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Dual-acting 5-Fluorodeoxyuridine (FUdR) Prodrugs: Cell Death Induced by 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine and 5'-*O*-[bis(2,2,2,-trichloroethyl) phosphoryl-3'-*O*-butanoyl]-5-fluoro-2'-deoxyuridine

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Abstract

Several novel 5-fluoro-2'-deoxyuridine (FUdR) prodrugs with postulated dual mechanisms of anticancer activity based on nucleoside cytotoxicity and cell differentiation induced by retinoic acid/butyric acid have been reported. The *O*-retinoyl- and *O*-butanoyl- esters of FUdR were reported to be more potent and had broader anticancer spectra than either FUdR or 5-fluorouracil (FU) against a bank of human cancer cell lines. The induction of necrosis or apoptosis in HL-60 cells by 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine (RFUdR) and the masked butyryl ester nucleotide, 5'-*O*-*bis*(trichloroethyl)phosphoryl-3'-*O*-butanoyl-5-fluoro-2'-deoxyuridine (BTCEP-BFUdR), is now reported. Apoptosis was the major pathway of HL-60 cell death caused by RFUdR (1×10^{-5} M), independent of the exposure time. In contrast, apoptosis and necrosis were equally evident after exposure to BTCEP-BFUdR (1×10^{-5} M) for 48 h, which is similar to the effect of FUdR. These *in vitro* data support a cytotoxicity model in which release of the nucleoside (FUdR) and the cell differentiator (all-*trans* retinoic acid, RA or butyrate, NaBu) from the respective prodrugs act synergistically to induce greater cell killing.

Keywords: HL-60 cells; Dual-acting prodrugs; Fluorodeoxyuridine; Fluorouracil; Cell death; Retinoic acid; Butyric acid; Apoptosis; Necrosis

Abbreviations: FUdR: Fluorodeoxyuridine; FU: Fluorouracil; RA: Retinoic Acid; BA: Butyric Acid; RFUdR: 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine; BTCEP-BFUdR: 5'-*O*-*bis*(trichloroethyl)phosphoryl-3'-*O*-butanoyl-5-fluoro-2'-deoxyuridine; FUR-TP: 5-fluororibouridine triphosphate nucleotide; FdUMP: Fluorodeoxyribouridine monophosphate nucleotide; FdUTP: Fluorodeoxyribouridine triphosphate nucleotide; TS: Thymidylate synthase; PI: Propidium iodide

Introduction

Cell death pathways are important targets for anticancer drugs [1], and induction of differentiation to activate these pathways represents an attractive approach to chemotherapy [2,3]. The induction of apoptosis, a normal aspect of cell development and homeostasis [4,5], is an important phenomenon in cancer chemotherapy and chemotherapy resistance [6,7]. Most, if not all, anticancer agents kill tumor cell through apoptosis [4,6,8]. In the case of FUdR, interaction with specific biochemical targets may disrupt the cell cycle [9], which in turn may initiate an apoptotic cascade due to induced mistakes or imbalances of regulatory protein molecules [10]. Since apoptosis is closely linked to the normal differentiation pathway [11], induction of malignant cell differentiation may favor progression of the cell to apoptosis [12].

A number of drugs are capable of inducing malignant cell differentiation *in vitro*, including retinoic and butyric acids, and some of their chemical derivatives. The influence of retinoids on cell differentiation in embryology and cancer is well established [13], but the molecular biology of these effects remains the subject of intense research [14]. All-*trans*-retinoic acid (RA) treatment of Acute Promyelocytic Leukemia (APL) pioneered differentiation therapy, and while encouraging, there were several limitations, including its severe, even life-threatening, toxicity [15]. Butyric acid (BA) also induces malignant cell differentiation and inhibits neoplastic cell proliferation in a broad spectrum of neoplastic cells, but its ED_{50} in malignant cells *in vitro* is usually in the mM range [16-18]. Clinical trials of BA in cancer patients have been unsuccessful, possibly due to the difficulty in reaching and maintaining effective plasma concentrations *in vivo* [19].

For potent, cytotoxic, drugs like 5-fluoro-2'-deoxyuridine (FUdR) and its nucleobase, 5-fluodouracil (FU), the complex mechanisms of molecular cell death involve both apoptosis and necrosis, and are still under investigation [20]. FU itself is anabolized intracellularly to several cytotoxic species. Thus, the FU anabolite ribotriphosphate nucleotide (FUR-TP) is incorporated into RNA effecting point mutations, its deoxyribo monophosphate nucleotide (FdUMP) inhibits Thymidylate (TS), and its deoxyribotriphosphate nucleotide (FdUTP) is incorporated into DNA to form multiple point/pairing mutations; of these anabolites, FdUTP; the latter contributes the least to overall cytotoxicity [21]. FUdR has limited clinical utility because its poor bioavailability [22] necessitates intra-arterial infusion [23]. The maintenance of therapeutic FUdR blood levels is difficult because it is rapidly catabolised to FU by pyrimidine phosphorylases in blood and cleared via the urine [24].

