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Cytogenetic genotoxicity of antiherpes virostatics in Chinese hamster V79-E cells. I. Purine nucleoside analogues

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Abstract

The antiherpes virostatics acyclovir (ACV), valaciclovir (VACV), penciclovir (PCV), famciclovir (FCV) and ganciclovir (GCV), which belong to the group of purine acyclic nucleoside analogues, were tested for clastogenic and sister chromatid exchange (SCE)-inducing activity in Chinese hamster V79-E cells upon chronic application with and without a recovery period. ACV induced borderline effects in both cytogenetic assays, a dose-dependent reduction of the mitotic index and an increasing cell cycle delay. With VACV and PCV only a decrease of the mitotic index and an increase of cell cycle delay were observed. FCV was negative with respect to the four parameters studied, presumably due to the incapacity of the target cells of metabolizing FCV to PCV.

GCV was a very potent genotoxin in both assays. It induced a statistically significant SCE response even in the range of the cytomegalovirus IC_{50} of $< 10\ \mu M$. By variation of the experimental protocol it was shown that SCEs are induced in the second cell cycle following exposure to GCV but not in the first one.

It is assumed that the drugs under study are metabolized to their respective triphosphates and then inhibit DNA replication as detected by decreasing mitotic index and increasing cell cycle delay. In the case of GCV it is suggested that GCV-TP is incorporated into the target cell DNA and that chromosomal aberrations and SCEs are secondary lesions due to repair processes at the substituted template.

Keywords: Virostatics; Antiherpes nucleosides; Purine analogues; Chromosome damage

1. Introduction

Infections with herpesviruses such as herpes simplex virus types 1 and 2, varicella zoster virus, cytomegalovirus (CMV) and Epstein-Barr virus show, in general, a relatively mild course in immunocompetent patients. But they are gaining an increasing importance as the number of immunocompromised patients (AIDS cases, transplant recipients and tumor patients under chemotherapy) is increasing, in which a herpesvirus infection may cause a life-threatening condition. Most of the drugs that are at present available in clinical practice to control these diseases are nucleoside analogues. The mode of action of these agents

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relies on interference with viral nucleic acid synthesis. Their specificity is based on preferential activation/phosphorylation by virus-encoded thymidine kinases and subsequent inhibition of virus-encoded DNA polymerases by nucleoside analogue triphosphates (for recent reviews, see Cameron, 1993; De Clercq, 1994; Kulikowski, 1994). But this specificity is not absolute and it is suggested that agents which interfere with nucleic acid metabolism induce genetic damage not only in viruses but also in the genome of eukaryotic cells and, in the case of therapeutic drugs, might pose a genetic risk to patients.

Although all licensed antiviral drugs have passed preclinical toxicology testing including screening for mutagenicity and genotoxicity, findings on this topic have scarcely been published. In the present paper, we report on cytogenetic effects of five purine acyclic nucleosides that are used for treatment of herpesvirus infections.

2. Materials and methods

2.1. Chemicals

The test agents and their sources were: acyclovir (9-[(2-hydroxyethoxy)-methyl]-guanine/ACV) and valacivlovir (l-valyl ester of acyclovir/VACV) from Wellcome (London, UK), penciclovir (9-(4-hydroxymethylbut-1-yl)guanine/PCV) and famciclovir (9-[4-acetoxy-3(acetoxymethyl)but-1-yl]-2-aminopurine/FCV) from SmithKline Beecham Pharma (Munich, Germany), and ganciclovir (9-[(1.3-dihydroxy-2-propoxy)-methyl]-guanine/GCV) from Syntex Arzneimittel GmbH (Aachen, Germany). With the exception of PCV, the compounds were directly dissolved at a concentration of 2×10^{-3} M in complete culture medium, then further diluted and added to the cell cultures. PCV was available as free acid. It was predissolved in 130 μl 0.3 N NaOH under addition of culture fluid giving a maximum concentration of 2×10^{-3} M. If necessary, the medium was adjusted to pH 7.2 by 0.1 N HCl.

2.2. Cell cultures

Chinese hamster strain V79-E cells (Thust, 1979) were used as the target system. The cells were cultured in Eagle's minimun essential medium (MEM), supplemented by 15% newborn calf serum and penicillin/streptomycin. Asynchronously growing (3×10^5) cells were plated per 25-cm^2 flask under a humidified CO_2-atmosphere. One day later, the experiments were started.

