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5′-[2-(2-Nitrophenyl)-2-methylpropionyl]-2′-deoxy-5-fluorouridine as a Potential Bioreductively Activated Prodrug of FUDR: Synthesis, Stability and Reductive Activation

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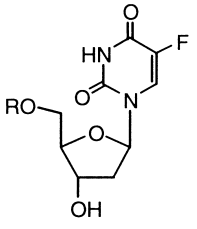
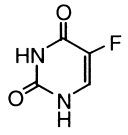
Abstract—5′-[2-(2-Nitrophenyl)-2-methylpropionyl]-2′-deoxy-5-fluorouridine was synthesized as a potential bioreductively activated prodrug of 5-fluoro-2′-deoxyuridine (FUDR). The target compound was stable in both phosphate buffer and human serum and was found to release quickly the parent drug FUDR in quantitative yield upon mild chemical reduction.

5-Fluoro-2′-deoxyuridine (FUDR, **1**) is a fluoropyrimidine nucleoside used in the treatment of a variety of tumors.[1] It exerts its anticancer effects mainly by suppression of DNA synthesis. It is converted by a single thymidine kinase to the active metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate (**2**), which disrupts DNA synthesis via its inhibition of thymidylate synthase and its incorporation into DNA.[2] The simplicity of this pathway provides a strong rationale for the use of FUDR in preference to 5-fluorouracil (5-FU, **3**), which undergoes extensive metabolism to both active fluoropyrimidine nucleotides and other inactive degradation products.[2] Unfortunately, FUDR has not shown consistently superior therapeutic results.[3] It suffers from a number of drawbacks including high toxicity, rapid blood clearance, rapid conversion to 5-FU, poor oral activity and lack of selectivity.



1 R = H
2 R= $PO_3^=$



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Because of the primitive state of tumor vasculature, solid tumors often develop regions of chronic or acute hypoxia as a result of chronic or transient deficiencies of blood flow. Such oxygen deficiency often leads to resistance to ionizing radiation and to many chemotherapeutic drugs.[4–6] Hypoxia also appears to accelerate malignant tumor progression and increase metastasis.[6] Hypoxic tumor cells are known to have a greater capacity for reductive reactions as compared to well-oxygenated normal cells.[6] This unique feature of solid tumors provides an attractive target for selective anticancer chemotherapy. Several bioreductively activated nitro compounds, quinones and aromatic *N*-oxides are currently in clinical trials as hypoxia-selective cytotoxins and could potentially be developed into selective anticancer prodrugs.[7]

Prodrug design is an important strategy that has been proven to work for many drugs in improving their undesirable physico-chemical and biological properties.[8–10] Several strategies based on intramolecular cyclization reactions have been reviewed.[11] Recently, prodrug strategies have also been used in targeted drug delivery including antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT). In these approaches, an enzyme is delivered site-specifically by chemical conjugation or genetic fusion to a tumor specific antibody or by enzyme gene delivery systems into tumor cells. This is then followed by the administration of a prodrug, which is selectively activated by the delivered enzyme at the tumor cells. A number of these systems are in development and have been reviewed.[12–16] Among the enzymes

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under evaluation is a bacterial nitroreductase from *Escherichia coli* B. This FMN-containing flavoprotein is capable of reducing certain aromatic nitro groups to the corresponding amines or hydroxylamines in the presence of a cofactor NADH or NADPH.[17–19]

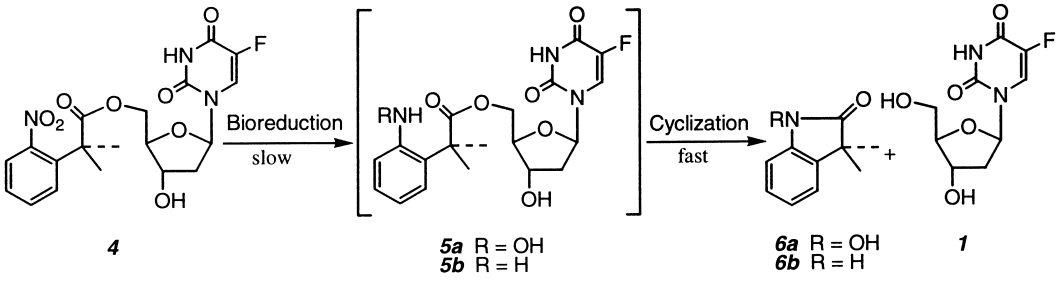
To improve the oral bioavailability and tumor-selectivity of FUDR, we designed and synthesized 5′-[2-(2-nitrophenyl)-2-methylpropionyl]-2′-deoxy-5-fluorouridine (**4**) as a bioreductively activated FUDR prodrug that could be used to target hypoxic cells or used in the enzyme prodrug therapies mentioned above. Scheme 1 shows the potential mechanism of activation of the target compound **4**. After reduction in the hypoxic tumor cells or by an enzyme such as the bacterial nitroreductase, the resulting hydroxylamine **5a** (R=OH) or amine **5b** (R=H) could undergo facile cyclization reaction forming the lactam **6a** or **6b** and, at the same time, releasing the active drug FUDR (**1**). The two methyl groups attached to the α-position of the carbonyl are designed to restrict the rotational freedom of the conformation of the molecule and place the carbonyl group in a more favorable position with respect to the nucleophilic amine or hydroxylamine.[20] In addition, the two methyl groups might inhibit the esterase-catalyzed hydrolysis reaction of the ester bond and provide the necessary stability in human serum, which is required for the compound to be useful. In this communication, we report the synthesis, stability in phosphate buffer and in human serum, and the reductive activation of target compound **4**.

