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Conjugated 5-fluorouracil with mitochondria-targeting lipophilic cation: design, synthesis and biological evaluation†‡

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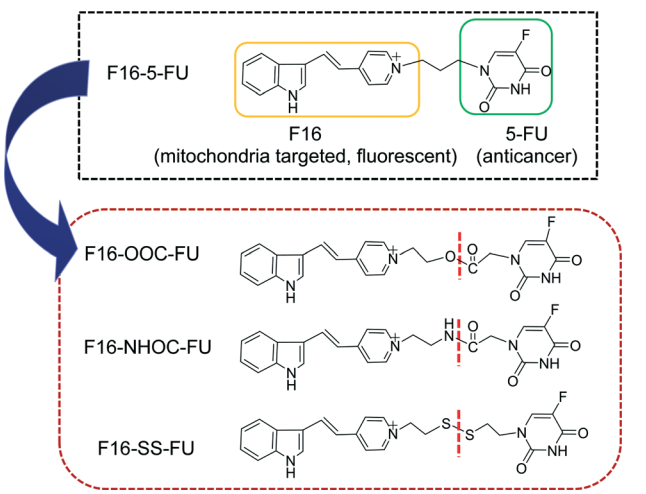
5-Fluorouracil (5-FU) was linked with F16 by vulnerable bonds to selectively target cancer mitochondria which resulted in conjugated compounds, including F16–5-FU, F16–OOC-FU, F16–NHOC-FU and F16–SS-FU. F16–OOC-FU decreased the antiproliferative activity of 5-FU on the nontumor cell line, and the cytotoxicity of F16–SS-FU significantly increased when administered with dithiothreitol (DTT).

As pluripotent organelles, mitochondria constitute the most prominent source of ATP and are implicated in multiple anabolic and catabolic circuitries, controlling cell death as well as several aspects of cell survival.[1] The pioneering work, which started in the early 1990s, demonstrated that mitochondria-localized antiapoptotic proteins could be successful targets for cancer therapy.[2] Since cancer cell mitochondria are structurally and functionally different from their normal counterparts,[3] mitochondria-targeting agents emerge as a means to selectively target cancer for therapeutic purpose.[4]

5-Fluorouracil (5-FU) was introduced in 1957 as a rationally designed anti-cancer agent.[5] It has been widely used in the treatment of a range of cancers, including colorectal, breast and head and neck cancers, and cancers of the aerodigestive tract. However, 5-FU displays nonspecific cytotoxicity for tumor cells and poor distribution to tumor sites. The clinical use of 5-FU would result in inhibition of rapidly dividing tissues such as the bone marrow hematopoietic cells and the gastrointestinal mucosal cells. Well described side effects of 5-FU include myelosuppression, stomatitis, nausea, emesis, and diarrhea.[6]

In order to reduce the side effects of 5-FU and enhance its selectivity towards cancer cells, we conjugated 5-FU with F16 ((*E*)-4-(1*H*-indol-3-ylvinyl)-*N*-methylpyridinium iodide), a delocalized lipophilic cationic (DLC) compound exhibiting mitochondria-specific accumulation in a variety of cancer cells and resulting in cytotoxicity by triggering apoptosis or necrosis.[7] Moreover, F16 shows excellent optical properties with fluorescence emission in the visible region.[7] Considering the specific mitochondria-targeting ability of F16, coupling 5-FU with F16 may improve the selectivity of 5-FU between tumor and nontumor cells and provide the novel compounds with optical imaging ability.

The first conjugated compound designed and synthesized by our team was F16–5-FU, and the structure is shown in Scheme 1.[8] However, the toxicity of F16–5-FU on the human gastric carcinoma SGC-7901 cell line was reduced and did not show selectivity between tumor and nontumor cell lines (Table 1), which was beyond our initial expectation. The mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme



Scheme 1 Conjugating 5-fluorouracil (5-FU) with F16 by three kinds of vulnerable bonds to facilitate the release of 5-FU after using F16 as a carrier for selectively targeting mitochondria in cancer cells.