One approach to circumvent the pharmacokinetic limitations and rapid metabolism of BA, RA and FUdR is to chemically combine them using biodegradable ester linkages [25]. These combinations may improve their individual properties (e.g., reduced catabolism, delayed elimination, reduced toxicity), give rise to synergistic effects, and improve the pharmacokinetics of delivery to target tissue [26].

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The *in vitro* anticancer mechanisms of 3'-*O*-retinoyl-5-fluoro-2'-deoxyuridine (RFUdR) and the masked butyryl ester nucleotide, 5'-*O*-*bis*(trichloroethyl)phosphoryl-3'-O-butanoyl-5-fluoro-2'-deoxyuridine (BTCEP-BFUdR), shown in Figure 1, are now reported.

Materials and Methods

3'-*O*-Retinoyl-5-fluoro-2'-deoxyuridine (RFUdR) and 3'-*O*-butanoyl-5'-*O*-bis(2,2,2,-trichloroethyl)phosphoryl-5-fluoro-2'-deoxyuridine (BTCEP-BFUdR) (Figure 1) were synthesized by literature methods [25]. Retinoic acid (RA), Sodium butyrate (NaBu) and the reagents used for *in vitro* tests were cell culture quality. Fetal Bovine Serum (FBS), trypan blue and Waymouth cell culture medium were purchased from GIBCO BRL Co., and RPMI 1640 cell culture medium was kindly provided by Dr. M. Suresh (Faculty of Pharmacy and Pharmaceutical Sciences, U. Alberta).

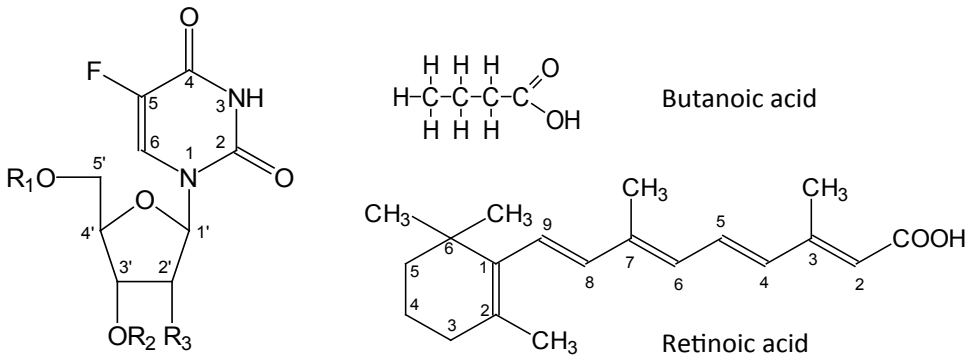
Human acute promyelocytic leukemia cells (HL-60 cells; ATCC) were cultivated in suspension in RPMI 1640 medium supplemented with 10% of fetal bovine serum. The cells were grown at 37°C in a humidified air atmosphere with 5% CO_2. Cell density was determined by a Coulter counter (Coulter Electronics, Ltd.) and cell viability was estimated by trypan blue dye exclusion. Cells were harvested by centrifugation and re-suspended in the growth medium at a density of 2×10^5 cells/mL, then dispensed into 24-well culture plates (1 mL/well). After pre-incubation (24 h), freshly prepared test compounds dissolved in DMSO were added at concentrations of 10^{-6} M and 10^{-5} M for RFUdR, BTCEP-BFUdR, RA and FUdR, and 10^{-3} M for NaBu. The controls contained DMSO only. The final concentration of DMSO was less than 0.1%. Culture plates were incubated for 1, 2 and 7 days, at 37°C in a humidified air atmosphere with 5% CO_2.

Qualitative changes in cell morphology were observed by microscopic observation of Wright-stained cytospin preparations (on slides), which show normal HL-60 cells as typical promyelocytes with large round nuclei (Plate 1).