2.3. Clastogenicity and SCE assays

In general, the cultures were exposed to freshly prepared solutions of the test agents for 25 h, either with or without an additional recovery period of 25 h. Flasks provided for detection of SCEs received 5-bromodeoxyuridine (BrdU, 10 μg/ml) during this period. Deviations from this treatment schedule are described in Section 3. Test agent-free cultures served as negative controls. As no exogenous metabolizing system was used whose activity must be checked, and simultaneously a series of alkylating cytostatics was studied (Preuss et al., 1996) which in all cases confirmed the high sensitivity of the assays, positive controls were not considered to be necessary. At 3 h prior to chromosome preparation, colcemid (0.05 μg/ml) was added for metaphase arrest, and the chromosome preparations were made in the usual manner after trypsinization of the cultures. For clastogenicity and mitotic index evaluation the preparations were stained by conventional Giemsa staining. BrdU substitution and SCEs were visualized by the reverse SCE staining technique (Takayama and Sakanishi, 1977).

Clastogenicity was evaluated in 100 consecutive stemline metaphases (22 chromosomes) per sample. The aberration types scored are given in the Tables. Premature chromosome condensations (PCCs) were included in the evaluation because they are micronucleus-derived and may indicate clastogenic or aneugenic effects occurring in previous cell cycles. The mitotic index was calculated from the metaphase ratio in 10 arbitrarily chosen microscopical fields at low magnification (100 ×). SCEs were enumerated in 30 stemline metaphases per sample. Cell cycle progression was estimated

by scoring 100 metaphases per sample for incomplete chromatid contrast (<2 S metaphases), complete chromatid contrast (2 S metaphases) or partial bifilar BrdU substitution on both sister chromatids (>2 S metaphases). The SCE test was assessed to be positive when the highest agent concentration induced at least a doubling of the background SCE rate. Statistical significance (Student's t-test) per se of the SCE values was considered insufficient as a criterion of genotoxic activity. All exposure conditions were checked at least twice in independent experimental series and yielded consistent results.

3. Results

With the exception of GCV the antiherpes drugs tested gave either borderline effects, like ACV in some experiments, or were completely devoid of activity in the clastogenicity assay, even at concentrations which provoke a distinct reduction of the mitotic index (Table 1). FCV did not reduce the mitotic index. Obviously, the target cells are unable to activate this compound to its intermediary metabolite penciclovir. Variation of the treatment conditions (recovery period, etc.), as done in the case of GCV (see below), did not change the genetic activity of the test agents (data not shown).

GCV showed a potent dose-dependent clastogenic activity at concentrations higher than 2.2×10^{-4} M after a 25-h exposure without a recovery period. Concomitantly, there was a distinct reduction of the mitotic index (Table 1). Chromosome preparations made after 25 h cultivation in fresh medium following the 25-h exposure to GCV revealed a dramatic increase of clastogenicity even at the lowest test concentration which did not alter the mitotic index (Table 2). The majority of aberrations were complex translocations, and SCE assays conducted in parallel to these experiments showed that a large proportion of cells with such aberrations had already passed 2 S phases during the posttreatment period, but chromatid breaks occurred in first and second metaphase cells as well. Moreover, it is remarkable that at the highest test concentration, 2×10^{-3} M, under these conditions chromatid breaks are the predominating aberration type (Table 2). In neither case was an increase of premature chromosome condensations observed, thus suggesting that clastogenic effects were not induced in previous mitoses.

ACV, VACV and FCV caused only a marginal SCE response at the maximum concentrations which permitted passage through two cell cycles under the test conditions chosen, but, according to our assessment criteria as described in Section 2, PCV was a weak SCE-inducing agent in V79-E cells (Table 3).

GCV, however, was a very potent genotoxin in the SCE assay (Table 4). When the cells were exposed to GCV for 25 h and allowed to recover for 25 h in drug-free medium, the lowest test concentration (1.1×10^{-6} M, experiment 'b') still induced a statistically significant SCE increase.

