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# The combination of adenosine deaminase inhibition and deoxyadenosine induces apoptosis in a human astrocytoma cell line

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ABSTRACT

Alterations in the functions of astrocytes contribute to the appearance of a variety of neurological pathologies. Gliomas, especially those of astrocytic origin, are particularly resistant to chemotherapy and are often characterized by a poor prognosis. Neuroblastoma is the tumour with the higher incidence in infants. Anticancer drugs can induce apoptosis and their cytotoxic effect is often mediated by this process. We have previously demonstrated that the combination of deoxycoformycin, a strong adenosine deaminase inhibitor, and deoxyadenosine is toxic for a human astrocytoma cell line. In fact, after 15 h of treatment, this combination increases both mitochondrial reactive oxygen species and mitochondrial mass, induces apoptosis as indicated by cytochrome c release from mitochondria and activation of caspase-3. These events are preceded by reduction in lactate release in the medium. In this work we demonstrate that after 8 h of incubation with deoxyadenosine and deoxycoformycin, caspase-8 is activated, mitochondrial mass increases and mitochondrial reactive oxygen species decrease. The addition of baicalein to the incubation medium reduces cell death and caspase-3 activity induced by deoxycoformycin and deoxyadenosine in combination. This protective effect is correlated to an increase of lactate released in the medium, a decrease in the intracellular levels of dATP, and an increase in ATP levels, as compared with the cells subjected to the treatment with deoxycoformycin and deoxyadenosine without any further addition. The effect of baicalein appears to be related to an inhibition of deoxyadenosine phosphorylation, rather than or in addition to the well known antioxidant activity of the compound. This work indicates that an astrocytoma cell line, reported to be resistant to mitochondria-dependent pathways of apoptosis, is indeed very sensitive to a manipulation affecting the balance of cellular purine metabolite concentrations. The same treatment is also cytotoxic on a neuroblastoma cell line, thus suggesting long term implications for cancer therapy



## 1. Introduction

The deficiency of adenosine aminohydrolase (EC 3.5.4.4) (adenosine deaminase, ADA), the enzyme that catalyses the hydrolytic deamination of adenosine (Ado) and deoxyadenosine (dAdo), is associated with severe immunodeficiency and abnormalities in the functioning of many organs including nervous system (Camici et al., 2010). Neurological abnormalities, rarely reported in early infancy (Hershfield, 2001), include various degrees of motor coordination disorders, learning disability, hyperactivity, seizures, attention and hearing deficits (Hönig et al., 2007). Immune system impairments but not the neurological problems may be reverted by allogenic bone marrow transplantation (Hönig et al., 2007). Indeed, even though lower than in untreated patients, the level of Ado and dAdo appears to be higher than normal in ADA-deficient treated patients (Hirschhorn et al., 1981). It is conceivable that the described neurological manifestations arise from an adverse effect exerted by Ado,

*Abbreviations:* Ac-IETD-pNA, Ac-Ile-Glu-Thr-Asp-paranitroaniline; ADA, adenosine deaminase; ADF, human astrocytoma cell line; Ado, adenosine; AdoK, adenosine kinase; dAdo, deoxyadenosine; dATP, deoxyATP; dCF, deoxycoformycin; DMEM, Dulbecco's modified Eagle's medium; DEVD-pNA, Asp-Glu-Val-Asp-paranitroanilinine; DMSO, dimethylsulfoxide; FBS, foetal bovine serum; IAP, inhibitor of apoptosis proteins; LDH, lactic dehydrogenase; MT-ROS, MitoTracker Red CM-$H_2$XROS; MT-Green, MitoTracker Green; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide); NDGA, nordihydroguaiaretic acid; $NH_2$dAdo, 5' amino-5'deoxyadenosine; PBS, phosphate buffered saline; pNA, paranitroanilinine; ROS, reactive oxygen species; SH-SY5Y, human neuroblastoma cell line; TMRM, tetramethylrhodamine methyl ester; $\Delta\Psi_m$, mitochondrial membrane potential.

