The cytostatic activity of 5-(1-azidovinyl)-2′-deoxyuridine (AzVDU) against herpes simplex virus thymidine kinase gene-transfected FM3A cells is due to inhibition of thymidylate synthase and enhanced by UV light (λ = 254 nm) exposure

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Received 17 July 1995

Abstract 5-(1-Azidovinyl)-2′-deoxyuridine (AzVDU) and a series of 5-[1-azido-2-halogenoethyl]-derivatives of β-D-arabinofuranosyluracil (AU) proved markedly inhibitory to the replication of herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV), but not thymidine kinase (TK)-deficient HSV-1 and VZV strains. None of the compounds were cytostatic. However, AzVDU, but not the 5-[1-azido-2-halogenoethyl]-AU derivatives became highly cytostatic against HSV-1 and HSV-2 TK gene-transfected FM3A tumor cells. The molecular target for the cytostatic effect of AzVDU proved to be thymidylate synthase. Short exposure of AzVDU-treated FM3A TK⁻/HSV-1 TK⁺ cells to irradiation at λ = 254 nm enhanced the cytostatic activity of AzVDU by 5-fold.

Key words: Azidovinyldeoxyuridine; Herpes simplex virus; Thymidine kinase; Gene therapy; Thymidylate synthase

1. Introduction

We have recently reported that selective anti-herpetic agents such as (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU, brivudine) [1–3] and various related derivatives of BVDU (i.e. (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVDU) (1), (*E*)-5-(2-bromovinyl)-2′-deoxy-4′-thiouridine (S-BVDU) [4], 2′-fluoro-substituted IVDU (IVFRU) [5] and 5-(furan-2-yl- and thien-2-yl)-substituted derivatives of 2′-deoxyuridine [6]) exhibit a markedly increased cytostatic activity against murine mammary carcinoma FM3A cells transfected with the HSV-1 or HSV-2 thymidine kinase (TK) gene. We also demonstrated that the cytostatic activity of these anti-herpetic drugs against HSV TK gene-transfected tumor cells is based on the inhibition of thymidylate synthase (TS) [2–6]. In 1980, we demonstrated that thymidylate synthase is susceptible to photoaffinity labelling upon administration of light-exposed (*E*)-5-(3-azidostyryl)-2′-deoxyuridine, a compound that provides a highly reactive functional group which interacts with the bound TS enzyme to give enzyme-inactivation [7]. Based on: (i) the current interest in anti-herpetic compounds that can be applied as chemotherapeutic agents against tumor cells in which the thymidine kinase gene of herpes simplex virus has been introduced [8–12]; (ii) the identification of thymidylate synthase as the target enzyme for the cytostatic activity of BVDU and related compounds against HSV TK gene-transfected tumor cells [1–6]; and (iii) our observations that thymidylate synthase may become susceptible to irreversible inactivation by light-exposed azido-substituted 2′-deoxyuridine analogues [7], we designed several novel azido-substituted 2′-deoxyuridine analogues and evaluated their anti-herpetic and cytostatic activity against HSV TK gene-transfected FM3A tumor cells. We found 5-(1-azidovinyl)-2′-deoxyuridine (AzVDU) to be a potent and non-toxic anti-herpetic compound that showed a markedly increased cytostatic activity against HSV TK gene-transfected tumor cells. This cytostatic activity could further be enhanced upon short irradiation of the drug-treated tumor cells at λ = 254 nm.

2. Materials and methods

2.1. Cells

FM3A cells (designated FM3A/0) were derived from a spontaneous murine mammary carcinoma in a C3H/He mouse [13]. FM3A TK⁻ cells, selected for resistance against 5-bromo-2′-deoxyuridine and lacking host cell TK activity, were maintained in RPMI 1640 culture medium containing 10% fetal calf serum and 2 mM L-glutamine, as described previously for the wild-type FM3A/0 cells [13,14]. The FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cell lines, lacking cellular TK activity but containing the HSV-1 and HSV-2 TK genes, respectively, were derived from FM3A TK⁻ cells as reported earlier [14,15]. The culture conditions for the cells were as described above.

2.2. Compounds

The synthesis of the test compounds (AzVDU, AzClEAU, AzIEAU and AzBrEAU) (Fig. 1) will be described elsewhere.