Chemical Synthesis

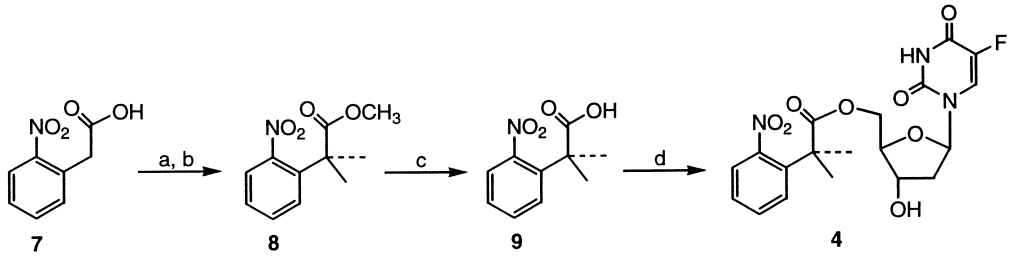
The synthesis of 5′-[2-(2-nitrophenyl)-2-methylpropionyl]-2′-deoxy-5-fluorouridine (**4**) is shown in Scheme 2 starting from the commercially available 2-nitrophenylacetic acid (**7**). After treatment with thionyl chloride ($SOCl_2$) in methanol, the corresponding methyl ester was dialkylated using methyl iodide and sodium hydride in the presence of catalytic amount of 18-crown-6 to give methyl 2-nitrophenyl-2-methylpropionate (**8**). Sodium hydroxide-mediated hydrolysis converted the methyl ester **8** to the corresponding acid **9**. Coupling of the acid **9** to the 5′-primary hydroxyl group of FUDR was accomplished by a Mitsunobu reaction (treatment with diethyl azodicarboxylate (DEAD) and triphenyl phosphine (PPh_3)).[21,22] A major advantage of this approach is that the condensation reaction can be effected under mild and neutral conditions. Furthermore, the reaction is selective for the 5′-primary hydroxyl over the 3′-secondary hydroxyl of the 2′-deoxyribonucleosides, thus avoiding the requirement for protection and subsequent deprotection of the 3′-hydroxyl group.[21] All new compounds were fully characterized by ^{1}H NMR and high-resolution MS.[23]

Stability Test

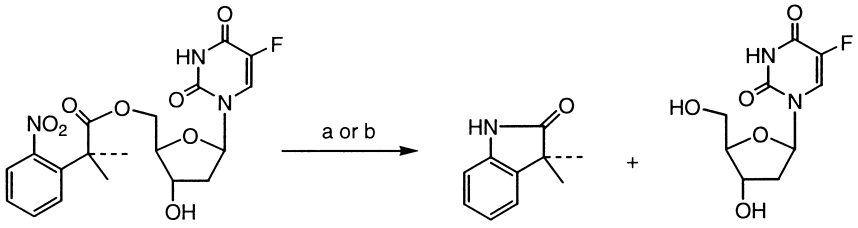
One concern in using compound **4** as a prodrug is the presence of the ester linkage that might make it vulnerable to hydrolysis by esterases present in the blood. We, therefore, tested the stability of compound **4** by incubating it in sodium phosphate buffer (100 mM, pH 7.4) and in human serum at 37 °C. Acetonitrile was used to help dissolve compound **4** and the concentration of acetonitrile in the incubation mixture was kept below 5%. At different time intervals, aliquots (25 μL) were withdrawn and analyzed by HPLC on a C_{18} reversed phase column using acetonitrile/water/TFA as the mobile phase. In the case of human serum, the esterase activity was quenched with 7% $HClO_4$ (100 μL) immediately after the aliquots were withdrawn. Our results indicate that compound **4** is stable in both phosphate buffer and human serum and no significant hydrolysis was observed after three days of incubation. Derivatives



Scheme 1. Proposed mechanism of activation of the FUDR prodrug **4**.



Scheme 2. Synthesis of FUDR prodrug **4**. (a) $SOCl_2$, MeOH (97%); (b) MeI, NaH, 18-Crown-6, 5 °C (89%); (c) NaOH, MeOH (90%); (d) DEAD, PPh_3, FUDR, dioxane, rt (57%).