From the BrdU substitution pattern of cells exposed to 6.7×10^{-4} and 2×10^{-3} M GCV for 25 h in the presence of BrdU it was concluded that they had passed just one cell cycle. Therefore, further experiments were conducted in which the cells were prelabeled with BrdU for one normal cell cycle period (12 h) prior to GCV exposure in the presence of BrdU (25 h). As expected, most mitoses were 2 S metaphases but, surprisingly, under these conditions the SCE rates induced by 6.7×10^{-4} or 2×10^{-3} M GCV were essentially in the range of untreated controls (data not shown). This observation raised the suspicion that GCV provokes SCE induction almost exclusively in the second or later cell cycle after agent exposure. Experiment 'c' (Table 4) shows that this assumption was correct: an SCE induction is only detectable when the cells had undergone two cell cycles during and after GCV treatment (schedule 'c1') and not after the first cell cycle (schedule 'c2'). Clastogenicity and SCE induction by GCV were proportional to the duration of exposure (data not shown). The SCE rates following schedule 'c1' were therefore much lower than in experiment 'b' at similar test concentrations.

Table 1
Clastogenicity of acyclovir (ACV), valaciclovir (VACV), penciclovir (PCV), famciclovir (FCV) and ganciclovir (GCV) with 25 h exposure, no recovery

Agent/Concentration (M)	Aberrant cells (%) −Gaps/+Gaps	Aberration types									MI (%)
		G′	G″	B′	B″	Ex	Dic	R	Extr	PCC	
ACV											
Control	1/2	1	–	–	–	–	–	–	–	–	20.6
7.3×10^{-6}	0/2	2	–	–	–	–	–	–	–	–	18.6
2.2×10^{-5}	1/5	2	2	–	–	1	–	–	–	1	16.1
6.6×10^{-5}	2/2	–	–	2	–	–	–	–	–	–	16.0
2.0×10^{-4}	3/6	3	–	1	–	1	–	–	–	1	8.4
6.0×10^{-4}	6/10	8	1	4	–	–	1	–	–	1	6.3
2.0×10^{-3}	No mitoses										
VACV											
Control	0/1	1	–	–	–	–	–	–	–	–	19.2
1.1×10^{-4}	1/1	–	–	–	–	–	–	–	–	1	19.7
3.3×10^{-4}	2/3	1	–	–	–	–	–	2	–	–	14.8
1.0×10^{-3}	3/3	1	–	2	2	–	–	–	–	–	5.8
PCV											
Control	1/1	–	–	–	–	–	–	–	–	1	16.8
2.5×10^{-5}	2/3	1	–	–	–	1	–	–	–	1	15.1
7.4×10^{-5}	1/3	2	2	1	–	–	–	–	–	–	18.0
2.2×10^{-4}	0/0	–	–	–	–	–	–	–	–	–	7.5
6.7×10^{-4}	5/6	–	1	4	1	–	–	–	–	–	3.7
2.0×10^{-3}	1/2	–	1	1	–	–	–	–	–	–	4.3
FCV											
Control	1/1	–	–	–	–	–	–	–	–	1	16.2
2.5×10^{-5}	0/0	–	–	–	–	–	–	–	–	–	15.5
7.4×10^{-5}	1/1	–	–	–	–	–	–	–	–	1	14.3
2.2×10^{-4}	0/0	–	–	–	–	–	–	–	–	–	17.5
6.7×10^{-4}	0/1	1	–	–	–	–	–	–	–	–	18.2
2.0×10^{-3}	3/4	1	–	1	–	1	1	–	–	–	12.8
GCV											
Control	1/1	–	–	–	–	–	–	–	–	1	15.7
2.5×10^{-5}	1/1	–	–	1	–	–	–	–	–	–	17.3
7.4×10^{-5}	2/2	–	–	–	–	1	1	–	–	–	13.6
2.2×10^{-4}	2/2	1	1	1	–	1	–	–	–	–	9.3
6.7×10^{-4}	12/13	1	7	5	6	1	–	–	–	2	4.3
2.0×10^{-3}	28/35	8	10	25	3	–	–	1	–	–	1.6

Aberrant cells: −Gaps, gaps excluded; +Gaps, gaps included.
Aberration types (total numbers): G′, chromatid gap; G″, isochromatid gap; B′, chromatid break; B″, isochromatid break; Ex, exchange figure; Dic, dicentric chromosome; R, ring chromosome; Extr, extremely aberrant metaphase; PCC, premature chromosome condensation; MI, mitotic index.

