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Evaluation of the mutagenic and genotoxic activities of anti-hepatitis B analogs of β-L-adenosine by the Ames test and the Comet assay

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Abstract

β-L-2′-deoxyadenosine (β-L-dA), β-L-2′,3′-dideoxyadenosine (β-L-ddA) and its two bis (*S*-acyl-2-thioethyl; SATE) phosphotriester derivatives, β-L-2′,3′-dideoxyadenosine-5′-monophosphate-bis(MeSATE) and β-L-2′,3′-dideoxyadenosine-5′-monophosphate-bis(tButylSATE) have been previously shown to exhibit potent and selective anti-hepatitis B activity in vitro. None of the four compounds was mutagenic up to 100 μg in the Ames test (microtechnique) using *Salmonella typhimurium* strains TA 97a, TA 98, TA 100 and TA 102, with and without metabolic activation. In addition, the genotoxicity of β-LdA and the three other compounds was evaluated in human lymphocytes using the Comet assay, at doses up to 5 μg with or without the addition of a microsomal S9 fraction. None of the four compounds induced DNA strand breakage with and without metabolic activation. In summary, the data clearly demonstrate that the purine nucleoside β-L-dA, β-L-ddA and the two prodrugs, β-L-ddAMP-bis(MeSATE) and β-L-ddAMP-bis(tButylSATE) are not mutagenic in the Ames test and do not induce DNA damage in human lymphocytes, as assessed by the Comet assay.

Keywords: Purine nucleosides; Genotoxicity; Ames test; Comet assay; Hepatitis B

1. Introduction

It has been estimated that, ≈5% of the world's population is chronically infected with the hepatitis B virus (HBV; Hoofnagle, 1998). Of these chronic carriers, 25–40% will ultimately develop cirrhosis or hepatocellular carcinoma (Sherker

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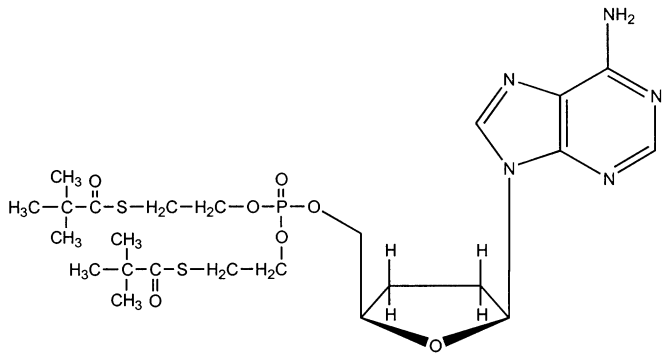
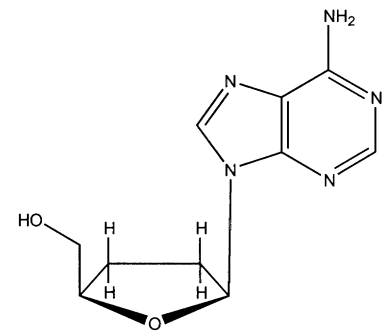
and Marion, 1991). A few years ago, HBV vaccination was implemented to prevent the development of new infections, but very limited effective treatments are currently available for the chronic carriers. Several nucleoside analogs have been discovered to be potent inhibitors of HBV replication (Shaw and Locarnini, 1995). Nucleoside analogs per se do not possess anti-hepatitis B activity, and must be metabolized to their respective 5′-triphosphate derivatives. These intracellular metabolites are either inhibitory to the HBV polymerase or incorporated into the growing viral DNA chain and terminate replication. Because of the chronicity of HBV infection maintained by the persistence of the viral covalently circular closed DNA (cccDNA) in hepatocytes, short-term monotherapy will not be sufficient to clear viral infection. Therefore, long-term administration of a combination of several HBV DNA polymerase inhibitors will probably be required for seroconversion. It has been suggested that a minimum of 5-yr therapy will be required to possibly eliminate viral infection (Nowak et al., 1996), however, this strategy will place the patient at risk of developing drug toxicity and particularly drug-induced genotoxicity (Lee, 1995).

