a period of 48 h. Then the growth inhibition (%) values were determined.

general, boronic ester 1a demonstrated larger effects than the boronic acid analogue 1b (SI, Table S1). We reasoned that the differences in anticancer effects between compounds 1a,1b may be due to the more favorable membrane permeability of the boronic ester 1a.

We further evaluated the anticancer effects of compounds 1a,1b in detail using the breast cancer MCF7 cells and normal MEC1 cells (Figure 4). Compound 1a exerted potent but

Figure 4. Anticancer effects and selectivity of 5FU and compounds 1a–1c. Cell viability was analyzed after the breast cancer MCF7 cells (A), and normal MEC1 cells (B) were treated with 5FU or compounds 1a–1c at different concentrations. Data are presented as mean $\pm$ SD, $n = 4$.

lower antiproliferative activity as compared to 5FU, while compound 1b indicated much weaker effects. We reasoned that the decreased anticancer effects of the prodrugs may be due to the diminished cell membrane permeability. Cellular uptake of 5FU is facilitated by organic anion transporter 2 (OAT2) and reduced folate carrier 1 (RFC1),³⁷ which would probably not contribute to the penetration of compounds 1a,1b. On the other hand, compared to 5FU, both prodrugs 1a,1b demonstrated no toxicity toward the normal MEC1 cells (cell viability >95%) at concentrations up to 100 μ M. These differential killing results highlight the specificity of the new

prodrugs 1a,1b for cancer cells over normal cells. Moreover, the control compound 1c, without the ROS-reactive group in its structure, exhibited no obvious antiproliferative effects against the MCF7 cells, indicating that prodrugs 1a,1b achieved their anticancer effects through the reactivity of the boronic acid or ester groups toward the ROS. To further verify the role of ROS in the drug release process, we performed the MTT assays using MCF7 cells pretreated with the ROS scavenger *N*-acetyl-cysteine (NAC). Upon pretreatment of NAC, the anticancer effects of prodrugs 1a,1b decreased significantly (SI, Figure S1), confirming that the new prodrugs exerted their cytotoxicity depending on ROS activation.

To understand the mechanism of action for prodrugs 1a,1b, we examined the impact of the treatment of compounds 1a,1b on γ H2AX formation in MCF7 cells using an immunofluorescence assay. The γ H2AX formation is a sensitive marker to visualize and quantify DNA double-strand breaks and the consequential cell apoptosis.^{38,39} As shown in Figure 5A, upon treatment of compounds 1a,1b, the cells showed dramatical increases in γ H2AX formation, which was consistent with the effect of their parent 5FU. In addition, we also evaluated the effects of prodrugs 1a,1b on the PARP1 and caspase 3 cleavage in MCF7 cells using Western blots assays (Figure 5B). Activation of PARP1 and caspase 3, through cleavage, leads to

Figure 5. Effects of compounds 1a,1b in causing cell apoptosis. (A) Immunofluorescence of DNA cleavage protein γ H2Ax was enhanced by using 5FU and prodrugs 1a,1b. (B) Immunoblot analysis of MCF-7 cells treated with 5FU, prodrugs 1a,1b for apoptosis markers PARP1, and caspase 3. The cells were treated for 48 h with the indicated concentrations of the compounds.

cell apoptosis.^{40,41} Compared to vehicle-treated cells, treatment of prodrugs **1a**, **1b**, similar to 5FU, reduced the levels of both PARP1 and caspase 3 in MCF7 cells. Interestingly, the same treatments of compounds **1a**, **1b**, similar to that of 5FU, significantly increased the levels of cleaved PARP1 (CL-PARP1) and cleaved caspase 3 (CL-caspase 3) in MCF7 cells, as compared with those in the vehicle-treated controls. Therefore, prodrugs **1a**, **1b**, similar to 5FU, can induce MCF7 cell apoptosis, which might contribute to their pharmacological actions.

To evaluate the safety profile of the new prodrugs, we tested the acute toxicity of compound **1a** in wild-type C57BL/6 male mice (Figure 6). While treatment with a high dose (100 mg/

Figure 6. Survival rate (A) and body weight (B) curves of the mice received vehicle, 5FU, or **1a**. The 21 mice were randomly distributed into three groups (seven mice in each group): vehicle (5% DMSO and 15% polyethylene glycol in saline), 100 mg/kg of 5FU, and 100 mg/kg of prodrug **1a**. Mice were treated every other day by intraperitoneal (ip) injection for 15 days, and the survival and body weights of the mice were recorded during the treatment.

kg) of 5FU killed all mice on the twelfth day (Figure 6A), the same dose of compound **1a** did not cause animal death or abnormality in eating, drinking, or activity throughout the period. While body weights of the mice in the 5FU treated group significantly decreased as compared to those of the vehicle group, no obvious changes were observed in the prodrug **1a** treated group (Figure 6B). These results indicate that, compared with the parent 5FU, prodrug **1a** had more favorable drug safety, exhibiting reduced toxicity in normal tissues. This prodrug would benefit further research on its potential as an effective targeted therapy.

In conclusion, we have designed, synthesized, and evaluated novel arylboronate-based 5FU prodrugs **1a**, **1b**. These prodrugs are activated by high levels of ROS in cancer cells and efficiently release 5FU. Prodrugs **1a**, **1b** exhibited high specificity for cancer cells over normal cells. Furthermore, prodrug **1a** demonstrated a more favorable safety profile as compared to parent 5FU. These findings suggest that ROS-activated prodrugs offer an effective way to improve the therapeutic effectiveness and selectivity of current anticancer chemotherapeutic agents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.8b00539.

Experimental procedures and compound characterization for all new compounds (PDF)

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Notes

The authors declare the following competing financial interest(s): Y.S. is a co-founder for and owns equity in Optivia Biotechnology.

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