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Inhibition of ATP synthase by chlorinated adenosine analogue

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ABSTRACT

8-Chloroadenosine (8-Cl-Ado) is a ribonucleoside analogue that is currently in clinical trial for chronic lymphocytic leukemia. Based on the decline in cellular ATP pool following 8-Cl-Ado treatment, we hypothesized that 8-Cl-ADP and 8-Cl-ATP may interfere with ATP synthase, a key enzyme in ATP production. Mitochondrial ATP synthase is composed of two major parts; F_O intermembrane base and F1 domain, containing α and β subunits. Crystal structures of both α and β subunits that bind to the substrate, ADP, are known in tight binding ($\alpha_{dp}\beta_{dp}$) and loose binding ($\alpha_{tp}\beta_{tp}$) states. Molecular docking demonstrated that 8-Cl-ADP/8-Cl-ATP occupied similar binding modes as ADP/ATP in the tight and loose binding sites of ATP synthase, respectively, suggesting that the chlorinated nucleotide metabolites may be functional substrates and inhibitors of the enzyme. The computational predictions were consistent with our whole cell biochemical results. Oligomycin, an established pharmacological inhibitor of ATP synthase, decreased both ATP and 8-Cl-ATP formation from exogenous substrates, however, did not affect pyrimidine nucleoside analogue triphosphate accumulation. Synthesis of ATP from ADP was inhibited in cells loaded with 8-Cl-ATP. These biochemical studies are in consent with the computational modeling; in the $\alpha_{tp}\beta_{tp}$ state 8-Cl-ATP occupies similar binding as ANP, a non-hydrolyzable ATP mimic that is a known inhibitor. Similarly, in the substrate binding site ($\alpha_{dp}\beta_{dp}$) 8-Cl-ATP occupies a similar position as ATP mimic ADP-BeF_3^-. Collectively, our current work suggests that 8-Cl-ADP may serve as a substrate and the 8-Cl-ATP may be an inhibitor of ATP synthase.

1. Introduction

The nucleoside analogues currently used in the clinic are largely DNA-directed and act by inhibiting DNA synthesis [1]. These analogues either incorporate into DNA and/or affect metabolic enzymes such as ribonucleotide reductase [2], purine nucleoside phosphorylase [3], and adenosine deaminase [4,5] to perturb intracellular deoxynucleotide pools, resulting in decreased DNA synthesis in cells. In contrast, 8-chloroadenosine (8-Cl-Ado) contains a ribose sugar and is unique because it is RNA-directed. The advantage of RNA-directed agents such as 8-Cl-Ado is that they provide a valuable strategy for targeting quiescent cancers that do not actively synthesize DNA. Two RAID (Rapid Access to Interventional Development) contracts were awarded by the National Cancer Institute for the development of 8-Cl-Ado, and the drug is being tested in the first Phase I clinical trial for the treatment of chronic lymphocytic leukemia (CLL), an indolent leukemia. Ultimately, this agent will be used for other quiescent malignancies such as multiple myeloma and solid tumors.

In preclinical pharmacological studies using multiple myeloma (MM) cell lines and primary leukemia cells, 8-Cl-Ado was demonstrated to be phosphorylated into its triphosphate form (8-Cl-ATP) [6], which inhibits transcript synthesis by incorporation into RNA [7] and by inhibition of polyadenylation [8]. In addition, a decline in intracellular ATP is observed in cells treated with 8-Cl-Ado, however, by unknown mechanisms. Studies using cell lines that are proficient or deficient in adenosine kinase and in vitro kinase assays demonstrated that 8-Cl-Ado is monophosphorylated (8-Cl-AMP) by adenosine kinase [6,9]. 8-Cl-AMP is further metabolized to 8-Cl-ADP and 8-Cl-ATP, which are presumed to be catalyzed by monophospho-kinase and diphospho-kinase, respectively. We previously demonstrated that 8-Cl-ATP accumulates at high levels (>400 μM) [6,10] while ATP levels decline to 50% of control after 6 h of treatment. The endogenous ATP concentration was reduced from ~1.7 mM to 0.65 mM a 12-h

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incubation with 8-Cl-Ado in cell lines, and similar phenomenon was observed in primary leukemia cells obtained from the peripheral blood of patients with CLL [10].

The majority of cellular ATP is synthesized using respiratory chain oxidative phosphorylation by ATP synthase, which is the last enzyme in the respiratory chain [11]. ATP synthase catalyzes the synthesis of ATP from recycling ADP [12–15], and it has been associated with a number of human diseases including cancer (for a review, see [16]). Studies suggest that cell surface ATP synthase can function as a receptor for ligands involved in several cellular processes including regulation of cell proliferation and differentiation, and immunological tumor recognition [17,18]. Elevated expression of ATP synthase on endothelial cell surfaces has been reported to play an important role during angiogenesis, and angiostatin action is in part via the inhibition of cell surface ATP synthase [19]. As such, recent developments have highlighted ATP synthase as a potential cancer target for therapeutics. Aurovertin B, an ATP synthase inhibitor, is currently under investigation for the treatment of breast cancer and has been shown to inhibit proliferation and induce apoptosis [20]. More recently, monoclonal antibodies against ATP synthase was reported to inhibit proliferation and colony formation in human vascular endothelial cells and reduce tumor growth in xenograft models [21,22].