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Table 1 Cytotoxicity of F16–OOC-FU, F16–NHOC-FU, F16–SS-FU, F16–5-FU and 5-FU on the tumor cell line SGC-7901 and the nontumor cell line GES-1. The ratio represents the cell viability of GES-1 compared to that of SGC-7901

Drug treatment (48 h)	SGC-7901 Cell viability	GES-1 Cell viability	Ratio
DMSO	1 ± 0.035	1 ± 0.044	1
10 μM F16–OOC-FU	0.707 ± 0.029	0.822 ± 0.030	1.16
50 μM F16–OOC-FU	0.597 ± 0.042	0.561 ± 0.051	0.94
10 μM F16–NHOC-FU	0.977 ± 0.042	1.055 ± 0.052	1.08
50 μM F16–NHOC-FU	0.888 ± 0.041	1.069 ± 0.034	1.20
10 μM F16–SS-FU	1.108 ± 0.043	0.978 ± 0.049	0.88
50 μM F16–SS-FU	1.021 ± 0.043	0.895 ± 0.036	0.88
50 μM F16–5-FU	1.019 ± 0.043	1.072 ± 0.053	1.05
10 μM 5-FU	0.986 ± 0.055	0.733 ± 0.031	0.74
50 μM 5-FU	0.600 ± 0.022	0.530 ± 0.028	0.88

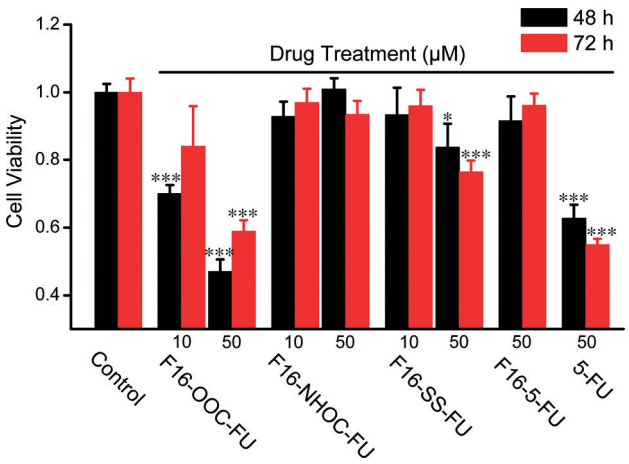

Fig. 1 The cell viability of SGC-7901 cells treated with various concentrations of F16–OOC-FU, F16–NHOC-FU, F16–SS-FU, F16–5-FU or 5-FU for 48 h or 72 h. An asterisk indicates $P < 0.05$ compared to the control group (ANOVA). *, $P < 0.05$; ***, $P < 0.001$.

thymidylate synthase (TS),[9] while F16 concentrates in the mitochondrial matrix and is capable of inhibiting oxidative phosphorylation, inducing mitochondrial transmembrane depolarization and permeability transition.[7] Since the action mechanisms of 5-FU and F16 are different, it may be reasonable to uncouple 5-FU from F16 after using F16 as a carrier for the selective delivery of 5-FU to cancer cells. Therefore, based on optimizing the structure of F16–5-FU, we designed and successfully synthesized three other conjugated compounds linked by vulnerable bonds, including F16–OOC-FU linked by an ester bond, F16–NHOC-FU linked by an amido bond and F16–SS-FU linked by a disulfide bond.

The antiproliferative activities of the four conjugated compounds were preliminarily investigated by testing their antiproliferative effects on human gastric carcinoma (SGC-7901) and human fetal gastric epithelial (GES-1) cell lines. The figures in Table 1 show the viability of SGC-7901 and GES-1 cells with the treatment of these compounds. It was indicated that F16–OOC-FU and F16–NHOC-FU exhibited stronger antiproliferation ratios in SGC-7901 than in GES-1 cells, compared to 5-FU. Moreover, F16–OOC-FU showed the strongest cytotoxicity among these compounds (Fig. 1).

