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Synthesis of novel spin-labeled derivatives of 5-FU as potential antineoplastic agents

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Abstract

Chemotherapy is a general treatment option for various cancers, including lung cancer. In order to find compounds with superior bioactivity and less toxicity against lung cancer, novel spin-labeled 5-fluorouracil (5-FU) derivatives (**3a-f**) were synthesized and evaluated against four human tumor cell lines (A-549, DU-145, KB, and KBvin). Two promising compounds **3d** and **3f** exhibited IC₅₀ values of 2.76 and 2.38 μ M, respectively, against non-small cell lung carcinoma cell line A-549. These compounds were twofold more cytotoxic than 5-FU and less toxic against other tested cell lines. Compound **3f** exhibited seven times more selective cytotoxicity against A-549 than 5-FU. Our results suggest that compounds **3d** and **3f** merit further investigation for development into clinical trial candidates for non-small cell lung cancer.

Keywords

5-Fluorouracil; Spin-labeled; Nitroxide; Cytotoxicity

Introduction

5-Fluorouracil (5-FU, **1**) is a widely used clinical treatment for solid tumors, such as colorectal, breast, liver, gastric, and other cancers. 5-FU is used as a combination chemotherapy for lung cancer (Ghoshal and Jacob, 1997; Huang *et al.*, 2007; Isanbor and O'Hagan, 2006). As an antimetabolite, 5-FU was first synthesized in 1957 (Heidelberger *et al.*, 1957). It is a pyrimidine analogue of DNA and RNA, with a fluorine atom at the C-5 position in place of the hydrogen of uracil (Zhang *et al.*, 2008). 5-FU is an irreversible inhibitor of thymidylate synthetase and consequently interferes with the formation of thymidylic acid from deoxyuridylic acid to inhibit DNA synthesis. As a uracil analogue, 5-FU may be incorporated into RNA to give an aberrant form of RNA, leading to cytotoxicity and cell death (Bosch *et al.*, 1958; Cohen *et al.*, 1958; Goldberg *et al.*, 1966; Madoc-Jones and Bruce, 1968; Kufe and Major, 1981). However, 5-FU is poorly selective toward tumors, and it causes some undesirable side effects, such as gastrointestinal tract reaction, bone marrow depression, dermatologic changes, alopecia, leucopenia, thrombocytopenia, neurotoxicity, cardiotoxicity, and even death (Kennedy, 1999). These side effects have interfered significantly with 5-FU's clinical application. Accordingly, it is necessary to continuously design and synthesize 5-FU analogues that have broad-spectrum or selective antineoplastic activities and can overcome the clinical limitations.

Furthermore, the introduction of a stable nitroxyl radical into a pharmaceutical molecule can, to some extent, reduce toxicity and augment antineoplastic activities (Gadjeva, 2002; Jin *et al.*, 2006; Zheleva and Gadjeva, 2001). In our previous studies, we successfully prepared several series of spin-labeled antineoplastic drugs by modifying different bioactive molecules. When compared with the parent compounds, many of the spin-labeled

compounds showed significant antineoplastic activity with markedly decreased toxicity (Liu *et al.*, 2007, 2012; Liu and Tian, 2005; Tian *et al.*, 2002; Wang *et al.*, 1993). In addition, L-amino acids are actively transplanted into mammalian tissue, have good water solubility, and are often used as carrier vehicles for some drugs. Based on the above facts, we introduced the nitroxyl radical moiety into 5-FU via a hydrophilic amino acid spacer, in an attempt to simultaneously circumvent 5-FU's limitations and develop superior pharmacological profiles. Herein, we report the synthesis and structure–cytotoxicity relationships of a series of novel spin-labeled derivatives of 5-FU as potential antitumor agents.

Results and discussion

Design and synthesis of novel 5-FU analogues

The synthetic route to the *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-amino acids **9a–f** is depicted in Scheme 1. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**5**) was synthesized in 83 % yield by catalytic oxidation of 4-hydroxy-2,2,6,6-tetramethylpiperidine (**4**) with sodium tungstate–hydrogen peroxide–EDTA (Rozantsev, 1964). The reaction of **5** with *N,N*-carbonyldiimidazole gave *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyloxycarbonyl)-imidazole (**6**) (Staab and Mannschreck, 1962). Without purification, this intermediate (**6**) was reacted with *p*-toluenesulfonic acid monohydrate to give a highly reactive tosylate (**7**). When dissolved in an aqueous solution of sodium azide, **7** was converted instantaneously into the alkoxycarbonyl azide (**8**). Compounds **9a–f** were obtained in good yields by reaction of **8** with the appropriate amino acids in the presence of MgO (Hankovszky *et al.*, 1979).