In order to understand the function and mechanism of ATP synthase, X-ray crystal structures of both F_O and F1 domains have been obtained by others [12–15,23]. ATP synthase is composed of two major parts: the F_O domain, an intermembrane proton channel, and the F1 domain, the catalytic complex. The nucleotide binding/catalytic sites are within F1 domain, which is composed of five different subunits with stoichiometry $\alpha 3\beta 3\gamma\delta\varepsilon$. The crystal structures demonstrate that α and β subunits are structurally similar, and each subunit consists of three domains: a small N-terminal domain, a nucleotide binding domain and a helical C-terminal domain [12,13,23]. Both α and β subunits bind nucleotides but only the β subunit participates in the catalysis. It has been found that the three catalytic sites in the three β subunits have different affinities for nucleotides, and these together with other results have been used to propose a binding change mechanism of β units during the catalytic cycle of the enzyme [12–15]. It was proposed that rotation of the central F_O stalk interconverted the three binding sites of F_1 domain from open (low affinity binding) to tight (high affinity binding), from tight to loose (intermediate affinity binding), and from loose to open [23,24]. The structures of the F1-ATPase provided insight into the binding change mechanism: the different conformations of β-subunits account for the conformations proposed to occur during the catalytic cycle [12,13,23].

We hypothesized that 8-Cl-ADP may serve as a substrate of ATP synthase and that 8-Cl-ATP may be an inhibitor to this key enzyme based on the following prior observations. First, the structural similarities between Ado and 8-Cl-Ado or ATP and 8-Cl-ATP. Second, the use of 8-Cl-Ado or 8-Cl-ATP by the same enzymes as Ado and ATP, and third, a decline in cellular ATP pool after incubation of cells with 8-Cl-Ado. Using biological, biochemical, and computational molecular modeling studies our current work tested this hypothesis.

2. Materials and methods

2.1. Cell lines

All experiments were conducted using an exponentially growing multiple myeloma cell line, MM.1S [25,26]. All cells were routinely tested for *Mycoplasma* infection using a commercially available kit (Invitrogen, Carlsbad, CA).

2.2. Drugs and other chemicals

8-Cl-Ado was purchased initially from BioLog (La Jolla, CA) and then obtained from Dr. V. Rao at the Drug Development Branch of the NCI. [^{3}H]8-Cl-Ado and [^{3}H]Ado were purchased from Moravek Biochemicals (Brea, CA). 8-Cl-ATP was custom-synthesized by BioLog. Oligomycin was purchased from Sigma-Aldrich (St. Louis, MO). Coformycin (CF) and deoxycoformycin (dCF) were obtained from Dr. Robert Schultz at the NCI (Bethesda, MD). All other chemicals were reagent grade.

2.3. Oxygen consumption assay

Oxygen consumption is measured using a Hansatech Oxytherm (Hansatech Instrument, England). Drug treated cells are placed in sealed respiration chamber containing 1 mL of fresh culture medium pre-equilibrated with 21% oxygen. The sample temperature was regulated with a thermostat control and stirred with a micro-stirrer [27,28]. Oxygen levels were measured polarographically using a Hansatech oxygen electrode disc and the manufacturer's software.

2.4. Accumulation of Ado and 8-Cl-Ado metabolites

To quantitate the phosphorylated metabolites of Ado and 8-Cl-Ado, MM cells were incubated with oligomycin (2 μg/mL) for 30 min and then 10 μM [^{3}H]Ado or [^{3}H]8-Cl-Ado was added. The adenosine deaminase inhibitors CF and dCF (0.1 μM) were added to prevent deamination. The cellular nucleotides were extracted using the perchloric acid extraction procedure [6] and analyzed on a gradient that separates free nucleoside, mono-, di-, and triphosphate forms [29].

2.5. Measurement of intracellular nucleoside mono-, di-, and triphosphates by HPLC

For non-radioactive material, the cellular extracts were applied to an anion-exchange Partisil-10 SAX column as described before [6]. The column eluate was monitored by UV absorption at 256 nm, and 8-Cl-ATP was identified by comparing its retention profile and absorption spectrum with those of an authentic standard. The intracellular concentration of nucleotides contained in the extract was calculated from a given number of cells of a determined mean volume. The cell number was determined using Coulter counter (Coulter Electronics, Hialeah, FL). This equipment is attached to a channelizer which was used to estimate the mean volume of cells in a given cell population. This volume was used to quantitate the concentration of nucleotides. The lower limit of sensitivity of this assay was 10 pmol in an extract of 5×10^6 cells corresponding to a cellular concentration of 1 μM. For radioactive 8-Cl-Ado a Radiomatic Flow-through HPLC system (Packard, Downers Grove, IL) was used. The eluate from the anion-exchange column passed through an automatic radiometric detector along with liquid scintillation fluid (Ultima Flo, Packard) and tritium counts were recorded for each radioactive peak.

2.6. ATP synthase molecular docking studies

The ATP synthase structures (1BMF [23] and 2CK3 [24]) were obtained from Protein Data Bank (PDB [30]). In these crystal structures, ADP and ANP (an non-hydrolyzable ATP analogue) were observed at the interfaces of tight binding subunits ($\alpha_{dp}\beta_{dp}$) and loose binding subunits ($\alpha_{tp}\beta_{tp}$), respectively [23,24]. As such, both subunits from 1BMF were extracted to conduct the modeling studies. ADP and 8-Cl-ADP were docked into the catalytic site between $\alpha_{dp}\beta_{dp}$ while $\alpha_{tp}\beta_{tp}$ was used to probe its interactions