To investigate the stability of the four conjugated compounds, F16–5-FU, F16–OOC-FU, F16–NHOC-FU and F16–SS-FU were incubated in PBS or DMEM (supplemented with FBS) for various hours, and then the fluorescence intensity was tested and compared to that for 0 h (Fig. S1‡). It was observed that the four compounds were stable in PBS for as long as 72 h. Moreover, F16–5-FU, F16–OOC-FU and F16–NHOC-FU were relatively stable for 48 h in DMEM, as their fluorescence intensities didn't change much. Meanwhile, the stability of F16–SS-FU in DMEM might be influenced by proteins and the fluorescence intensity obviously increased as the incubation time extended. This indicated that prolonged treatment time might not be beneficial for conjugates displaying activities.

The optical properties of these synthetic compounds were studied at a concentration of 10 μM in water. Based on the spectra shown in Fig. 2A, the four conjugated compounds all inherited F16's fluorescent characteristic and exhibited close absorption and emission wavelengths, while they displayed red shifts of the maximum absorption wavelength and decreased absorption compared to F16. We took advantage of the optical properties to investigate the cellular localization of these compounds. The results are shown in Fig. 2B and S2.‡ The green fluorescence signal representing F16–OOC-FU overlapped well with the red fluorescence signal produced by the mitochondrion-selective dye MitoTracker® Red CM-H_2-XRos, indicating the mitochondrion-targeting property. F16–SS-FU also exhibited selective accumulation in the mitochondria. On the other hand, the green fluorescence signals representing F16–5-FU and F16–NHOC-FU could hardly be

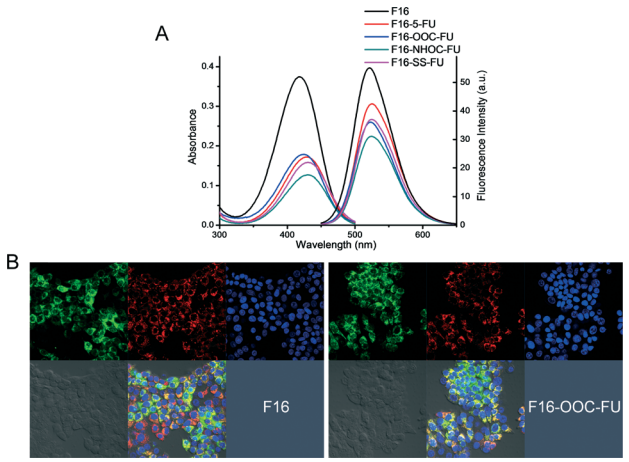

Fig. 2 A: UV-vis absorption and fluorescence spectra of F16, F16–5-FU, F16–OOC-FU, F16–NHOC-FU, and F16–SS-FU (in water). B: Colocalization assay. MCF-7 cells were pre-incubated with 2 μM F16 (green) or 5 μM F16–OOC-FU (green), and then were stained with MitoTracker® Red CM-H_2XRos (red) and Hoechst 33342 (blue).

observed in the SGC-7901 cells, indicating that the two compounds might not enter into cells.

The tumor cell uptake of these conjugated compounds was studied by incubating SGC-7901 cells with the compounds for various hours and then analyzing the cellular fluorescence signal intensity by using a flow cytometer (Fig. 3). Like F16, F16–OOC-FU displayed good uptake after incubating with SGC-7901 cells for 4 h, and F16–SS-FU showed a similar performance. F16–5-FU displayed poor uptake, and F16–NHOC-FU almost could not be found in the SGC-7901 cells. The result is identical with that of cellular localization. The difference in uptake might be attributed to the fact that F16–5-FU and F16–NHOC-FU were predicted to have weaker lipophilicities (clog P −1.25 and −1.65, respectively) compared to F16–OOC-FU and F16–SS-FU (clog P −1.04 and 0.01, respectively).