2.3. Radiochemicals

[5-^{3}H]dCyd (specific radioactivity 20.1 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, UK) and Moravek Biochemicals Inc. (Brea, CA).

2.4. Anti-HSV-1, HSV-2, VZV and CMV activity of the test compounds in cell culture

The procedure for measuring antiviral activity in human skin embryo fibroblast (E_6SM) and human embryonic lung fibroblast (HEL) cells has been described previously [17,18]. The assay was based on HSV-1 (strain KOS)-, HSV-1/TK⁻ (strain VMW-1838)- and HSV-2 (strain G)-induced cytopathicity in E_6SM monolayer cell cultures at day 3 post infection, or VZV (strains Oka and YS)-, VZV/TK⁻ (07/1 and YS/R)- and CMV(strains AD169 and Davis)-induced cytopathicity in HEL monolayer cells at day 7 post infection. The IC_{50} was defined as the compound concentration required to reduce virus-induced cytopathicity by 50%.

2.5. Inhibition of cell proliferation by anti-herpetic drugs

The methods for evaluating the cytostatic activity of the test compounds against the different FM3A cells were reported previously [16].

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SSDI 0014-5793(95)00994-9

Briefly, 5×10^4 cells suspended in growth medium were allowed to proliferate in 200-μl wells of microtiter plates in the presence of 5-fold dilutions of the test compounds at 37°C in a humidified CO_2-controlled atmosphere. After 48 h, the number of cells was counted in a Coulter counter (Harpenden, Hertz, UK). The IC_{50} was defined as the compound concentration required to inhibit FM3A cell proliferation by 50%.

2.6. Tritium release from [5-^{3}H]dCyd in FM3A cells

Thymidylate synthase activity was measured in intact FM3A/0 and FM3A TK$^-$/HSV-1 TK$^+$ cells by estimating tritium release from [5-^{3}H]dUrd, which itself had been formed intracellularly from exogenous [5-^{3}H]dCyd. This procedure has been previously described in detail [17].

2.7. Irradiation of AzVDU-treated FM3A cell cultures

FM3A/0 and FM3A/TK$^-$/HSV-1 TK$^+$ cells were seeded in microtiter plate wells in the presence of serial dilutions of the test compounds as described above. Then, the microtiter plate was exposed to irradiation with a Sylvania lamp at $\lambda = 366$ nm or 254 nm at a fixed intensity of approximately 16 lux for different time periods (i.e. 0, 1, 1.5, 2, 2.5, 3 or 5 min). After irradiation, the cell cultures were incubated in the dark at 37°C for 48 h after which the cell number was determined as described above.

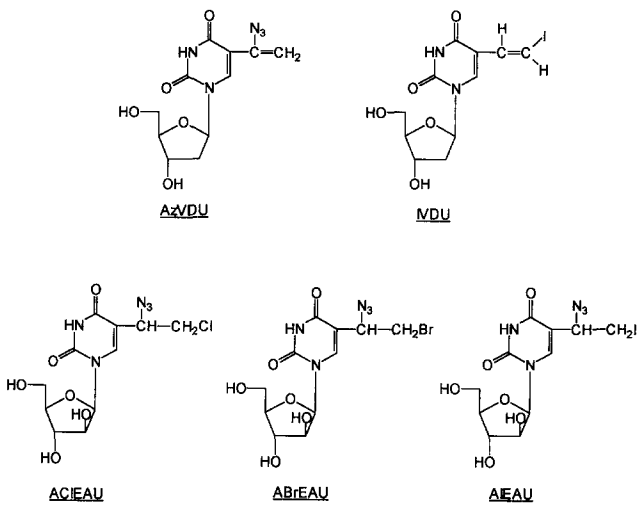

Fig. 1. Structural formulae of AzVDU, IVDU and 5-(1-azido-2-halogenovinyl)-2′-deoxyuridine analogues.