Quantitative cell death by apoptosis and necrosis were estimated by flow cytometry using a literature method in which the membrane-impermeant Propidium iodide (PI) stain is used to reflect changes in DNA morphology and the membrane permeant Hoechst 33342 (HO342) stain marks the structural integrity of the cell membrane as well as DNA [27]. This method is based on the concept that apoptotic cells will display morphological changes in their nuclei, but not display membrane damage until the final stages of cell death, whereas necrotic cells will demonstrate early loss of membrane function and structural integrity [28]. Following the requisite incubation period, the cells were harvested by gentle centrifugation and the medium was decanted. A stock solution of Hoechest 33342 (0.5 mM in distilled water and stored at 4°C) was diluted to 0.1 mM with Ca^{++}- and Mg^{++}-free Phosphate-buffered saline (PBS) just before use. PI stain (0.03 mM) was freshly prepared in PBS. Fixative was 25% (v/v) ethanol in PBS. The cells were mixed with PI (100 µL) and kept on ice for 30 min, then fixative (1.9 mL) was added with vortex mixing, followed by the addition of Hoechst 33342 (0.1 mM, 50 µL) again mixed by vortex for a few seconds. This cell preparation was then kept on ice for at least 30 min before the measurement of fluorescence intensity by flow cytometer (Coulter Elite Inc.).

Results

Microphotographs of Wright-stained HL-60 cells following incubation for 7 days in the presence of the test drugs are shown in Plate 1. In the absence of prodrug or drug the HL-60 APL cells appear predominantly as typical promyelocytes with large round nuclei (A). The morphologic changes in HL-60 cells induced by the prodrugs included irregularly-shaped nuclei and pyknotic changes in nuclear chromatin. However, the differentiated cell phenotypes, observed after treatment with the prodrugs, were not the same as those induced by RA or BA (B and C), displaying morphological characteristics of cell death, such as highly condensed chromatin, fragmented nuclei, toxic granulation and vacuolation. Cells treated with either RFUdR



Compound	R_1	R_2	R_3
FUdR	H	H	H
RFUdR	H	-retinoyl	H
BTCEP-BFUdR	$(Cl_3C\text{-}CH_2O)_2\text{-}P(=O)\text{-}$	-butanoyl	H

Figure 1: Chemical structures of FUdR, all-*trans*-retinoic acid, butanoic acid and the substitution patterns for RFUdR and BTCEP-BFUdR.

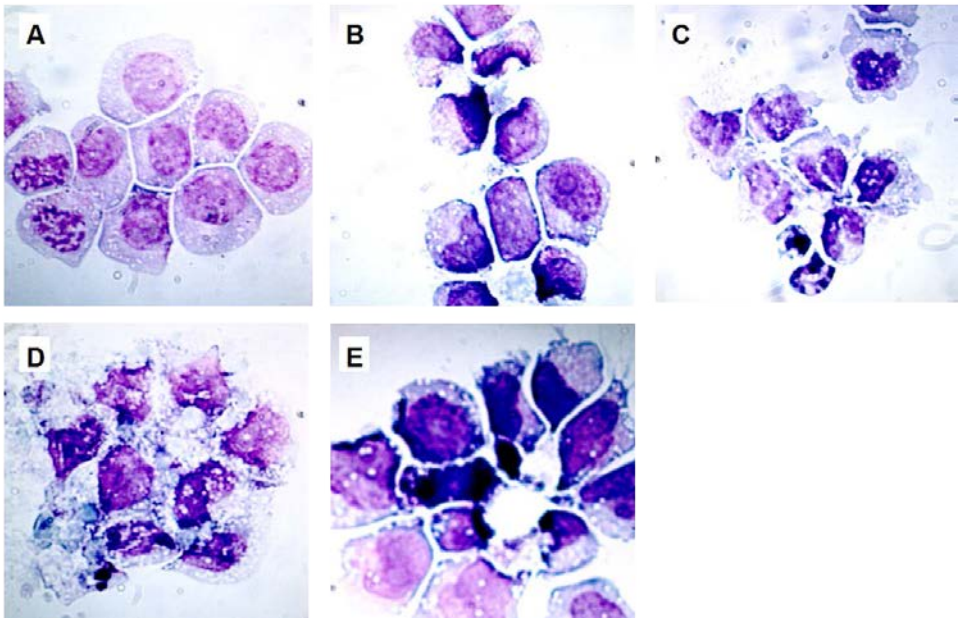


Plate 1: Representative morphology of HL-60 human acute promyelocytic leukemia cells on Wright-stained cytospin slides after 7 days of incubation (A) control; (B) with sodium butanoate (1×10^{-4} M); (C) with retinoic acid (1×10^{-5} M); (D) with RFUdR (1×10^{-5} M); (E) BTCEP-BFUdR (1×10^{-6} M).

or BTCEP-BFUdR thus appeared committed to cell death rather than terminal differentiation or maturation, which may be due to toxic synergism of FUdR or FdUMP released upon hydrolysis of RFUdR and BTCEP-BFUdR, respectively.