The above experiments revealed that F16–OOC-FU was the most promising compound among the four conjugates, thus we focused on F16–OOC-FU and further investigated its cytological effect. The IC_{50} values of F16–OOC-FU on two tumor and two nontumor cell lines are listed in Table S1.‡ The cytotoxicity of F16–OOC-FU was not very strong, while it still showed selectivity for the tumor cell lines. Fig. 4A shows the cytotoxicity of F16–OOC-FU on GES-1 cells with 5-FU serving as the control. F16–OOC-FU displayed less antiproliferative effect on GES-1 cells compared to 5-FU. The distinction was especially obvious at low concentrations. Annexin V-PE in conjunction with the viability dye 7-AAD was used to stain the cells, monitored using a flow cytometer to indicate cell death under treatment with F16–OOC-FU. Increasing the concentration of F16–OOC-FU resulted in an increase in cell death, indicated by a decrease in viable, nonapoptotic cells (PE-/7-AAD-) and an increase in the Annexin V-PE positive cell population (Fig. 4B).

Our previous study has shown that F16 would induce an increase in the number of cells in the S phase in SGC-7901 cells, while 5-FU resulted in G1 arrest. The effect of F16–OOC-FU on the cell cycle in SGC-7901 cells seemed to be a mixture of those of F16 and 5-FU. When the treatment concentration of F16–OOC-FU was lower than 20 μM, the percentage of cells in the G1 phase increased. However, as the treatment concentration of F16–OOC-FU further increased, the number of cells in the G1 phase decreased. The number of cells in the S phase was consistently increasing compared

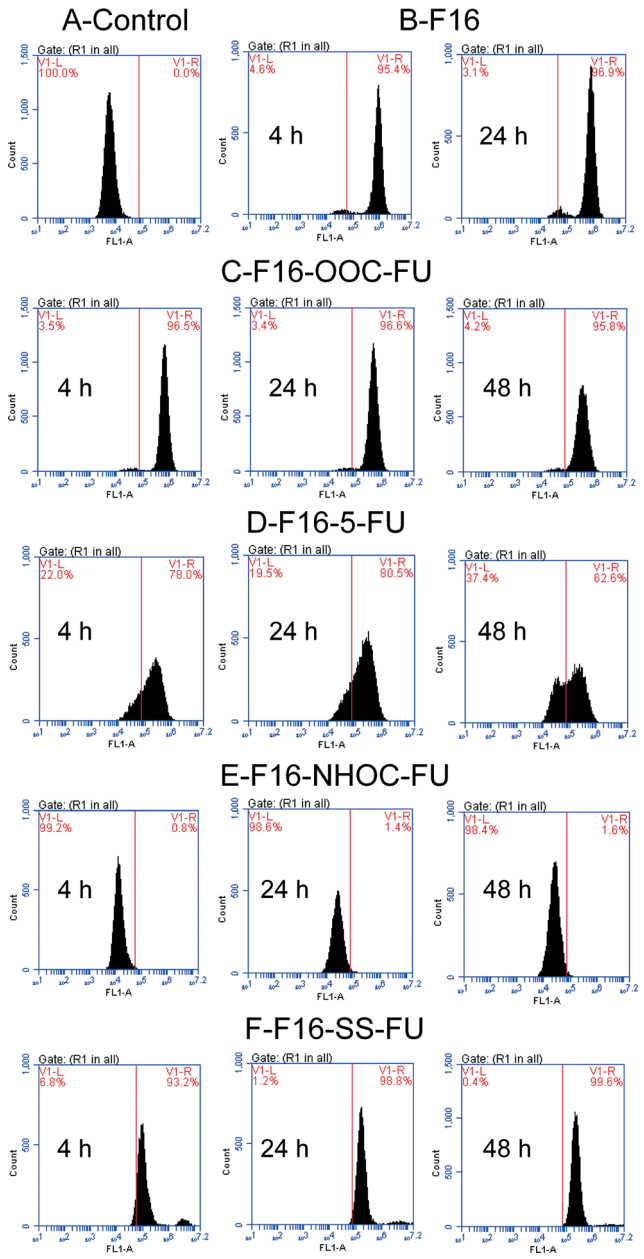

Fig. 3 The uptake of F16 (B), F16–OOC-FU (C), F16–5-FU (D), F16–NHOC-FU (E) and F16–SS-FU (F) in SGC-7901 cells at various time points analyzed by using a flow cytometer.