3. Results

3.1. Antiviral activity of azido-substituted 2′-deoxyuridine and 1-β-D-arabinofuranosyluracil analogues in cell culture

5-(1-Azidovinyl)-2′-deoxyuridine (AzVDU) and 5-(1-azidoethyl)-1-β-D-arabinofuranosyluracil analogues containing a chloro (AzClEAU), bromo (AzBrEAU) or iodo (AzIEAU) substituent in the C-2 position of the ethyl moiety were synthesized and evaluated for their anti-herpetic properties. As a rule, all the test compounds proved inhibitory to the replication of HSV-1 and VZV strains in human E_6SM and HEL cell cultures, but not thymidine kinase-deficient (TK$^-$) HSV-1 and VZV strains. The IC_{50} values of the test compounds ranged between 0.4 and 1.8 μM for HSV-1 and 0.02 and 0.23 μM for VZV. Whereas the EAU derivatives were inhibitory to the CMV strains at an IC_{50} that ranged between 1.45 and 10.4 μM, AzVDU and IVDU proved inactive against CMV at 20 μM. None of the test compounds were markedly inhibitory to the proliferation of E_6SM or HEL cells (CC_{50}: 92 to >100 μM).

3.2. Inhibitory activity of azido-substituted 2′-deoxyuridine and 1-β-D-arabinofuranosyluracil analogues against murine mammary carcinoma FM3A cells

The 5-(1-azido-2-halogeno)-EAU derivatives showed poor cytostatic activity against wild-type FM3A/0 and the HSV-1- and HSV-2 TK gene-transfected FM3A TK$^-$/HSV TK$^+$ cell lines. Their IC_{50} ranged between 235 and 814 μM (Table 2).

However, in contrast with the EAU derivatives, AzVDU showed an increased cytostatic activity against FM3A TK$^-$ cells (~30-fold), and, even more so, against the HSV-1 and HSV-2 TK gene-transfected FM3A TK$^-$ cells (~2000-fold and 8000-fold, respectively) (Table 2). The cytostatic activity of AzVDU against the latter two cell lines was thus 3–4 orders of magnitude higher than against the parental FM3A/0 cells. In this respect, AzVDU behaved like IVDU, which also inhibited the growth of the HSV TK gene-transfected tumor cells at a 3,000- to 10,000-fold lower concentration than the parental tumor cells (Table 2).

3.3. Molecular target of the cytostatic activity of AzVDU against HSV TK gene-transfected FM3A cells

The molecular target of the cytostatic action of IVDU had been previously identified as thymidylate synthase [2–5]. The activity of this enzyme in the intact cells can be quantified by measuring the tritium release from [5-^{3}H]dCyd that is added to drug-exposed cell cultures [18]. Indeed, the tritium atom at the C-5 position of the cytosine ring is, upon deamination of dCyd

Table 1
Antiviral activity of 5-(1-azidovinyl)-2′-deoxyuridine and 5-(1-azido-2-halogenoethyl)-1-β-D-arabinofuranosyluracils

Compound	IC_{50} (μM)[a]						CC_{50} (μM)[b]
	HSV-1 (KOS)	HSV-2 (G)	TK$^-$/HSV-1 (VMW 1837)	VZV (Oka,YS)	TK$^-$/VZV (07/1,YS/R)	CMV (AD169,Davis)	(HEL)
AzIEAU	0.46	161	460	0.11–0.21	>11	2.3–4.6	92
AzClEAU	0.58	>580	≥580	0.02–0.04	>15	1.45–3.48	100
AzBrEAU	1.8	390	≥260	0.08–0.234	>13	2.9–10.4	>100
AzVDU	0.4	150	>400	0.1	>50	>20	>200
IVDU	0.02	70	10	0.001–0.002	10–>50	>20	90

[a]50% inhibitory concentration or compound concentration required to inhibit HSV-1- and HSV-2-induced cytopathicity in human E_6SM and VZV- and CMV-induced cytopathicity in human embryonic lung (HEL) fibroblasts by 50%.
[b]50% cytotoxic concentration or compound concentration required to inhibit HEL cell growth by 50%. All data represent means for at least 2 independent experiments.

Table 2
Inhibitory effects of [5-(1-azidovinyl)-2′-deoxyuridine and 5-(1-azido-2-halogenoethyl)-1-β-D-arabinofuranosyluracils on the proliferation of murine mammary carcinoma (FM3A) cell lines transfected with the HSV-1 or HSV-2 thymidine kinase gene

Compound	IC_{50} (μM)[a]			
	FM3A/0	FM3A/TK⁻	FM3A TK⁻/ HSV-1 TK⁺	FM3A TK⁻/ HSV-2 TK⁺
AzIEAU	534	–	235	313
AzClEAU	658	–	400	531
AzBrEAU	814	–	449	382
AzVDU	183	5.5	0.087	0.022
IVDU	18	0.40	0.005	0.002

[a]50% inhibitory concentration or compound concentration required to inhibit FM3A cell proliferation by 50%. Data represent means for 2–3 independent experiments.