Subsequently, commercially available 5-FU (**1**) was heated with 37 % aqueous formaldehyde for about 50 min at 60 °C to yield 1-hydroxymethyl-5-fluorouracil (**2**) (Ahmad *et al.*, 1987; Ouyang *et al.*, 2011). The target esters **3a–f** were obtained in excellent yields by the straightforward acylation of intermediate **2** with the corresponding *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-amino acids **9a–f** in the presence of *N,N*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) (Scheme 2). The presence of the nitroxide group in the target compounds **3a–f** was confirmed from ESR and IR spectra. In the ESR spectra, the nitroxide group showed a triplet with hyperfine coupling constants (a_N) in the range of 15.90–16.28 G due to interaction between an unpaired electron and nitrogen nucleus of the aminoxyl radical (g value = 2.006). In the IR spectra, bands characteristic of the nitroxyl moiety appeared at $1,370 \pm 7 \text{ cm}^{-1}$ as shown in Table 1. Furthermore, melting point and high-resolution mass spectrometry (ESI) data also characterized the target compounds **3a–f** (Table 1).

Effects of novel 5-FU analogues on tumor cell growth

Target compounds **3a–f** were evaluated for in vitro cyto-toxicity against four tumor cell lines, human alveolar adenocarcinoma (A-549), human prostate carcinoma (DU-145), human nasopharyngeal carcinoma (KB), and human vincristine-resistant nasopharyngeal carcinoma (KBvin). The parent compound 5-FU (**1**) was included as a positive control, and the obtained IC₅₀ values are shown in Table 2. The selectivity index (SI) against A-549 was

calculated as mean IC₅₀ against DU-145, KB, and KBvin divided by IC₅₀ against A-549. Our results demonstrated that **3f** showed the best SI (7.5) against A-549.

5-FU and its spin-labeled derivatives showed the same order of cell line sensitivity: A-549 > DU-145 > KB > KBvin (decreasing potency of test compound). Against the A-549 cell line, compounds **3d** and **3f**, with IC₅₀ values of 2.762 and 2.38 μ M, respectively, were twofold more potent than 5-FU, with an IC₅₀ value of 5.09 μ M. Furthermore, these compounds exhibited good selectivity against A-549, suggesting less toxicity for normal cells. Against the DU-145, KB, and KBvin cell lines, compound **3e**, with IC₅₀ values of 11.36, 11.60, and 11.71 μ M, respectively, was as or slightly more potent than 5-FU, with IC₅₀ values of 10.97, 12.79, and 13.70, respectively. Against the indicated cell lines, the rank orders of activity based on the different amino acid linkages were as follows: for A-549, L-proline > L-phenylalanine > L-methionine > L-leucine > L-alanine > L-valine; for DU-145, L-methionine > L-leucine > L-phenylalanine > L-proline > L-alanine > L-valine; for KB L-methionine > L-leucine > L-phenylalanine > L-proline > L-valine > L-alanine; and for KBvin, L-methionine > L-leucine > L-phenylalanine > L-valine > L-proline > L-alanine. These results showed that the structures of the L-amino acids can have potential effects on the bioactivity of these compounds.

Thus, we have successfully introduced a stable nitroxyl radical into 5-FU via an L-amino acid linkage. Based on the cytotoxicity results, this modification might result in synergistic action against certain tumor cell lines. Further biological evaluation is in progress to better define the antineoplastic activity of these compounds and to clarify whether spin-labeled 5-FU analogues might display decreased side effects compared with 5-FU.

Conclusion

We have synthesized novel spin-labeled derivatives of 5-FU and evaluated their cytotoxic effects against four tumor cell lines by the SRB method. Among all tested compounds, compounds **3d** and **3f** were more cytotoxic than 5-FU against the A-549 lung cancer cell line and merit further investigation for development into clinical trial candidates against non-small cell lung cancer.

Experimental

Chemistry

Melting points were taken on a Kofler melting point apparatus and uncorrected. IR spectra were obtained on NIC-5DX spectra photometer, mass spectral analysis was taken on ZAB-MS and Bruker Daltonics APEXII49e instruments, and ESR spectra were obtained with a Bruker ER-200D-SRC X-band spectrometer. The synthetic compounds were purified by flash chromatography on Merck silica gel (70–230 mesh). Thin-layer chromatography (TLC) was performed on silica gel plates with a fluorescent indicator (Merck Silica Gel 60 F2540.25 mm thick). The *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxy carbonyl)-amino acids (**9a–f**) (Hankovszky *et al.*, 1979) and 1-hydroxymethyl-5-fluorouracil used for the experiments were prepared by modifications of previous procedures (Ahmad *et al.*, 1987; Ouyang *et al.*, 2011).

General procedure for the synthesis of target compounds (3a–f)

A mixture of a *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyloxycarbonyl)-amino acid (0.001 M), 1-hydroxymethyl-5-fluorouracil (0.001 M), and dimethylaminopyridine (DMAP, 0.1 g) was stirred in dichloromethane (10 mL) for 5 min at room temperature under nitrogen. *N,N*-Dicyclohexylcarbodiimide (DCC, 0.21 g, 0.001 M) was added, and the reaction mixture was stirred for 2 h. The reaction mixture was filtered, and the filtrate was evaporated under reduced pressure. The residue was separated by flash column chromatography (gradient elution with mixtures of dichloromethane–acetone) on silica gel and monitored by TLC. Synthesized target compounds **3a–f** were characterized by m.p., ESR, IR, and HRMS spectroscopic analyses.