4. Discussion

Published findings on the genotoxicity of the antiherpes virostatics studied in the present paper are so far available only for ACV and, partially, for GCV. The ACV data were reviewed by Clive et al. (1983). The drug was inactive in the Ames assay in five Salmonella strains up to a concentra-

Table 2
Clastogenicity of ganciclovir after 25 h exposure and a recovery of 25 h

Concentration (M)	Aberrant cells (%) −Gaps/+Gaps	Aberration types									MI(%)
		G′	G″	B′	B″	Ex	Dic	R	Extr	PCC	
Control	2/2	–	–	1	–	–	–	–	–	1	16.8
1.0×10^{-4}	16/16	3	–	1	–	15	–	–	–	–	15.4
2.2×10^{-4}	49/49	1	2	16	–	75	–	–	3	–	12.5
6.7×10^{-4}	70/74	19	2	45	6	74	–	–	6	1	7.6
2.0×10^{-3}	78/78	35	4	108	8	29	–	–	2	1	6.3

For explanation, see Table 1.

tion of 3000 μg/ml, inactive for gene mutation in mouse lymphoma L5178Y (OUA and HPRT assays) and in CHO cells (OUA, HPRT and APRT assays), but induced small-type colonies in the L5178Y TK$^{\pm}$ assay at concentrations >400 μg/ml. It is remarkable that other nucleoside analogues with antiviral activity are active in the L5178Y TK$^{\pm}$ assay as well, e.g. (E)-5-(2-bromovinyl)-2′-deoxyuridine (Oshiro et al., 1992), but negative in other mammalian cell gene mutation tests. This assay appears to differ crucially from single gene mutation assays in that it detects large deletions at the heterozygous TK locus (for review, see Glatt, 1994) and, thus, reflects 'chromosomal mutations' revealed as small colonies. Furthermore, ACV was inactive in the C3H 10T1/2 transformation test, in the dominant-lethal test in the mouse and for clastogenicity in the rat in vivo, but ACV was positive in the BALB/c-3T3 transformation assay at 50 μg/ml, and clastogenic in human peripheral blood lymphocytes at >125 μg/ml (Clive et al., 1983). We conclude that these data and those presented here, where ACV induced borderline effects in both cytogenetic assays, are insufficient to classify this drug as genotoxic. Cytogenetic monitoring of patients with recurrent genital herpes medicated with ACV also did not reveal any clastogenic effects in comparison with lifestyle and pretreatment controls, or placebo treatment (Clive et al., 1991). The search for an ACV derivative which combines the safety profile of ACV with a more convenient dosage schedule for the patient recently succeeded with the development of valaciclovir which has an oral bioavailability about 4 times better than ACV. Investigations on genotoxicity of VACV have not been published so far, but based on its mode of activation there is no reason to assume any differences from ACV. The only effect seen in the present study is a distinct reduction of the mitotic index and cell cycle delay at the highest concentrations tested. This reduction is somewhat lower than with ACV and can be explained by a limited capacity of the target cells to cleave this valyl ester by esterases, although V79-E cells possess an intrinsic esterase activity (Thust and Schneider, 1989).

Hitherto no data on the screening of FCV and PCV for genotoxicity have been published. FCV shows a better oral absorption than PCV and is metabolically converted to PCV by an aldehyde oxidase and an esterase. The clinical benefit of both drugs relies on the longer intracellular half-life of 7–20 h, depending on the virus type, of the ultimately reactive virostatic metabolite PCV-TP compared with ACV-TP (Morse et al., 1993). FCV was completely negative in our assays, presumably because V79-E cells are devoid of the oxidase activity necessary to convert it to PCV. PCV was non-clastogenic but reduced the mitotic index and caused a strong cell cycle delay at concentrations higher than 2.2×10^{-4} M. The relevance of the doubling of the SCE rate at the maximum test concentration after a recovery of 25 h is still unclear and deserves further studies. Till now there seems to be no reason to classify these virostatics as genotoxic.

