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MODULATION OF APOPTOSIS IN HUMAN LYMPHOCYTES BY ADENOSINE ANALOGUES

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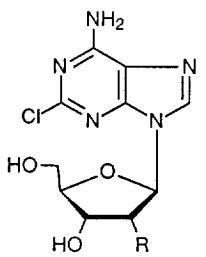
Abstract: We previously described that 2-chloroadenosine (2CA) and 2-chloro-2'-deoxyadenosine (2CdA) induced apoptosis in human peripheral blood mononuclear cells (PBMC). In this study we tested different adenosine analogues on PBMC and we found that the modifications introduced in the 2CA structure prevented the molecule from exerting its apoptotic effect. On the other hand, substitutions on 2CdA are tolerated, although with a significant decrease in activity.

Introduction

Adenosine is an endogenous nucleoside that is considered a key regulator of neuro-endocrine-immune functions, being involved in central and peripheral neurotransmission,[1] hormonal regulation[2] as well as in modulation of immune cell function.[3] In particular, in the immune system, adenosine can act as an immunosuppressor and inhibitor of tumor necrosis factor (TNF-α) production,[4] and it has been implicated as a physiological signal in the apoptotic deletion of T-cells during intrathymic cell selection, a process which functions to prevent autoimmunity.[5]

The mechanisms by which adenosine affects immune cells are not clearly understood yet.[6,7] Apoptosis by adenosine analogues is relatively well characterized in neural cells, where both receptor-mediated and receptor-independent mechanisms have been demonstrated.[8]



R = OH 2-chloroadenosine (2CA, **19**)

R = H 2-chloro-2'-deoxyadenosine (2CdA, **1**)

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In particular, we have previously characterized the apoptosis induced by a P_1 receptor non-selective hydrolysis-resistant adenosine analogue, 2-chloroadenosine (2CA, **19**), on rat astroglial cells. We have shown that cell death by this agent seems to involve an atypical adenosine receptor, does not occur through activation of poly(ADP-ribose)polymerase or radical generation, and is not apparently due to early damage of the mitochondria.[9] Furthermore, it has been observed that 2CA induces apoptosis also in human normal peripheral blood mononuclear cells (PBMC) through a mechanism partially A_{2A} receptor-dependent and partially exerted intracellularly.[10] Moreover, its deoxy-derivative 2-chloro-2'-deoxyadenosine (2CdA, **1**), currently used in the treatment of chronic lymphoid malignancies (e.g. chronic lymphocytic leukemia, hairy cell leukemia and cutaneous T cell lymphoma),[11] is able to induce apoptosis too on human PBMC, cells rather insensitive to apoptosis.[10]

On this basis, in an attempt to verify which part of the molecule could be fundamental for the 2CA and 2CdA actions, we compared the apoptotic effect of 2CA and 2CdA on PBMC with that exerted by 23 adenosine derivatives, obtained by modifying different parts of the parent nucleosides.

Experimental Procedures

Lymphocyte cultures. Peripheral blood from healthy volunteers was collected aseptically; mononuclear cells were separated by centrifugation in Lymphoprep gradient,[12] washed extensively with Hanks' medium and resuspended at a concentration of 10^6 cells/ml in RPMI 1640 medium supplemented by 10% heat-inactivated FCS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cell suspensions were seeded into culture plates (Corning) and substances were added at the concentrations indicated in Table I. Cultures were incubated for 24, 48 and 72 h in a humidified atmosphere of 5% CO_2 in air at 37°C, with or without adenosine analogues. At each time point, cells were harvested and analyzed by flow cytometry. Apoptosis detected in the absence of drugs was lower than 10%.

Drugs and Treatments. 2CA, 2CdA and propidium iodide (PI) were purchased from Sigma Chemical Co., St. Louis, USA. Lymphoprep was obtained from Nycored Pharma AS, Oslo, Norway. RPMI 1640, Hanks' Balanced Salts Solution (HBSS), L-Glutamine and Penicillin-Streptomycin were from Gibco, Paisley, Scotland, U.K. Adenosine analogues utilised in this study were previously synthesized and the references[13-23] are listed in Table 1.

