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Inhibition of adenosine kinase by phosphonate and bisphosphonate derivatives

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Abstract

The enzyme adenosine kinase (AK) plays a central role in regulating the intracellular and interstitial concentration of the purine nucleoside adenosine (Ado). In view of the beneficial effects of Ado in protecting tissues from ischemia and other stresses, there is much interest in developing AK inhibitors, which can regulate Ado concentration in a site- and event-specific manner. The catalytic activity of AK from different sources is dependent upon the presence of activators such as phosphate (Pi). In this work we describe several new phosphorylated compounds which either activate or inhibit AK. The compounds acetyl phosphate, carbamoyl phosphate, dihydroxyacetone phosphate and imidodiphosphate were found to stimulate AK activity in a dose-dependent manner comparable to that seen with Pi. In contrast, a number of phosphonate and bisphosphonate derivatives, which included clodronate and etidronate, were found to inhibit the activity of purified AK in the presence of Pi. These AK inhibitors (*viz*. clodronate, etidronate, phosphonoacetic acid, 2-carboxyethylphosphonic acid, *N*-(phosphonomethyl)-glycine and *N*-(phosphonomethyl)iminodiacetic acid), at concentrations at which they inhibited AK, were also shown to inhibit the uptake of ^{3}H-adenosine and its incorporation into macromolecules in cultured mammalian cells, indicating that they were also inhibiting AK in intact cells. The drug concentrations at which these effects were observed showed limited toxicity to the cultured cells, indicating that these effects are not caused by cellular toxicity. These results indicate that the enzyme AK provides an additional cellular target for the clinically widely used bisphosphonates and related compounds, which could possibly be exploited for a new therapeutic application. Our structure–activity studies on different AK activators and inhibitors also indicate that all of the AK activating compounds have a higher partial positive charge (δ^{+}) on the central phosphorous atom in comparison to the inhibitors. This information should prove helpful in the design and synthesis of more potent inhibitors of AK. (Mol Cell Biochem **283:** 11–21, 2006)

Key words: adenosine, adenosine kinase, bisphosphonates, enzyme inhibitors, structure–activity studies

Abbreviation: Ado, adenosine; AK, adenosine kinase; ADA, adenosine deaminase; EHNA, erythro-9-(2-hydroxyl-3-nonyl) adenine; AP, acetyl phosphate; CP, carbamoyl phosphate; DHAP, dihydroxyacetone phosphate; IDP, imidodiphosphate; MPA, methylphosphonic acid; DEPAA, diethylphosphonoacetic acid; PAA, phosphonoacetic acid; PFA, phosphonoformic acid; CEPA, 2-carboxyethylphosphonic acid; PMG, *N*-(phosphonomethyl)-glycine; PMIA, *N*-(phosphonomethyl)iminodiacetic acid; AR, adenosine receptor; 5′NH_2dAdo, 5′-amino, 5′-deoxyadenosine; 5IT, 5-iodotubercidin; 5′d-5IT, 5′-deoxy, 5-iodotubercidin; MOPAC, molecular orbital package; MNDO, modified neglect of diatomic overlap

Introduction

Adenosine kinase (AK; ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20) is a monomeric protein that catalyses the transfer of γ-phosphate from ATP to adenosine (Ado) [1–5]. As a purine salvage enzyme, AK plays an important role in the regulation of intracellular and interstitial Ado concentration [6]. The steady-state concentrations of Ado in tissues are

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kept in the nanomolar range by the combined action of enzymes AK and adenosine deaminase (ADA). Since AK has a much lower K_m for Ado than that of ADA, AK is likely to have a principal role in regulating Ado metabolism [6, 7].

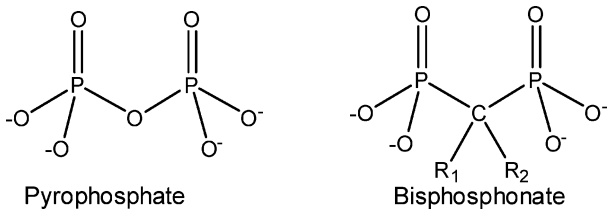
A great deal of recent interest in Ado metabolism and AK functioning is due to the discovery that Ado is involved in the protection of cells undergoing stress and trauma [8–13]. As an endogenous inhibitory neuromodulator, Ado acts to limit tissue damage and restores normal function by reducing cellular excitability. The local levels of Ado increase at sites of tissue injury and inflammation, where Ado binds to the members of the P1 receptor family (A_1, A_{2A}, A_{2B}, A_3 receptors) [14, 15]. Binding of Ado to these receptors activates a number of signaling pathways, which eventually result in various tissue-protective effects including inhibition of excitatory amino acid (glutamate) release, suppression of free radical formation and neutrophil adhesion [12, 16, 17]. For example, Ado protects the myocardium from ischemic insults by initiating the protective phenomenon known as preconditioning [11, 18, 19].