β-L-2′-Deoxyadenosine (β-L-dA), β-L-2′,3′-dideoxyadenosine (β-L-ddA) and its two bis (*S*-acyl-2-thioethyl; SATE) phosphotriester derivatives, β-L-2′,3′-dideoxyadenosine-5′-monophosphate-bis(MeSATE) and β-L-2′,3′-dideoxyadenosine-5′-monophosphate-bis(tButylSATE) exhibit potent and selective antiviral activity against HBV replication ranging from 0.08 to 5 μM (El Alaoui et al., 1996; Bryant et al., 2001; Placidi et al., 2001). Because of their chemical structure these adenosine derivatives could potentially be incorporated into DNA during replication (Marec and Gelbic, 1994). Indeed, there is some evidence that nucleoside analogs such as 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxyguanosine (ddG), or 2′,3′-dideoxyadenosine (ddA) are incorporated into mtDNA and therefore, induce mitochondrial toxicity and production of increased amounts of lactic acid (Tsai et al., 1994). The genotoxic and mutagenic properties of this series of purine nucleoside derivatives were therefore investigated using the Ames assay and the Comet assay, the latter being of particular use in evaluating the mutagenic potential of nucleoside analogs (Matsuda et al., 1990; Oshiro et al., 1992a,b).

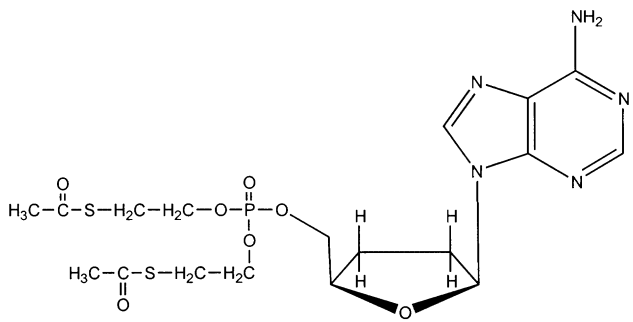
Salmonella typhimurium strains TA 97a, TA 98, TA 100 and TA 102 were kindly provided by Dr. B.N. Ames (Berkeley, CA). The bacterial strains were grown overnight in Oxoid Nutrient Broth no. 2 supplemented with antibiotics (ampicillin, 25 μg/ml for TA 97a, TA 98, and TA 100; ampicillin 25 μg/ml and tetracyclin 2 μg/ml for TA 102). Mutagenicity assays were performed by the Laboratory of Biogentoxicology and Environmental Mutagenesis, at the University de la Mediterranee, Marseille, France, according to the method of Maron and Ames (1983) modified by De Méo et al. (1996). Compounds purity was measured by elemental analysis (C, H, N) and was always 99%. Nucleosides were than dissolved in spectro grade DMSO at their solubility limits (1–10 mg/ml) and kept at −80°C until tested. Based on dose-range fitting spot test in TA 100 and TA 102 strains, each nucleoside derivative was tested at increasing concentrations, with or without the addition of rat liver S9 metabolic activation fraction. The S9 fraction was prepared from 200 g Sprague–Dawley rats previously treated with Aroclor 1254 (500 mg/kg body weight). Assay reaction mixtures contained 0.1 ml of S9 fraction (28 mg/ml) if required, increasing concentrations of β-L-dA, β-L-ddA, β-L-ddAMP-bis(meSATE) or β-L-ddAMP-bis(tbutylSATE; Fig. 1), and 0.1 ml of the overnight culture. The mixtures were incubated at 37°C for 60 min. Each assay was performed in triplicate in two independent experiments. Then, 2 ml volumes of molten top agar were added to the tubes and were subsequently poured onto Vogel-Bonner minimal salt agar plates and incubated at 37°C for 48 h in the dark. Spontaneous and induced revertants per plate were determined for each dose with a bacterial colony counter (Spiral System Instruments Inc., Bethesda, MD). The potential toxicity of the analogs was determined by the condition of the bacterial lawn. Seeded plates with slightly reduced or totally absent background were induced by a toxic effect of compounds and were not included in the final analysis. For each experiment, the controls included:



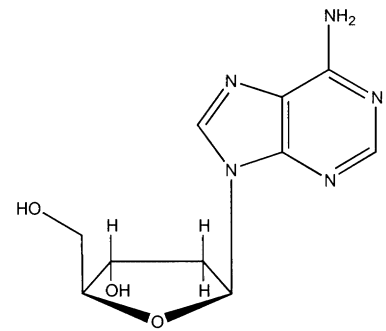
L-ddAMP-bis(tButylSATE)



L-ddA



L-ddAMP-bis(MeSATE)



L-dA

Fig. 1. Chemical structure of L-dA, L-ddA, L-ddAMP-bis(MeSATE) and L-ddAMP-bis (tButylSATE).