Cell culture and determination of cytotoxic activity

A-549 (non-small cell lung carcinoma), DU145 (androgen-independent prostate cancer), and KB (nasopharyngeal carcinoma) cell lines were obtained from the Lineberger Comprehensive Cancer Center (UNC-CH). KBvin (vincristine-resistant KB) cell line was a generous gift of Professor Y.-C. Cheng, Yale University. Cells were cultured in RPMI 1640 medium containing 25 mM HEPES and 2 mM L-glutamine (Mediatech), supplemented with 10 % heat-inactivated fetal bovine serum (Hyclone), 100 IU penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Mediatech) in 5 % CO₂ and 95 % air at 37 °C.

Antiproliferative activity was determined by the sulfo-rhodamine B (SRB) colorimetric assay as previously described (Nakagawa-Goto *et al.*, 2011). In brief, cells ($3\text{--}5 \times 10^3$ cells/well) were seeded in 96-well plates filled with culture medium containing various concentrations of sample and incubated for 72 h. At the end of the exposure period, cells were fixed with cold 50 % trichloroacetic acid followed by staining with 0.04 % SRB (Sigma Chemical Co.). The absorbance of solubilized SRB was measured at 515 nm on a Microplate Reader ELx800 (Bio-Tek Instruments, Winooski, VT) with Gen5 software. All results are representative of three or more experiments.

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Scheme 1.

Synthesis of compounds **9a–f**. Reagents and conditions: *i* Na₂WO₄/H₂O₂/EDTA; *ii* *N,N'*-carbonyl-diimidazole/THF; *iii* *p*-toluenesulfonic acid monohydrate; *iv* NaN₃/H₂O; *v* amino acids/MgO, 24 h

Scheme 2.

Synthesis of target compounds **3a–f**. Reagents and conditions: *i* HCHO; *ii* **9a–f**/DCC/DMAP, 2 h

Table 1

Physical and spectroscopic data of compounds **3a–f**

Cmpd.	Molecular formula	mp (°C)	HRMS ([M + NH ₄] ⁺)		IR (cm ⁻¹)	ESR	
			Calcd.	Found		a _N (G)	g
3a	C ₁₈ H ₂₆ FN ₄ O ₇	85–87	447.1792	447.1786	3,330 (N–H), 1,698 (C=O), 1,364 (N–O), 1,244 (C–F)	16.16	2.0060
3b	C ₂₀ H ₃₀ FN ₄ O ₇	64–66	475.2437	475.2440	3,341 (N–H), 1,717 (C=O), 1,367 (N–O), 1,241 (C–F)	15.81	2.0058
3c	C ₂₁ H ₃₂ FN ₄ O ₇	87–89	489.2593	489.2603	3,338 (N–H), 1,717 (C=O), 1,368 (N–O), 1,263 (C–F)	16.04	2.0058
3d	C ₂₄ H ₃₀ FN ₄ O ₇	73–75	523.2437	523.2442	3,341 (N–H), 1,715 (C=O), 1,365 (N–O), 1,259 (C–F)	16.10	2.0061
3e	C ₂₀ H ₃₀ FN ₄ O ₇ S	69–71	507.2157	507.2162	3,333 (N–H), 1,716 (C=O), 1,365 (N–O), 1,246 (C–F)	15.88	2.0060
3f	C ₂₀ H ₂₈ FN ₄ O ₇	83–85	473.2280	473.2275	3,440 (N–H), 1,715 (C=O), 1,370 (N–O), 1,245 (C–F)	16.02	2.0060

Table 2

Cytotoxic activity of **3a–f** against four human cancer cell lines

Compounds	IC ₅₀ (μM)				SI ^a
	A-549	DU-145	KB	KBvin	
3a	12.39 ± 0.91	19.58 ± 0.64	> 20	> 20	1.7
3b	13.10 ± 1.81	> 20	> 20	> 20	1.5
3c	11.14 ± 0.77	12.87 ± 0.32	14.30 ± 0.23	17.08 ± 1.66	1.3
3d	2.76 ± 0.02	13.15 ± 0.34	17.62 ± 0.75	19.09 ± 0.78	6.0
3e	10.28 ± 0.62	11.36 ± 0.71	11.60 ± 0.57	11.71 ± 0.12	1.1
3f	2.38 ± 0.12	15.53 ± 0.97	18.29 ± 0.21	> 20	7.5
5-FU	5.09 ± 0.46	10.97 ± 0.45	12.79 ± 0.22	13.70 ± 0.46	2.4

^a Selectivity index (fold selective against A-549): (mean IC₅₀ against DU-145, KB, and KBvin)/IC₅₀ against A-549