Prolonged exposure to all test compounds decreased cell survival, coincident with an increase in cell apoptosis, as determined by flow cytometry (Table 1). A 24 h exposure to either RFUdR or BTCEP-BFUdR, or to their component active moieties (FUdR, RA, BA) had little effect on cell survival, but cell survival dropped markedly after 48 h and especially after 7 days. BTCEP-BFUdR was less toxic than RFUdR or FUdR at 1 x 10^{-6} M concentrations, but there were no clear distinctions regarding mechanisms of death among the compounds other than for BA, which had a negligible toxicity at the concentrations studied.

Discussion

The prodrugs of FU, including the orally stable and well-tolerated FU substitute capecitabine, are effective largely by virtue of their *in vivo* catabolism to FU [29]. Resistance to FUdR can result from the depletion of activated pyrimidine kinases which hydrolyse FUdR to FU [30], high activity of pyrimidine phosphorylase [24], and Thymidylate synthase (TS) overproduction and/or TS structure variation [31]. Only FU, despite its own clinical limitations, is used routinely for anticancer chemotherapy [32]. The current work is based on the hypothesis that a prodrug that can deliver FUdR into the target cell without conversion to FU will have the advantage of facile conversion to active forms, especially FdUMP and FdUTP. Furthermore, masking FUdR with a differentiation-inducing moiety will increase cytotoxicity to non-differentiated (cancer) cells.

Several cell lines have been treated successfully with such combined cytotoxic-differentiation therapy, even when the two drug moieties are not chemically linked, i.e., synergistic effects but not a single prodrug formulation). For example, induction differentiation of M1 myeloid leukemic cells by interleukin 6 increased their apoptotic response to 1-β-D-arabinofuranosylcytosine (ara-C) [33], and enhanced cell killing was observed in human colon carcinoma cells *in vitro* treated sequentially with FU and the differentiation inducer *N*-methylformamide [34]. It has also been reported that retinoids increased the therapeutic efficacy of FU *in vivo* in mice [35]. In initial *in vitro* anticancer studies, the cytotoxicities of two dual acting differentiation-cytotoxic prodrugs, FUdR *O*-retinoyl ester (RFUdR) and a masked FUdR monophosphate *O*-butanoyl ester (BTCEP-BFUdR), were greater and had broader anticancer spectra than either FUdR, RA or BA alone [25]. Apart from the cytotoxic action of FUdR or FdUMP released upon hydrolysis of RFUdR and BTCEP-BFUdR, respectively, the second metabolite (RA or BA) of these double-barreled prodrugs was expected to induce malignant cell differentiation and synergistic induction of cell apoptosis with the cytotoxic metabolite (FUdR or FdUMP). To confirm this postulate, cell morphology and flow cytometric methods were used to monitor the cell differentiation and cell death in human HL-60 acute promyleocytic leukemia cells after exposure of the cells to RFUdR and BTCEP-BFUdR.

The current observations of concentration dependency and complex mechanisms of cell death are rationalized on the basis that these prodrugs might be enzymatically hydrolyzed very slowly [36], therefore, in the drug screening time (48 h), hydrolysis may be incomplete. Following Michaelis-Menten calculations, the hydrolysis velocity is $V=V_{max}[S]/([S]+K_M)$, where V_{max} is the maximum reaction rate; [S] is the concentration of the prodrug; K_M is the Michealis constant, and assuming that the condition satisfies $K_M >> [S]$, we would have $V \cong V_{max}[S]/K_M$, namely, $V \propto [S]$. Therefore, the hydrolytic rate would be slower at lower concentration. Furthermore, these prodrugs release two active drugs with different anticancer mechanisms, each having its own pharmacologically effective concentration; if the concentration of either active drug is lower than the required minimum concentration, it would look like only one of these drugs was effective.