- 10 μl of DMSO to determine the frequency of spontaneous revertants,
- a positive control for each strain which consisted in:
  20 ng of 2-methoxy-6-chloro-9(32-chloroethyl)aminopropylamino acridine (ICR 191) for tester strain TA 97a,
  20 ng of 2,4,7-trinitro-9-fluoreone (TNFone) for tester strain TA 98,
  0.5 μg of sodium azide for tester strain TA 100, and
  20 ng of mitomycin C for tester strain TA 102, and
- 0.5 μg of benzo[a]pyrene with all strains as a quality control measure of the S9 fraction.

The following criteria were considered to determine the mutagenicity of the tested analogs:

- The compound investigated induced at least a 2-fold increase in the mean number of spontaneous revertants/plate.
- A dose response relationship can be calculated and is statistically significant by analysis of variance of the fitted model.
- The dose-response can be reproduced at least twice.

Results of these assays are presented in Table 1. Neither the β-L-dA nor the three other compounds exhibited mutagenic or genotoxic activities in the Ames test at pharmacologically relevant concentrations. Metabolic activation did not increase their mutagenicity.

The Comet assay was performed as previously described (Singh et al., 1988) using the dried slide technique (Eriksson and Nyrgen, 1995). Conven-

Table 1
Mutagenic activities of β-L-2′-deoxyadenosine derivatives using the Ames test[a]

Compound	Concentration (μM)*	Strain TA 97a		Strain TA 98		Strain TA 100		Strain TA 102	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
DMSO	704	170 ± 11	220 ± 27	15 ± 3	24 ± 1	98 ± 7	121 ± 1	316 ± 24	455 ± 123
L-ddAMP-tBuSATE	193.5	204 ± 7	189 ± 3	11 ± 1	25 ± 1	115 ± 29	115 ± 20	352 ± 17	425 ± 12
	290.5	153 ± 32	216 ± 2	14 ± 3	20 ± 1	95 ± 13	225 ± 1	276 ± 18	477 ± 16
	387.5	143 ± 21	206 ± 11	13 ± 2	23 ± 1	93 ± 10	139 ± 2	290 ± 28	457
	481	147 ± 4	194 ± 8	13 ± 5	22 ± 1	98 ± 12	114 ± 6	359 ± 29	459 ± 19
L-ddAMP-MeSATE	373.5	168 ± 3	198 ± 3	11 ± 3	24 ± 2	91 ± 12	108 ± 5	301 ± 8	421 ± 10
	560.5	164 ± 2	214 ± 32	18 ± 3	14 ± 1	86 ± 7	128 ± 20	297 ± 35	424 ± 18
	747.0	149 ± 11	216 ± 15	14 ± 1	22 ± 4	78 ± 6	117 ± 12	300 ± 3	447 ± 1
	934.0	154 ± 4	210 ± 16	15 ± 1	23 ± 1	86 ± 3	115 ± 7	267 ± 5	447 ± 40
L-ddA	850	169 ± 16	206 ± 28	15 ± 1	22 ± 4	105 ± 20	124 ± 2	317 ± 24	449 ± 7
	1025.0	133 ± 20	215 ± 15	13 ± 1	22 ± 2	126 ± 7	103 ± 4	314 ± 6	403 ± 8
	1700.5	153 ± 10	214 ± 26	16 ± 4	21 ± 5	103 ± 4	120 ± 17	282 ± 2	411 ± 34
	2125.5	130 ± 18	192 ± 1	14 ± 1	24 ± 2	117 ± 12	100 ± 4	301 ± 44	451 ± 44
L-dA	646	182 ± 20	213 ± 10	12 ± 1	26 ± 1	95 ± 12	96 ± 17	360 ± 14	411 ± 27
	1194.0	148 ± 17	236 ± 17	15 ± 3	22 ± 2	91 ± 2	112 ± 9	280 ± 16	413 ± 7
	1592.0	143 ± 8	243 ± 13	13 ± 3	23 ± 5	97 ± 6	107 ± 8	309 ± 39	450 ± 5
	1990.0	162 ± 1	252 ± 3	17 ± 1	26 ± 2	89 ± 12	88 ± 5	322 ± 10	439 ± 4
ICR191	0.9	2679	NT	NT	NT	NT	NT	NT	NT
TNFone	1.3	NT	NT	925	NT	NT	NT	NT	NT
Dimetri.	35.4	NT	NT	NT	NT	484	NT	NT	NT
Mit. C	0.3	NT	NT	NT	NT	NT	NT	2524	NT
BaP	9.9	NT	1111	NT	345	NT	1377	NT	1418