Table 3
SCEs and cell cycle delay induced by acyclovir (ACV), valaciclovir (VACV), penciclovir (PCV), and famciclovir (FCV) after 25 h exposure in the presence of BUdR, with no recovery

Agent/Concentration (M)	SCEs/cell			Cell cycle state (%)		
	Mean	S.D.	*P*	<2S	2S	>2S
ACV						
Control	7.63	2.34	–	9	91	0
7.3×10^{-6}	6.97	2.80	>0.05	7	93	0
2.2×10^{-5}	6.80	2.20	>0.05	18	82	0
6.6×10^{-5}	7.13	2.10	>0.05	34	66	0
2.0×10^{-4}	8.40	2.80	>0.05	90	10	0
4.0×10^{-4} [a]	11.20	4.60	<0.001	1	89	10
6.0×10^{-4}	N.d.			100	0	0
1.0×10^{-3} [b]	7.00	2.51	>0.05	2	94	4
VACV						
Control	6.67	2.77	–	1	97	2
1.1×10^{-4}	7.27	2.64	>0.05	2	96	2
3.3×10^{-4}	7.27	2.82	>0.05	5	95	0
1.0×10^{-3}	8.37	3.02	<0.05	74	26	0
PCV						
Control	6.77	2.46	–	1	97	2
2.5×10^{-5}	7.00	2.18	>0.05	2	96	2
7.4×10^{-5}	7.47	3.87	>0.05	5	95	0
2.2×10^{-4}	6.80	2.94	>0.05	14	86	0
6.7×10^{-4}	7.50	2.81	>0.05	52	48	0
2.0×10^{-3}	N.d.			100	0	0
2.0×10^{-3} [b]	16.67	5.02	<0.001	85	15	0
FCV						
Control	6.63	1.94	–	1	96	3
2.5×10^{-5}	7.17	2.59	>0.05	1	96	3
7.4×10^{-5}	6.47	2.86	>0.05	0	98	2
2.2×10^{-4}	7.07	2.42	>0.05	0	98	2
6.7×10^{-4}	7.47	2.85	>0.05	0	98	2
2.0×10^{-3}	9.63	2.25	<0.001	0	100	0

Abbreviations: N.d., not detectable in consequence of cell cycle delay.
[a] Culture prelabeled with BUdR for one cell cycle (12 h) prior to drug exposure.
[b] 25-h exposure without BUdR, 25-h drug-free recovery with BUdR.

GCV has been checked for SCE induction in human blood lymphocytes by De Clercq and Cassiman (1986) and was found positive at a concentration of 3.5×10^{-5} M (48 h exposure). According to the manufacturer's information (Syntex, package insert) it "may cause hereditary damage" and "should be considered as potentially carcinogenic". The present investigations have shown that GCV is a very potent genotoxin in mammalian cells and corroborate the assessment by the manufacturer. The antiviral activity and selectivity of the nucleoside analogues is exerted on two levels. First, these drugs are efficiently phosphorylated by herpesvirus-encoded nucleoside kinases (or, for CMV, protein kinase; for review, see De Clercq, 1995) due to their less stringent substrate specificity compared with homologous cellular enzymes. Further metabolization of the nucleoside monophosphates is accomplished by cellular and, partially, viral nu-

Table 4
SCEs and cell cycle delay induced by ganciclovir under different treatment conditions

Experiment	Concentration (M)	SCEs/cell			Cell cycle state (%)		
		Mean	S.D.	*P*	<2S	2S	>2S
(a)	Control	7.17	2.67	–	2	96	2
	2.5×10^{-5}	10.17	3.67	<0.001	4	94	2
	7.4×10^{-5}	17.67	4.61	<0.001	14	86	0
	2.2×10^{-4}	31.27	7.63	<0.001	44	56	0
	6.7×10^{-4}	N.d.	–	–	100	0	0
(b)	Control	6.77	2.44	–	0	95	5
	1.1×10^{-6}	10.40	3.42	<0.001	3	95	2
	3.3×10^{-6}	10.42	3.02	<0.001	2	98	0
	1.1×10^{-5}	16.20	4.85	<0.001	3	97	0
	3.3×10^{-5}	28.27	6.62	<0.001	13	87	0
	1.0×10^{-4}	61.80	14.74	<0.001	42	58	0
	2.2×10^{-4}	93.55	16.71	<0.001	95	5	0
(c)	Control	7.33	1.95	–	0	96	4
(c1)	2.2×10^{-4}	27.43	5.54	<0.001	10	90	0
(c2)	2.2×10^{-4}	7.37	2.31	>0.05	0	97	3
(c1)	6.7×10^{-4}	47.67	11.13	<0.001	35	65	0
(c2)	6.7×10^{-4}	8.30	2.64	>0.05	0	96	4