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dAdo and their derivatives on the development, differentiation, and functioning of nervous system. In this regard, it is known that deficiency of ADA results in the accumulation of deoxyadenosine triphosphate (dATP), which affects normal methylation reactions, induces apoptosis in thymic lymphocytes, and inhibits ribonucleotide reductase thus interfering with normal DNA synthesis (Takeda et al., 1991). In a previous paper (Garcia-Gil et al., 2012) we have demonstrated that the inhibition of ADA in the presence of dAdo in a human astrocytoma cell line (ADF) induces apoptosis, interferes with glucose metabolism, since a reduction in lactate released in the medium is observed in treated cells as an event preceding caspase-3 activation and cellular death, and produces in long-term incubations an increase in mitochondrial ROS. Astrocytoma cells demonstrate both basic metabolic mechanisms of astrocytes as well as tumours in general, e.g. they show higher glycolytic rate, lactate production, ability to grow under hypoxia, suppression of cell death pathways. Therefore, on one hand, our cellular model may mimic a condition of ADA deficiency, on the other hand, ADF cells offer the possibility to study the effect of the inhibition of ADA activity, a manipulation affecting purine metabolism, on a cellular model deriving from a type of tumour reported to be particularly resistant to chemotherapeutic approaches and often characterized by a poor prognosis (Ohgaki and Kleihues, 2007). Indeed, over the past years, it has become evident that anticancer drugs can induce apoptosis and that their cytotoxic effects are often mediated by this process (Ferreira et al., 2002). However, the success of chemotherapy is often hampered by the appearance of broad drug resistance, which in several cases is due to defective common apoptosis-inducing pathways. These defects are probably at the basis of the initial expansion of the population of neoplastic cells eventually originating the tumour (Igney and Krammer, 2002). Thus, it is very important to identify the biochemical mechanisms leading to apoptosis of a specific tumour to predict the possible success of a given drug treatment and to plan more focused and effective therapies. In this respect, in this paper we have furthered our investigation, trying to understand the link between the production of lactate, therefore glucose metabolism, and the onset of the apoptotic program in astrocytoma cells. In addition, we have also tested the effect of the combination of ADA inhibition and dAdo on the viability of a neuroblastoma cell line, in order to assess whether this treatment could be effectively applied to a tumour of different origin.

## 2. Experimental procedures

### 2.1. Materials

Asp-Glu-Val-Asp-paranitroanilinine (DEVD-pNA, caspase-3 substrate) Ac-Ile-Glu-Thr-Asp-paranitroaniline (Ac-IETD-pNA, caspase-8 substrate) and calpain inhibitor III were from Calbiochem, Merck (Nottingham, UK); Brilliant blu, protease inhibitor cocktail, 5′-amino-5′-deoxyadenosine ($NH_2$dAdo), baicalein, deoxyadenosine (dAdo), nordihydroguairetic acid (NDGA), 3-(4,5-dimethyll-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), pepstatin A, [8-$^{14}$C] deoxyadenosine (10,000 dpm/nmol), L-lactate: $NAD^+$ oxidoreductase (EC 1.1.1.27) (lactic dehydrogenase, LDH), alcohol: $NAD^+$ oxidoreductase (EC 1.1.1.1) (alcohol dehydrogenase), and PD 146176, were from Sigma (Milan, Italy); dimethylsulfoxide (DMSO) from Carlo Erba (Milan, Italy); Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, penicillin, streptomycin and trypsin from Euroclone (Pero, Milan, Italy); MitoTracker Green (MT-Green), tetramethylrhodamine methyl ester (TMRM) and MitoTracker Red CM-$H_2$XROS (MT-ROS) from Molecular Probes, Invitrogen (San Giuliano Milanese, Milan, Italy); caspase-8 and caspase-3 activity kit were from Biovision Research Products (Mountain View, CA, USA); foetal bovine serum (FBS) and glutamine from Lonza (Basel, Switzerland); L-alanine: 2-oxoglutarate aminotransferase (EC 2.6.1.2) (glutamate–pyruvate transaminase) was from ICN Biomedicals (Irvine, CA, USA); deoxycoformicin (dCF) was from Tocris (Bristol, UK), the human astrocytoma cell line ADF and the human neuroblastoma cell line SH-SY5Y were generous gifts from Dr. W. Malorni (Istituto Superiore della Sanità, Rome, Italy) and Prof. A. Arcangeli (University of Florence, Italy), respectively.