[a] Mutagenicity is expressed as the mean number of revertants/plate found on the four strains of *S. typhimurium* ± SEM for two independent experiments ($n = 6$). Concentration calculated during the 60-min contact period. NT – not tested. ICR191 – 2-methoxy-6-chloro-9(32-chloroethyl)aminopropylamino acridine. TNFone – 2,4,7-trinitro-9-fluoreone. Dimetri. – dimetridazole. Mit. C – mitomycin C. BaP – benzo[a]pyrene.

tional slides with a dry layer of agarose in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) were used throughout this study. Volumes of 20 μl of whole blood were collected from the fingertips of one healthy volunteer then mixed with 1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and L-glutamine. One hundred microlitre volumes of Ficoll Hypaque were added carefully to the solution, which was then centrifugated at 3000*g* for 3 min. After centrifugation, lymphocytes were removed and resuspended in 1 ml RPMI 1640 medium containing 10% FCS and L-glutamine. Following centrifugation, cells were finally resuspended in 1 ml magnesium- and calcium-free PBS (pH 7.4). Increasing concentrations of nucleoside derivatives were then added to the reaction mixture and incubated at 37°C for 1 h. Following incubation, cells were sedimented and mixed with 75 μl of a solution of 0.5% low melting point agarose. Cell suspensions were then placed on the dry slides slides previously covered with 85 μl of 0.8% agarose solution in PBS. Coverslips were placed onto the slides to allow uniform spreading and hardening of the layers. After removing the coverslips, a 75 μl volume of a third layer of 0.6% agarose was added and allowed to solidify on ice. After hardening of this agarose layer, slides were dipped in a lysing solution containing 1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl (pH 10), Triton X-100 and 10% DMSO, for 1 h at 4°C. The slides were removed from the lysing solution and placed on an electrophoretic unit containing a 1 mM Na_2EDTA and 300 mM NaOH solution. DNA unwinding was allowed to proceed for 20 min. All steps were carried out under yellow light to prevent the occurrence of additional DNA damage. After a 20 min electrophoretic run (25 V, 300 mA), the slides were rinsed three times with 0.4 mM Tris buffer (pH 7.5), dipped into absolute methanol and air dried overnight. The dried slides were stained with 70 μl of a 2 μg/ml solution of ethidium bromide, then examined at 250 × magnification using a BH2-RFL fluorescent microscope equipped with a 20BG-W2 dichroic mirror and D Plan-Apo 20 × UV objective (oil immersion). Image analysis was performed by using the Fenestra Komet software (version 3.1, Kinetic Imaging, Liverpool, UK). DNA damage was defined by the increase of the tail moment (TM) which represents the product of the Comet length and the amount of DNA in the tail. Nonlinear regression analyses were performed on the normalized distribution frequencies of TM for each treated sample using a χ^2 function. The degree of freedom (n) of the model was used to describe DNA damage (Bauer et al., 1998). Table 2 summarizes the data obtained in the Comet test. β-L-dA, β-L-ddA, β-L-ddAMP-bis(MeSATE), and, β-L-ddAMP-bis(tButylSATE) were negative in this genotoxic assay using human lymphocytes. Cell viability was measured using the Trypan blue exclusion staining (Boyum, 1968). At the end of the contact period, the cell viability was over 90% for each tested dose in all experiments throughout this study.

Nonlinear regression analyses were performed on distribution frequencies of TM for each treated sample using a χ^2 function (Bauer et al., 1998). The fitted model was calculated using Table Curve 2D (jandel Scientific Software, version 3.0, Germany). As reported by Bauer et al. (1998), the calculated degree of freedom (n) of the function could be used as an indicator of DNA damage. The Comet assay for each analog was repeated twice from two independent donors and a Dunnet's multiple comparison test for each treatment group versus the control was conducted.