There are a number of potential strategies to harness the therapeutic benefits of Ado receptor (AR) mediated signaling pathways: (i) introduction of exogenous Ado, (ii) administration of AR agonists, and (iii) utilization of AK inhibitors. Unfortunately, administration of exogenous Ado is not a physiologically effective method, since Ado is rapidly phosphorylated in the plasma with a half-life on the order of seconds. AR agonists on the other hand, while producing positive effects in a number of animal pain models and seizure models [20–24], show severe side effects such as reductions in blood pressure and heart rate, hypothermia and motor depression [25–27].

Use of AK inhibitors seems more promising of a strategy, enabling one to harness the therapeutic benefits of AR activation while minimizing its side effects. This is because AK inhibitors affect Ado metabolism such that the level of extracellular Ado is increased in a site- and event-specific manner. The specificity of this action is due to the fact that extracellular Ado is produced primarily from breakdown of intracellular ATP [8]. Net ATP breakdown occurs in cells that are under cellular stress because repletion of ATP stores is not possible at a rate comparable with its utilization. Intracellular Ado passes out of the cells as its concentration increases, where they can activate nearby ARs. Agents that can decrease the metabolism of Ado, such as AK inhibitors, should therefore increase the level of extracellular Ado specifically within hypoxic regions of tissues and not in normoxic regions [28].

As reported previously, the catalytic activity of AK from various sources exhibits nearly complete dependence upon the presence of activators such as inorganic phosphate (Pi), arsenate or vanadate [29–31]. In a recent study, a number of additional compounds were identified to activate the enzyme, and these compounds included phosphonoformic acid (PFA), phosphocreatine (PC), phosphoenolpyruvate (PEP), phosphoribosyl pyrophosphate (PRPP) and inorganic pyrophosphate (PPi) [32]. Interestingly, a few other compounds, which share a close structural similarity with these activating compounds, were identified to inhibit AK activity in the presence of Pi. These inhibitors included phosphonoacetic acid (PAA), 2-carboxyethylphosphonic acid (CEPA), *N*-(phosphonomethyl)-glycine (PMG) and *N*-(phosphonomethyl)iminodiacetic acid (PMIA) [32]. The observation that inorganic pyrophosphate (PPi) is an effective stimulator of AK activity whereas phosphonic acid derivatives (*viz*. PAA, CEPA, PMG) are inhibitors of the enzyme, has prompted us to examine the effect of bisphosphonates on AK activity.

Bisphosphonates are structural analogues of PPi, of which the two phosphonate groups are linked to the central atom by phosphoether bonds (P─C─P structure, see the adjoining illustration).



They are an important group of drugs, which due to their marked ability to inhibit bone resorption, are widely used for treatment of different bone disorders including osteoporosis, Paget's disease, fibrous dysplasia and primary hyperparathyroidism [33, 34]. Additionally, they are also used for treatment and prevention of many bone-related problems (e.g. hypercalcemia, bone metastases) associated with several common malignancies such as breast cancer and multiple myeloma [33, 34].

In the present communication, we report identification of a number of additional AK activators and inhibitors. We describe for the first time that two of the bisphosphonate compounds that were studied in this work (*viz*. clodronate and etidronate), were both found to inhibit the activity of purified AK. The effect of various AK inhibitors, including bisphosphonates, was also examined on cultured mammalian cells. Structural comparisons of the activators and inhibitors reveal that the magnitude of the net partial charge (δ^+) on the core pentavalent atom of these compounds is a key factor in determining their ability to either activate or inhibit the enzyme.

Materials and methods

Adenosine kinase and its activity assay

Catalytically active recombinant AK from Chinese hamster ovary (CHO) cells was expressed and purified from *E. coli*

cells as previously described [31, 32, 35]. AK activity was measured by a radiochemical method using [2,8-^{3}H] adenosine as previously described [31, 32, 35].