### 2.2. Treatment of astrocytoma and neuroblastoma cells and viability assay

The viability assay is based on the ability of metabolically active cells to reduce the MTT tetrazolium salt, water-soluble, into a purple-coloured, water-insoluble MTT formazan salt. Human neuroblastoma (SH-SY5Y) and astrocytoma (ADF) cell lines were routinely grown in DMEM and RPMI medium, respectively, containing 10% FBS, 2 mM glutamine, 200 UI/mL penicillin and 200 μg/mL streptomycin at 37 °C in a humidified 5% $CO_2$/95% air atmosphere as described in Garcia-Gil et al. (2003, 2012). For the experiments, cells were seeded at 45,000 cell/cm$^2$ in 96-well plates, containing 100 μL of medium. Three days after, medium was removed and cells were preincubated for 30 min with 1 μM dCF and/or the different effectors in serum-free DMEM medium plus 10 mM glucose and then incubated in the same medium containing 120 μM dAdo, plus effectors when indicated. In control cells both preincubation and incubation were performed in the absence of effectors (20 μM NDGA, 25 μM baicalein, 100 μM pepstatin A, 10 μM $NH_2$dAdo, 50 μM calpain inhibitor III, 5 μM PD146176 for ADF and 10 μM $NH_2$dAdo for SH-5Y5Y). After 24 h (for ADF) and 48 h (for SH-SY5Y) of treatment in the presence or absence of dCF and dAdo and the different effectors, 0.5 mg/mL MTT in PBS was added to each well; cells were incubated at 37 °C in a humidified 5% $CO_2$/95% air atmosphere for 30 min, the reaction was stopped by replacing the MTT solution with 100 μL DMSO and the formazan salts were dissolved by gentle shaking for about 5 min at room temperature. Formazan salts were quantified spectrophotometrically by reading the absorbance at 570 nm using the Ultramark Microplate Systems (Bio-Rad, Hercules, CA, USA). Each experiment was performed in triplicate and repeated at least two times.

### 2.3. Determination of lactate and quantification of adenylate pool in the incubation medium of ADF cells

The human astrocytoma cell line (ADF) was routinely grown as described in Section 2.2. For the experiments, cells were seeded at 45,000 cell/cm$^2$. Three days after, medium was removed and cells were preincubated for 30 min with 1 μM dCF in serum-free DMEM medium plus 10 mM glucose and then incubated in the same medium containing 120 μM dAdo (250 μL in 24-well plates). In control cells both preincubation and incubation were performed in the absence of effectors. In order to test the effect of 20 μM NDGA or 25 μM baicalein or 10 μM $NH_2$dAdo, these compounds were added 30 min before dAdo. At different times of incubation, the medium was collected and subjected to the spectrophotometric assay at 340 nm for the determination of lactate (Martì et al., 1997). The reaction mixture contained in a final volume of 0.5 mL, 200 mM glycylglycine – 40 mM glutamate (pH 10), 4 mM $NAD^+$, 27 U of lactic dehydrogenase, 7 U of glutamate–pyruvate transaminase, 20 μL of culture medium, and 20 mM Tris–HCl pH 7.4.

For the determination of the intracellular adenylate pool (AMP, ADP, ATP, and dATP) the medium was removed, and the wells were washed twice with cold physiological solution, and incubated for 15 min with 0.1 mL perchloric acid 0.6 M. Cells were scraped and centrifuged 10,000 × *g* for 5 min. Supernatant was neutralized with 15 μL of 3.5 M $K_2CO_3$, centrifuged at 10,000 × *g* for 5 min and analysed by HPLC (Micheli et al., 1999).