Quantitative cell death was determined by the flow cytometry using a literature technique based on preservation of the integrity of

Compound	Conc. (logM)	1 Day			2 Days			7 Days		
		Apoptosis (mean%, n=2)	Necrosis (mean%, n=2)	Viable cells (%)	Apoptosis (mean%, n=2)	Necrosis (mean%, n=2)	Viable Cells (%)	Apoptosis (mean%, n=2)	Necrosis (mean%, n=2)	Viable Cells (%)
RFUdR	-5	0.7 (1.2)*	0.7 (0.4)	98.6	36.7 (0.1)	9.7 (0.1)	53.6	94.8 (1.0)	0 (0.1)	5.2
	-6	0.9 (0.8)	1.7 (3.3)	97.4	12.6 (1.5)	9.7 (1.4)	77.7	62.8 (29.0)	10 (9.7)	27.2
BTCEP-BFUdR	-5	0.1 (0.8)	0.2 (1.1)	99.7	21.6 (3.3)	16.3 (1.6)	62.1	84.8 (4.0)	2.5 (3.4)	12.7
	-6	0.3 (0.2)	0 (0.2)	99.7	0.3 (0.2)	0.2 (0.7)	99.5	1.5 (1.6)	1.5 (0.4)	97
FUdR	-5	0.6 (1.7)	0.5 (0.7)	98.9	27.5 (2.2)	19.8 (0.2)	52.7	83.9 (5.5)	6.9 (8.2)	9.2
	-6	0.5 (0.3)	1.8 (0.7)	97.7	19.9 (2.5)	17.8 (1.7)	62.3	38.7 (25.8)	8.9 (2.1)	52.4
RA	-5	0.0 (0.2)	0.0 (0.4)	100	0.5 (0.7)	0.0 (0.3)	99.5	56.9 (2.8)	5 (2.5)	38.1
	-6	$	$	$	$	$	$	23.0 (0.4)	4.7 (3.1)	58.9
NaBu	-3	0.3 (1.0)	0.0 (0.1)	99.7	0.5 (0.5)	0.0 (0.6)	99.5	1.5 (0.9)	1.2 (1.2)	97.3

*Data have been normalized with reference to the respective control (drug-untreated) data.
#Data in parentheses represents the differences between duplicate experiments.
$No discernible change.

Table 1: Relative contribution (%)* of apoptosis and necrosis to the cell death following exposure of HL-60 cells to RFUdR, BTCEP-BFUdR and reference compounds FUdR, RA and NaBu.

the plasma membrane in cells undergoing apoptosis until the final stages of the cell death [28]. In contrast, in cell necrosis, one of the earliest changes is loss of membrane function and structural integrity. Although novel fluorescent chromophores such as intrinsic reduced Nicotinamide adenine dinucleotide (NADH) can be used for cell death detection [37], the flow cytometry assay chosen for this work does demonstrate loss of cell membrane function using a DNA-binding fluorescent dyes (PI) and cell membrane integrity using Hoechst 33342. Cell death after treatment with RFUdR, BTCEP-BFUdR, RA, NaBu or FUdR was found to be both dose-dependent and time-dependent (Table 1). RA was weaker inducer of cell death than RFUdR, BTCEP-BFUdR or FUdR at the same concentration (1×10^{-5} M). NaBu was a very weak death inducer even at the high concentration (1×10^{-3} M) compared to the other compounds although it was previously shown to induce the cell differentiation [25] but to be effective *in vitro* only at mM concentrations [18,19].

It is well known that RA functions through several types/subtypes of retinoic acid nuclear receptors (RAR and RXR) that function as transcription factors that regulate the expression of specific genes [38,39]. These responses may be responsible for the proportional increase in differentiated cells, which is quickly followed by an increase in the proportion of cells displaying morphologic characteristics of apoptosis [40]. RA can induce apoptosis in a variety of cell lines [41,42], and in some cases, RA-mediated differentiation is a prerequisite for the induction of apoptosis [12].

Effects of prodrugs RFUdR and BTCEP-BFUdR on induction of cell differentiation and cell death in HL-60 acute promyelocytic leukemia cells show that these prodrugs can induce the malignant cell differentiation, but instead of terminal differentiation, the cells undergoing differentiation experienced enhanced cell death. Apoptosis was the major pathway of HL-60 cell death caused by RFUdR, whereas necrosis was an important part of the HL-60 cell death treated with prodrug BTCEP-BFUdR (as for FUdR), both in a time-dependent manner. *In vitro* data previously showed that some of the prodrugs with cytotoxic-differentiation mechanisms did potentiate cell apoptosis [25], which may be associated with the observed significant increase tumor growth delay *in vivo*. The *in vitro* experiments indicated that of these prodrugs, RFUdR was the most potent compound with the broadest spectrum of anticancer activity. RFUdR has been shown to be a bio-labile double-barreled prodrug releasing two active drugs *in vivo* with cytotoxic-differentiation synergism. RFUdR is potentially useful in cancer chemotherapy for its relatively low toxicity, broad spectrum of anticancer activity, potent induction of cell apoptosis and improved pharmacokinetics compared to either FDU or RA [25,26].

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