Ado uptake and incorporation assay

Approximately 1×10^5 CHO cells were seeded in 24-well plates in α-MEM medium supplemented with 5% dialysed fetal bovine serum (FBS). The cells were incubated overnight at 37 °C in a 95% air −5% CO_2 incubator. The following day, when the cells were nearly confluent, the medium was carefully aspirated and replaced with 1 ml of the medium containing 10 μCi/ml of [^{3}H]-adenosine, 10 μg/ml of the ADA inhibitor, erythro-9-(2-hydroxyl-3-nonyl) adenine (EHNA) and the desired concentrations of the compounds whose effect was to be tested. After 1 h incubation, the labeled medium was aspirated and the cells were washed three times with 1 ml of 1× PBS. After the final wash, the cells were dissolved in 0.5 ml of lysis buffer (0.4% deoxycholic acid in 0.1N NaOH). To quantify the total cellular uptake of adenosine, one-half of the cell lysate was counted after addition of 5 ml of scintillation fluid. To determine the amount of adenosine which had been incorporated into cellular macromolecules, the other half of the cell lysate was added to 1 ml of ice-cold 10% trichloroacetic acid. After 30 min, the precipitate was collected on glass microfibre filters, and after washing with 20 ml of ice-cold 10% trichloroacetic acid, they were dried and counted for radioactivity.

Cellular toxicity test

Approximately 5×10^5 CHO cells were seeded in 60 mm dishes. After overnight incubation, the medium was aspirated and the cells were washed with 1× PBS. The experiment was started with the addition of 2 ml of the medium containing various concentrations of AK inhibitor compounds. After 1 h incubation, cells were washed with 1× PBS, trypsinized and counted by using a coulter counter. In two 60 mm dishes, 250 cells from each treatment were seeded in growth medium without any drug and incubated at 37 °C in a 95% air–5% CO_2 incubator. After 7 days of incubation, the colonies formed were fixed and stained in 0.5% methylene blue in 50% methanol, and counted. The cloning efficiency of untreated cells was taken as 100% in calculating the relative plating efficiency of the drug-treated cells.

Calculation of partial charge

Partial charges on the phosphorus atoms of the activator and inhibitor compounds were calculated by using ChemOffice 2002. The molecular structures of the compounds were first constructed in ChemDraw Ultra 7.0, and then imported into Chem3D Pro 7.0, which converted the 2D structures into 3D models. With the MOPAC [36] integrated in Chem3D Pro 7.0, partial charges on the individual atoms were calculated by employing the Hamiltonian method MNDO [37]. Computation was done in duplicate, and the standard error values were determined subsequently.

Chemicals and reagents

[2,8-^{3}H] Adenosine (40 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. ATP was from Pharmacia Biotech (Canada). The compounds tested for AK activation and inhibition were mostly from Sigma-Aldrich Co. Etidronate and clodronate were obtained from the McMaster University Medical Centre pharmacy.

Results

Compounds which stimulate AK activity

As mentioned previously, the enzyme AK requires presence of inorganic phosphate or other activators for its catalytic function [29–32, 35]. Upon testing a number of compounds, which share similar molecular structures with these activators, we have identified four additional compounds that can activate AK. The effect of these compounds on AK activity is summarized in Fig. 1. As seen, in the absence of any added compound, only a low basal level of AK activity was observed. This activity was stimulated more than 10-fold upon addition of 10 mM phosphate. Acetyl phosphate (AP), carbamoyl phosphate (CP), dihydroxyacetone phosphate (DHAP) and imidodiphosphate (IDP) were all active in stimulating AK function in a dose-dependent manner. Except for IDP, 10 mM of these compounds resulted in similar levels of enzyme activity, which were comparable to the level of activation by inorganic phosphate.

Compounds which inhibit AK activity

Previously, a number of phosphono-compounds were reported to inhibit AK activity. These compounds included phosphonoacetic acid, 2-carboxyethylphosphonic acid, *N*-(phosphonomethyl)-glycine and *N*-(phosphonomethyl)iminodiacetic acid [32]. Methylphosphonic acid (MPA) and diethylphosphonoacetic acid (DEPAA), which are closely related to these compounds, were also found to inhibit AK (unpublished data). Since all of the AK inhibitors identified in our work are phosphonate derivatives, the effect of two of