Due to their chemical structure purine nucleosides and their analogs can be incorporated into DNA during replication and therefore have the potential to induce mutations or chromosome aberrations. For example, the anti-hepatitis B clinical candidate 9-(2-phosphonylmethoxyethyl)-adenine (PMEA) and two other adenine derivatives with potential antiviral activity, (R,S)-9-(2,3-dihydroxypropyl)adenine and D-eritadenine have been shown to induce chromosome aberrations in a dose-related fashion using the Drosophila wing spot test (Marec and Gelbic, 1994). Moreover, PMEA was shown to exhibit a moderate genotoxicity comparable to that shown with AZT (Ayers et al., 1996; Otova et al., 1997). Other adenine derivatives such as 2-amino-6-*N*-hydroxyadenine was found to induce multi loci mutations in mouse lymphoma and CHO cells (Moore

Table 2
Genotoxic activity of Comet assay β-L-2′-deoxyadenosine derivatives in human lymphocytes using the Comet assay[a]

Compound	Dose (μM)	n (−S9 mix; mean ± SEM)	n (+S9 mix; mean ± SEM)
Negative control	78.1	2.09 ± 0.08	2.06 ± 0.03
Positive control	13.2–4.0	8.04 ± 0.44*	12.68 ± 0.51*
	8.1	2.09 ± 0.08 (NS)[†]	2.07 ± 0.03 (NS)
β-L-ddAMP-bis(tbutylSATE)	16.1	2.09 ± 0.08 (NS)	2.06 ± 0.04 (NS)
	24.2	2.07 ± 0.05 (NS)	2.05 ± 0.03 (NS)
Negative control	78.1	2.11 ± 0.07	2.08 ± 0.04
Positive control	13.2–4.0	9.10 ± 0.63*	11.00 ± 0.48*
	9.3	2.23 ± 0.31 (NS)	2.06 ± 0.04 (NS)
β-L-ddAMP-bis(MeSATE)	18.7	2.09 ± 0.07 (NS)	2.04 ± 0.02 (NS)
	28.0	2.10 ± 0.08 (NS)	2.05 ± 0.03 (NS)
Negative control	78.1	2.11 ± 0.07	2.08 ± 0.04
Positive control	13.2–4.0	9.10 ± 0.63*	11.00 ± 0.48*
	21.3	2.11 ± 0.07 (NS)	2.04 ± 0.02 (NS)
β-L-ddA	42.5	2.09 ± 0.07 (NS)	2.05 ± 0.03 (NS)
	63.8	2.10 ± 0.08 (NS)	2.06 ± 0.04 (NS)
Negative control	78.1	2.12 ± 0.09	2.07 ± 0.05
Positive control	13.2–4.0	8.00 ± 0.32*	10.89 ± 0.43*
	11.9	2.10 ± 0.04 (NS)	2.06 ± 0.04 (NS)
β-L-dA	23.9	2.10 ± 0.06 (NS)	2.09 ± 0.09 (NS)
	35.8	2.07 ± 0.04 (NS)	2.07 ± 0.05 (NS)

[a] n – Degree of freedom calculated from regression analysis using a χ^2 function of normalized distribution of TM for each sample (mean ± SEM of two independent experiments). Dunnet's multiple comparison test for each treatment group versus the control was conducted for each compound. $P<0.001$; NS – not significant. Negative control – DMSO 78.1 μM. Positive controls – −S9 mix, H_2O_2 (13.2 mM), +S9 mix, benzo[a]pyrene (4.0 μM).

et al., 1991). In addition, some modified purine bases as 2-aminopurine and 2-amino-6-hydroxyaminopurine have been shown to be mutagenic using the Ames test (Oshiro et al., 1992a,b) and 2-(*p*-nitrophenyl)-adenine was strongly mutagenic using *Salmonella typhimurium* TA 98 and TA 100 without metabolic activation (Matsuda et al., 1990).

In conclusion, the present study clearly showed that the anti-HBV purine nucleoside, β-L-dA, and the related derivatives β-L-ddA, β-L-ddAMP-bis(MeSATE), and β-L-ddAMP-bis-(tButylSATE) are not mutagenic in the Ames test and do not induce DNA damage in human lymphocytes, as assessed by the Comet assay. In addition, metabolic activation with S9 fraction had no effect. Further analysis of these compounds as potent anti-HBV clinical candidates is warranted.

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