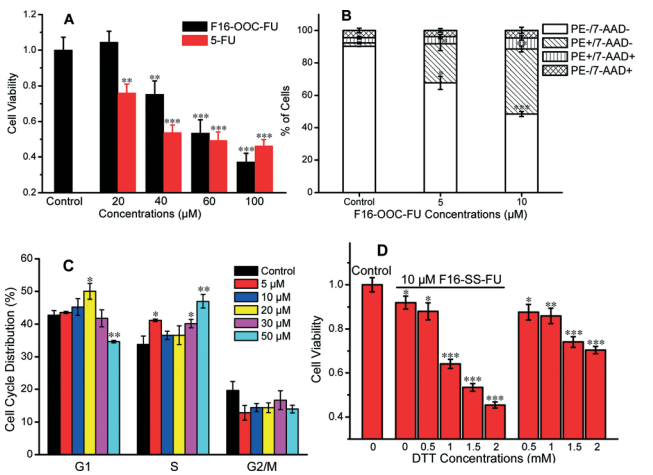

Fig. 4 A: Cytotoxicity of F16–OOC-FU on GES-1 cells in comparison with that of 5-FU. B: Annexin V-PE and 7-AAD double staining on SGC-7901 cells untreated or treated with various concentrations of F16-OOC-FU for 48 h. C: Cell cycle analysis of SGC-7901 cells after treating with increasing concentrations of F16–OOC-FU for 24 h. D: The addition of dithiothreitol (DTT) increased the cytotoxicity of F16–SS-FU on SGC-7901 cells. An asterisk indicates $P < 0.05$ compared to the control group (ANOVA). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

to the control without drug treatment. Despite inducing apoptosis and cell cycle arrest, F16–OOC-FU could also increase the intracellular reactive oxygen species (ROS) level, as indicated by right shifts of the fluorescent signal peaks of dihydroethidium (DHE) and MitoTracker® Red CM-H_2XRos (Fig. S3‡).

In the cellular uptake and localization experiments, the performance of F16–SS-FU was very similar to that of F16–OOC-FU, as they both showed good uptake in SGC-7901 cells and selectively accumulated in the mitochondria, while the antiproliferative effect of F16–SS-FU on the SGC-7901 cell line was not as obvious as that of F16–OOC-FU. Since the cleavage of a disulfide bond needs reduced sulfhydryl, we suspected that enhancing the level of reduced sulfhydryl in the mitochondria might stimulate the breakage of the disulfide bond in F16–SS-FU and thus increase its toxicity. Therefore, we chose to administer dithiothreitol (DTT) with F16–SS-FU (Fig. 4D). The SGC-7901 cells were incubated with F16–SS-FU first, and then DTT was added 4 h later to avoid reacting with F16–SS-FU in the medium. The results showed that DTT could strengthen the toxicity of F16–SS-FU and led to a striking decrease in cell viability. Compared to adding F16–SS-FU and DTT separately, it was found that the increased cytotoxicity was not the sum of the effects of the two compounds, but might result from DTT cleaving the S–S bond in compound F16–SS-FU.

We used F16 as a mitochondria-targeting group and linked it with 5-FU to enhance the cancer cell specificity of 5-FU, and thus successfully synthesized four conjugated compounds. These conjugates all inherited the fluorescent properties of F16, which were used to study the cellular uptake and localization status. In particular, F16–OOC-FU acquired the mitochondria-targeting ability of F16, which resulted in cell death, cell cycle arrest and an increased cellular ROS level in tumor cells and decreased the antiproliferative activity of 5-FU on the nontumor cell line GES-1. Also, the cytotoxicity of F16–SS-FU would significantly increase when administered with DTT. One thing to note is that these conjugates do not show obvious synergistic effects, which may be ascribed to the fact that conjugation causes an effect on the structure of F16 as well as 5-FU, suggesting the importance of designing the structure of the conjugate so as to achieve high cytotoxicity on cancer cells. This study will not only develop a novel way of enhancing the selectivity of 5-FU towards cancer cells, but also expand the usage of F16 as a probe for mitochondrion-targeting.

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