(or dCMP) to dUrd (or dUMP), replaced by a methyl group in the thymidylate synthase reaction (dUMP → dTMP + ^3H_3O) and thus inhibition of thymidylate synthase can be monitored by inhibition of tritium release. Whereas AzVDU was not markedly inhibitory to tritium release from [5-^{3}H]dCyd in FM3A/0 and FM3A TK⁻ cells, tritium release from [5-^{3}H]dCyd was substantially inhibited in the HSV TK gene-transfected FM3A tumor cells (Table 3). In fact, tritium release could be nearly 1000-fold more efficiently suppressed by AzVDU in the intact HSV TK gene-transfected FM3A tumor cells than in the parental tumor cells. Thus, AzVDU proved only 5- to 10-fold less potent an inhibitor of thymidylate synthase in FM3A TK⁻/HSV TK⁺ cells than IVDU. The data on inhibition of thymidylate synthase (tritium release) by both AzVDU and IVDU (Table 3) matched the cytostatic potency of the compounds (Table 2).

3.4. *Effect of light exposure on the cytostatic activity of AzVDU against HSV TK gene-transfected FM3A cells*

FM3A/0 and FM3A TK⁻/HSV-1 TK⁺ cell cultures to which AzVDU had been added were exposed to light of long wavelength (λ = 366 nm) or short wavelength (λ = 254 nm) for different time periods (0–5 min). Under our experimental conditions, longer light-exposure times (particularly at λ = 254 nm UV irradiation) resulted in a cytotoxic effect on the control cell cultures (data not shown).

Irradiation at λ = 366 nm did not affect the cytostatic activity of AzVDU against both FM3A/0 and FM3A TK⁻/HSV-1 TK⁺ cells (Table 4). Also, irradiation of AzVDU-treated FM3A/0 cells at λ = 254 nm (UV) light did not alter the cytostatic activity of the drug. However, exposure of AzVDU-treated FM3A TK⁻/HSV-1 TK⁺ cells to λ = 254 nm (UV) light during 1–5 min, after which the cell cultures were further incubated in the dark for 48 h, resulted in a markedly increased cytostatic activity, as compared to the drug-treated cell cultures that were not exposed to UV light. Upon irradiation at λ = 254 nm the cytostatic activity of AzVDU increased by 3- to 6-fold. This increase in cytostatic activity was even noted within one-minute of UV irradiation.

Table 3
Inhibitory effects of AzVDU and IVDU on tritium release from [5-^{3}H]dCyd in murine mammary carcinoma (FM3A) cell lines transfected with the HSV-1 or HSV-2 thymidine kinase gene

Compound	IC_{50} (μM)[a]			
	FM3A/0	FM3A TK⁻	FM3A TK⁻/ HSV-1 TK⁺	FM3A TK⁻/ HSV-2 TK⁺
AzVDU	56	59	0.050	0.097
IVDU	8.8	18	0.005	0.007

[a]50% inhibitory concentration or compound concentration required to inhibit tritium release from [5-^{3}H]dCyd in the intact FM3A tumor cells. Data represent means for 2–4 independent experiments.

Table 4
Effect of irradiation (λ = 254 or 366 nm) on the cytostatic activity of AzVDU against FM3A/0 and FM3A TK⁻/HSV-1 TK⁺ cells[a]

Time of irradiation (min)	IC_{50} (μM)[b]			
	FM3A/0		FM3A TK⁻/HSV-1 TK⁺	
	254 nm	366 nm	254 nm	366 nm
0	163	80	0.122	0.080
1	93	77	0.044	0.097
1.5	100	106	0.031	0.105
2	72	84	0.024	0.078
2.5	108	84	0.039	0.078
3	144	76	0.022	0.074
5	–	90	–	0.117

[a]Irradiation of the drug-exposed cell cultures was started immediately after addition of the test compound at various concentrations.
[b]50% inhibitory concentration or compound concentration required to inhibit FM3A cell proliferation by 50%. Data represent means for 3 independent experiments.