For abbreviations, see Table 3.
Experiments (treatment schedules): (a) 25-h exposure in the presence of BUdR, no GCV-free recovery; (b) 25-h exposure to GCV without BUdR, 25-h recovery in the presence of BUdR; (c1) 12 exposure with GCV + BUdR, 12-h GCV-free recovery with BUdR; (c2) 12-h GCV-free prelabeling with BUdR, 12-h exposure to GCV + BUdR.

cleotide phosphotransferases leading to the respective triphosphates. The second level of antiviral selectivity is at the DNA polymerase function. The nucleoside analogue triphosphates act versus dGTP as competitive inhibitors of viral DNA polymerase (for review, see Kulikowski, 1994). In contrast with ACV-TP which inevitably causes strand termination, the triphosphates of the other antiherpes drugs could, theoretically, be internally incorporated into DNA via formation of phosphodiester linkages. Obviously, this incorporation leads to the inhibition of further virus replication, but this mechanism is not yet fully elucidated.

The fact that all hitherto developed antiherpes nucleosides are cytotoxic to mammalian cells although, in general, at much higher concentrations than their efficiently virostatic concentrations, yields circumstantial evidence that these compounds are processed by mammalian enzymes as well and are probably incorporated into mammalian DNA. This has been supposed in many papers but quantitative data are still lacking. The ratio of cellular IC_{50} to viral IC_{50} (therapeutic index) can vary between 3000 for ACV and a very low value in the case of GCV. Our experiments have shown that GCV induces a significant SCE increase in the range of the CMV IC_{50} of < 10 μM.

Although so far no definitive proof exists, we suppose that the inhibition of proliferation as expressed by the reduced mitotic index and the cell cycle delay observed in the present study are mainly caused by inhibition of cellular DNA polymerases.

By their structure and mode of action it is unlikely that the acyclic nucleoside analogues studied, if genotoxic, exert adverse effects by DNA adduct formation as do 'typical' genotoxins. Possible explanations for genotoxic effects induced by these agents are nucleotide pool imbalances, disturbance of the fidelity of replication, or secondary lesions due to repair at the sites of incorporated modified nucleosides (for review, see

Negishi et al., 1994). We assume that the latter process is the most likely one to explain the adverse effects. Hitherto no direct proof exists for incorporation, but the effects observed with GCV in the present study yield circumstantial evidence. The delayed genotoxic effect induced by this agent strongly resembles our earlier findings with alkylating agents (Thust et al., 1980) which exerted their maximal clastogenic activity in the second or later posttreatment mitosis (for reviews, see Galloway, 1994; Galloway et al., 1994). We suggest that the delayed clastogenic effects are caused by continued replication from the template containing alkylated bases in the case of alkylating mutagens (Kaina and Aurich, 1985) or incorporation of abnormal nucleoside analogues (i.e. with the compounds studied here) followed by secondary lesions due to repair processes (postreplication repair) leading to double-strand breaks and chromatid and chromosome aberrations. The preponderance of chromatid breaks at the highest GCV concentration over translocations, which predominate at lower drug concentrations, is noteworthy (Table 2). As increasing GCV concentrations provoke an increasing cell cycle delay, it is suggested that the secondary lesions (double-strand breaks) first form chromatid breaks which are, in the course of ongoing cell cycle progression, secondarily ligated to translocations. This assumption needs further investigations with other methods. The observation that GCV induces SCEs only in the second cell cycle after exposure suggests that this damage type relies on secondary lesions as well. In contrast to GCV, the antiherpes pyrimidine analogue (E)-5-(2-bromovinyl)-2′-deoxyuridine exerts its major SCE-inducing capacity in the first cell cycle after treatment (Thust et al., unpublished data).

In conclusion, the present study on adverse cytogenetic effects of antiherpes acyclic nucleoside analogues confirms the statement made by Darby (1994) with respect to their antiviral properties: "In the world of nucleoside analogues, there is no such thing as a 'close' analogue. As soon as a nucleoside analogue is modified, there is the potential for considerable change in biological properties".

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