Detection of Apoptosis in Flow Cytomerty. 2CA and 2CdA-induced apoptosis was detected by reduced fluorescence of the PI - a DNA binding dye - in the apoptotic nuclei, as previously described.[24] Briefly, the 200 g centrifuged cell pellet (1 x 10^6 cells) was gently resuspended in 1 ml hypotonic fluorocrome solution [PI 50 μg/ml in 0.1% (w/v) sodium citrate plus 0.1% (v/v) Triton X-100 (Sigma) in bidistilled water]. Cells were analyzed by FACS after a minimum of 30 min of incubation in this solution. Red fluorescence due to PI staining of DNA was recorded on a logarithmic scale. Debris were gated out based on light scatter measurements before the single parameter histograms and the contour graphs were drawn.

Results and Discussion

All the nucleosides have been tested at 1, 10, 30, and 60 μM concentration and the percentage of PBMC apoptotic cells is reported in Table 1.

The structure-activity relationships of 2CdA have been evaluated by three approaches:

a - Modification of the hydroxy groups in the sugar moiety (compounds **2-4**)

b - Substitution of the amino group in 6-position (compounds **5-10**)

c - isosteric monosubstitution of a nitrogen atom of the pyrimidine ring by a methine group (deazaadenosine derivatives **11-18**).

The presence of a hydroxy group on C-3' is crucial for the activity of 2CdA; in fact both moving this group to the 2'-position (**2**) or removing it, as in the 2',3'-dideoxy derivative **3**, resulted in loss of apoptotic effect. The presence of the hydroxymethyl function in 5'-position is also required, since the 5'-N-methylcarboxamido derivative of 2CdA (**4**) is devoid of any apoptotic activity.

The introduction of cycloalkyl substituents on N^6 reduces the apoptotic effect, depending on the size of the ring. The 6-cyclopentylamino derivative **7** proved to be the most potent in this group, inducing 38.8% of apoptosis at 60 μM concentration. Removal of the chlorine atom in 2-position (compounds **6**, **8**, and **10**) proved to be detrimental for the apoptotic activity.

The isosteric substitution of the nitrogen atom in 1-position of 2CdA by a methine group led to 2'-deoxy-2-chloro-1-deazaadenosine (**11**), which did not trigger apoptosis. Moreover, the corresponding N^6-cycloalkyl derivatives **12**, **14**, and **16**, that in a previous study exhibited good anti-HIV-1 activity,[17] proved to be inactive in inducing apoptosis in human PBMC.

On the other hand, replacement of the nitrogen atom at the 3-position is better tolerated. In fact the resulting 2'-deoxy-2-chloro-3-deazaadenosine (**17**) induces 34.5% and 59.2% of apoptosis at 30 and 60 μM concentration, respectively.

The structure-activity relationships of 2CA (**20**) have been evaluated by three additional approaches:

a - Substitution of the chlorine atom by an iodine (compound **20**)

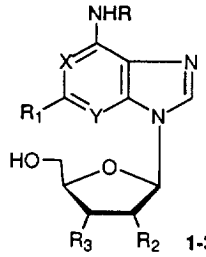
b - Substitution of the amino group at the 6-position (compound **21**)

c - Isosteric monosubstitution of a nitrogen atom of the pyrimidine ring by a methine group (deazaadenosine derivatives **22-25**).