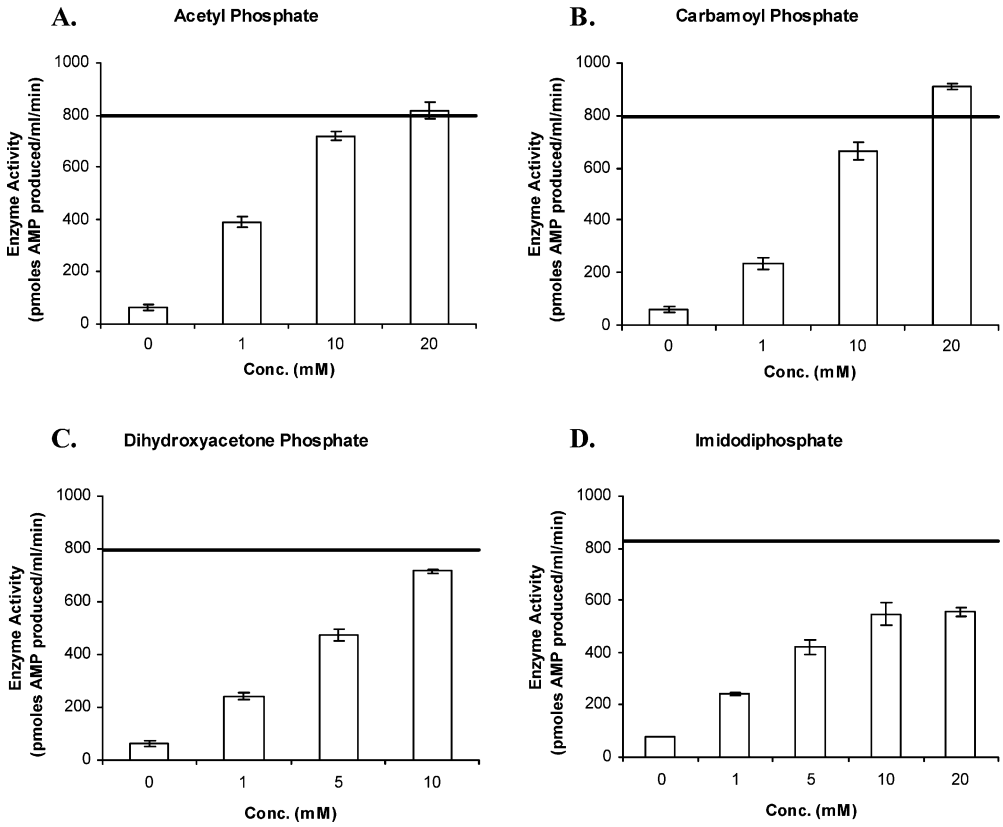


Fig. 1. Stimulation of AK activity by various compounds. Effect of different concentrations of the indicated compounds on AK reaction rate was determined. The thick straight line indicates AK activity in the presence of 10 mM inorganic phosphate without any other compound added.

the bisphosphonates, clodronate and etidronate, on AK activity was examined. As seen in Fig. 2, both these compounds inhibited AK activity in a dose-dependent manner in the presence of 10 mM inorganic phosphate. The enzyme inhibition curves for these compounds, as well as the IC_{50} values and the slope factors calculated from the plots are shown in Fig. 2.

Since our previous study indicated that PAA inhibits AK in a novel manner (noncompetitive with respect to Ado) [32], it was of interest to see whether the bisphosphonates also inhibited the enzyme in the same fashion. Thus, we have examined the effects of clodronate and etidronate on the kinetic properties of the enzyme (Fig. 3). As expected, increasing concentration of these compounds resulted in decreasing V_{max} of the enzyme. The maximum velocity of 2.39×10^3 pmoles/ml/min was reduced by 20, 38 and 45%, in presence of 4, 8 and 12 mM clodronate, respectively. Same concentrations of etidronate lowered the maximum velocity of the enzyme by 10, 43 and 67%. On the contrary, the K_m of the enzyme for Ado remained constant for different concentrations of clodronate and etidronate. The double-reciprocal plots in Fig. 3 show lines converging on the *x*-axis, indicating noncompetitive inhibition by these two compounds with respect to Ado. The K_i values for inhibition of AK by clodronate and etidronate were calculated to be 7.19 and 5.36 mM, respectively.

Ado uptake and incorporation assay

In view of the inhibitory effect of phosphono-compounds and bisphosphonates on purified AK, it was of interest to determine whether these compounds also inhibited the enzyme in the cellular environment. To test this possibility, effects of some of the inhibitors on the uptake of adenosine and its incorporation into macromolecules in CHO cells were investigated. In these experiments, cells were incubated with ^{3}H-adenosine in presence of different concentrations of the test compounds, and the amount of radioactivity retained and incorporated into the cells was measured (Section Materials and methods). The transport of adenosine across the cellular membrane is diffusive and equilibratory, and thus is driven by its intracellular metabolism. If the test compounds inhibit AK, AMP formation from Ado and its incorporation into macromolecules will be inhibited, and consequently, the