Scheme 3. Chemical reduction of FUDR prodrug **4**. (a) H_2 balloon, 10% Pd/C, MeOH, h to overnight; (b) $NaBH_4$, 10% Pd/C, MeOH-H_2O, 20 min.

without the two methyl groups at the α-position of the ester carbonyl or without the FUDR moiety (such as in a methyl ester) showed the same stability in phosphate buffer, but were significantly less stable than compound **4** in human serum (data not shown). We attribute this unusual stability of the ester bond in **4** to the steric hindrance provided by the two methyl groups α to the ester carbonyl as well as the deoxy-ribose sugar ring in FUDR.

Chemical Reduction

To test the feasibility of reductive release of the anticancer drug FUDR from compound **4**, we selected two mild chemical reduction conditions that mimick the bioreduction in hypoxic tumor cells and the enzymatic action of a nitroreductase. One was hydrogenation under normal atmospheric pressure in the presence of 10% Pd/C and the other was sodium borohydride ($NaBH_4$) reduction in the presence of 10% Pd/C.[24] Both reduction conditions were mild and neutral; and both would allow selective reduction of the nitro group without affecting other functional groups in the molecule. Both of these chemical reduction methods would only convert the nitro group to the final amino group. Bioreduction in hypoxic tumor cells or reduction by a nitroreductase would more likely stop at the intermediate hydroxylamine before reaching the final amino product. Kinetic studies have shown that cyclization of 2-nitroarylamides via the hydroxylamine intermediate is actually faster than that via the amino product.[25,26] Thus, the hydrogenation and $NaBH_4$ reduction reactions are simple, practical chemical tests of the cyclization-activation system.

Hydrogenation was found to be slower than sodium borohydride reduction. The former required hours of incubation to complete the reaction while the latter took only 20 min to finish. The only isolated products under both reduction conditions were the lactam **6b** and the parent drug **1** (Scheme 3). Both products were identified by ^1H NMR, MS and by comparison with authentic samples.[27] It should be noted that both reduction conditions were very clean and that the presumed intermediate **5b** was not observed. When we used the filtrate directly after partial hydrogenation (4 min) to test the cyclization process in pH 7.4 phosphate buffer at 37 °C, the cyclization was so fast that within seconds the absorbance at 249 nm became constant, making the calculation of a kinetic constant impossible. On the other hand, in a derivative without the two methyl groups, the cyclization process can be accurately monitored by observing the change in absorbance at 249 nm and has a calculated half life of 14 min at 37 °C (data not shown). These results suggest that the two methyl groups at the α-position of the ester carbonyl serve to restrict the rotational freedom of the conformation of the molecule and facilitate the intramolecular cyclization process as proposed in Scheme 1.

In summary, 5′-[2-(2-nitrophenyl)-2-methylpropionyl]-2′-deoxy-5-fluorouridine (**4**) was synthesized as a potential prodrug of FUDR to target hypoxic tumor cells or to be activated by a nitroreductase. Compound **4** is stable and resists the hydrolysis by human serum esterases present in the blood. The release of FUDR from **4** is very fast after conversion of the nitro group to the amino group. Compound **4** could potentially be used to treat hypoxic solid tumors or used in combination with a nitroreductase in antibody-directed or gene-directed enzyme prodrug therapy to improve the physico-chemical properties and the therapeutic effectiveness of FUDR in the treatment of cancer. Further work is underway to study its cytotoxicity towards hypoxic cancer cells or cancer cells in the presence of a nitroreductase and NAD(P)H.

Acknowledgements

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23. For compound **4**: ^{1}H NMR ($CDCl_3$): δ 1.67 (3H, s, CH_3), 1.72 (3H, s, CH_3), 2.22–2.38 (2H, m, 2′-CH_2), 4.11–4.48 (4H, m, 3′-CH, 4′-CH, 5′-CH_2), 6.18 (1H, t, 1′-CH), 7.37–7.65 (4H, m, Ar-H), 7.96 (1H, d, J = 7.8 Hz, 6-CH), 9.09 (b, 1H,-CONHCO-); MS (FAB^+): m/z (relative intensity) 438 (MH^+, 5.4), 308 (13.5), 289 (7.9); HRMS (FAB^+) [MH^+] calcd for $C_{19}H_{21}FN_3O_8$: 438.1313; found: 438.1321.
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27. For compound **6b**: ^{1}H NMR ($CDCl_3$): δ 1.41 (6H, s, $C(CH_3)_2$), 6.91–7.26 (4H, m, Ar-H), 8.20 (1H, b, -CONH-); MS (EI): m/z (relative intensity) 161 (M^+, 100), 146 (48); HRMS (FAB^+) [MH^+] calcd for $C_{10}H_{12}NO$: 162.0919; Found: 162.0898.