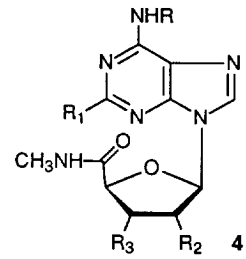
Both the iododerivative **20** and the 2-chloro-N^6-cyclopentyladenosine (CCPA, **21**) did not trigger apoptosis up to 60 μM concentration. Since CCPA is a potent and selective agonist of A_1 adenosine receptors,[20] this finding confirms the hypothesis that this subtype seems not to be involved in the regulation of apoptosis induced by 2CA.[10]

The 1-deaza and 3-deaza analogues of 2-chloroadenosine (**22** and **24**) and of adenosine (**23** and **25**) proved to be inactive in inducing apoptosis in human PBMC. The different result obtained with 2'-deoxy-2-chloro-3-deazaadenosine (**17**, 59.2% apoptosis at 60 μM concentration) and with the corresponding ribose derivative **24** supports the hypothesis that a different pathway is responsible for apoptosis induced by 2CdA and 2CA, respectively. Moreover, it is worthwhile to note that 3-deazaadenosine (**25**), which is reported to trigger apoptosis in HL60 and L1210 cell lines at concentrations above 25 μM,[25,26] was inactive in our experimental conditions.

Table 1. Apoptotic effect of adenosine analogues[a].



1-3, 5-25



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no	R	R_1	R_2	R_3	X	Y	1μM	10μM	30μM	60μM	Ref
1	H	Cl	H	OH	N	N	**91.0**	**91.2**	**93.8**	**94.7**	b
2	H	Cl	OH	H	N	N	7.7	13.0	11.4	10.4	13
3	H	Cl	H	H	N	N	4.7	8.5	9.6	11.4	13
4	H	Cl	H	OH	N	N	4.5	8.1	10.4	10.7	14
5	cC_3H_5	Cl	H	OH	N	N	7.8	7.7	17.9	**24.7**	15
6	cC_3H_5	H	H	OH	N	N	7.1	9.5	9.5	10.6	15
7	cC_5H_9	Cl	H	OH	N	N	4.7	10.0	18.8	**38.8**	15
8	cC_5H_9	H	H	OH	N	N	6.7	7.9	7.1	8.7	15
9	cC_7H_{13}	Cl	H	OH	N	N	8.2	7.9	12.3	**16.3**	15
10	cC_7H_{13}	H	H	OH	N	N	8.0	9.4	9.8	11.1	15
11	H	Cl	H	OH	CH	N	5.0	8.7	9.8	10.6	16
12	cC_3H_5	Cl	H	OH	CH	N	7.3	10.4	10.8	11.4	17
13	cC_5H_9	Cl	H	OH	CH	N	8.6	9.7	12.0	13.9	16
14	cC_5H_9	H	H	OH	CH	N	9.8	9.8	9.5	9.9	16
15	cC_7H_{13}	Cl	H	OH	CH	N	7.4	7.9	9.6	10.5	17
16	cC_7H_{13}	H	H	OH	CH	N	7.7	8.1	8.9	9.6	17
17	H	Cl	H	OH	N	CH	16.6	20.2	**34.5**	**59.2**	18
18	cC_5H_9	Cl	H	OH	N	CH	13.1	13.5	14.9	15.2	15
19	H	Cl	OH	OH	N	N	8.8	**41.8**	**82.7**	**91.9**	b
20	H	I	OH	OH	N	N	9.1	12.1	12.0	12.2	19
21	cC_5H_9	Cl	OH	OH	N	N	5.4	10.4	9.6	11.0	20
22	H	Cl	OH	OH	CH	N	10.2	10.4	11.1	12.1	21
23	H	H	OH	OH	CH	N	8.0	16.6	12.8	10.7	22
24	H	Cl	OH	OH	N	CH	9.6	9.0	9.6	10.3	23
25	H	H	OH	OH	N	CH	9.9	6.5	11.9	10.7	23

[a]Percentage of apoptotic cells after 72 h incubation. Values are means of at least 4 independent experiments. SEM is < 4. [b]Commercially available.

In conclusion, our study demonstrated that the modifications introduced in the 2CA structure prevented the molecule from exerting its apoptotic effect. On the other hand, substitutions on 2CdA are tolerated, although with significant decrease in activity.

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