4. Discussion

Novel azido-substituted pyrimidine nucleoside analogues that are endowed with potent and selective antiherpetic properties have been discovered.

AzVDU, as well as the 5-(1-azido-2-halogenoethyl)-substituted AU derivatives, are potent and selective inhibitors of herpes simplex virus type 1 and varicella zoster virus in cell cultures. In contrast with AzVDU and IVDU, AzIEAU, AzBrEAU and AzClEAU also proved to be relatively potent inhibitors of cytomegalovirus infection. The activity of the test compounds against HSV-1 and VZV is dependent on the viral TK-mediated phosphorylation, as indicated by the lack of activity against TK-deficient HSV-1 or VZV strains. The herpetic TK-dependence of the intracellular activation of the test compounds is further corroborated by their poor cytostatic activity against uninfected cells.

Interestingly, AzVDU, but not the 5-(1-azido-2-halogenoethyl)arabinouridine derivatives, displayed a markedly increased cytostatic activity against both HSV-1 and HSV-2 TK gene-transfected tumor cells. As previously demonstrated for BVDU and its structurally closely related derivatives (i.e. IVDU), the cytostatic activity of AzVDU against HSV TK gene-transfected tumor cells is due to a specific inhibition of thymidylate synthase, a well-recognized target enzyme for antitumor chemotherapy [18]. The cytostatic activity of AzVDU against these tumor cell lines could be further enhanced upon short-time exposure to UV light (λ = 254 nm). This may open interesting perspectives in the treatment of some cutaneous disorders such as malignant melanoma. Combination of HSV-1

TK gene therapy with anti-herpetic chemotherapy has been recently introduced in brain tumor-bearing animals that were first intra-tumorally exposed to murine fibroblasts (containing a retroviral vector in which the HSV-1 TK gene had been inserted), and then treated with the anti-herpetic drug ganciclovir [8–11]. This concept is now being further explored in experimental tumor models, including malignant melanomas.

Our findings that UV-light exposure of purified herpetic thymidine kinase in the presence of AzVDU did not affect the activity of the enzyme (data not shown) provide further evidence that the increased cytostatic activity observed in the irradiated drug-treated tumor cell cultures is not due to an altered TK activity (i.e. by increased phosphorylation of the test compound), but most likely to an increased inhibitory effect of the irradiated test compound on the thymidylate synthase in the HSV TK gene-transfected tumor cells.

From our earlier observations we know that BVaraU, the arabinosyl derivative of BVDU, is a potent anti-herpetic agent, being endowed with a high affinity for the HSV- and VZV-encoded TK. However, BVaraU is not markedly cytostatic against HSV TK gene-transfected FM3A cells. The inactivity of BVaraU against the HSV TK gene-transfected FM3A tumor cells could be ascribed to the virtual lack of affinity of BVaraU 5′-monophosphate against thymidylate synthase [19]. Therefore, the inactivity of the 5-(1-azido-2-halogenoethyl)-1-β-D-arabinofuranosyluracil derivatives included in this study against the FM3A TK^-/HSV-TK^+ cells could be most likely ascribed to the same phenomenon as observed for BVaraU. This failure of the arabinofuranosyluracil derivatives against the HSV TK gene transfected tumor cells to be endowed with a marked cytostatic potential provide further evidence for the role of thymidylate synthase as the target enzyme in the cytostatic activity of AzVDU against FM3A TK^-/HSV TK^+ cells.

In conclusion, our photoaffinity approach may open new perspectives for the treatment of cutaneous disorders such as malignant melanoma with respect to azido-based chemotherapeutic agents that show increased cytostatic activity against HSV TK gene-transfected tumor cells upon short (i.e. 1-min) UV light exposure.

Acknowledgements: We thank Lizette van Berckelaer, Anita Van Lierde, Frieda De Meyer and Anita Camps for excellent technical assistance and Inge Aerts, Dominique Brabants and Mieke Vandekinderen for dedicated editorial help. This work was supported by Project 3.0026.91 from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek, and Project 95/5 from the Belgian Geconcerteerde Onderzoeksacties, and Grant MT-17304 of the Medical Research Council of Canada.

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