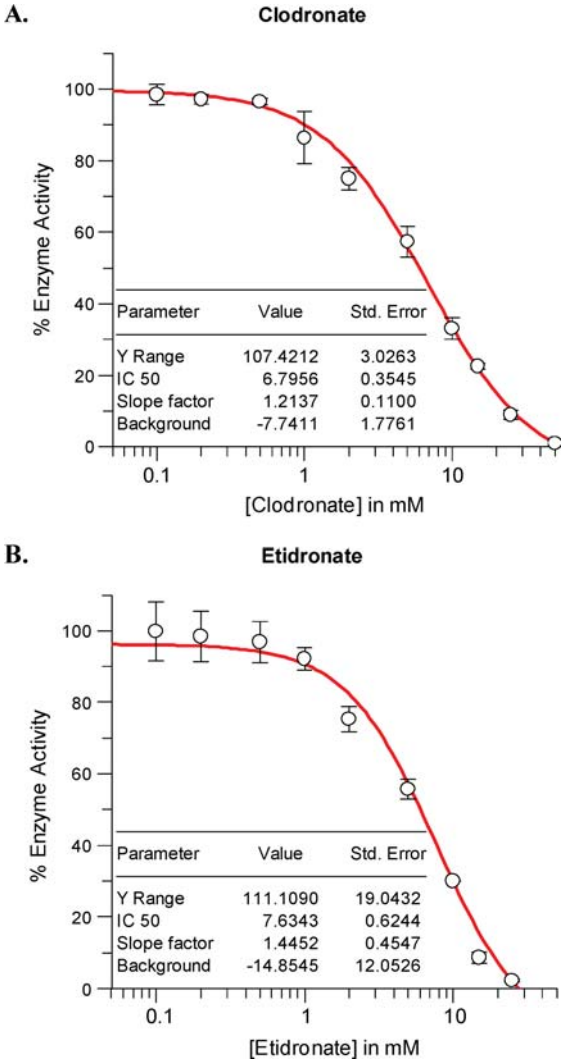


Fig. 2. Inhibition of AK activity by (A) clodronate and (B) etidronate. The enzyme assay mixture contained 10 mM of inorganic phosphate to activate the enzyme. The inhibition curves were plotted using Grafit 4, and the IC_{50} values and slope factors calculated are also shown in the figure. The assays were carried out in duplicate, and the standard error value for each data point is indicated on the plot.

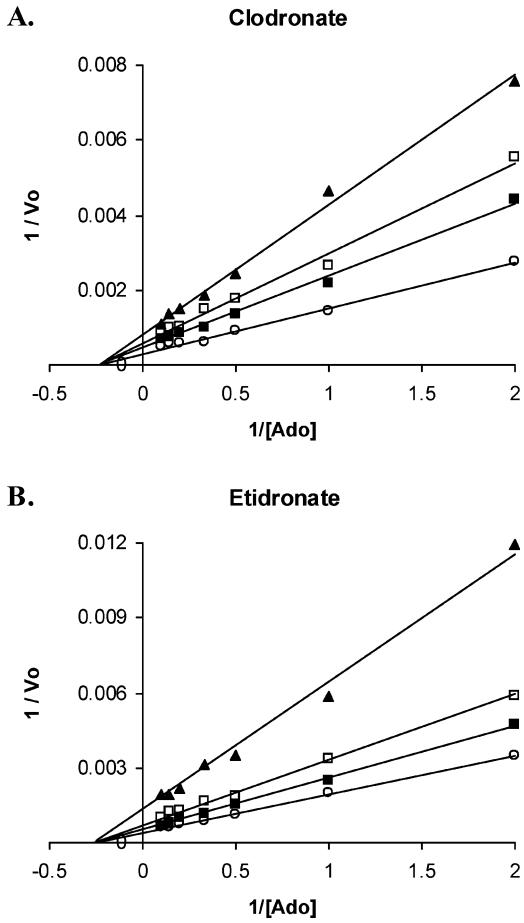


Fig. 3. Kinetic studies of the effect of (A) clodronate and (B) etidronate on AK catalytic activity. The enzyme assays were performed in presence of 10 mM inorganic phosphate in duplicate. Concentrations of the inhibitors used are: 0 mM (○); 4 mM (■); 8 mM (□); and 12 mM (▲).

cellular uptake and retention of Ado in these cells will also be inhibited. These experiments are carried out in presence of the ADA inhibitor EHNA to ensure that under the conditions of the experiment ^{3}H-adenosine is not deaminated and AK is the only enzyme responsible for its metabolism. When we tested several compounds including PAA, CEPA, PMG, PMIA, clodronate and etidronate, all of these compounds led to decreased Ado uptake in a dose-related manner (Fig. 4). In all cases, significant inhibition of ^{3}H-adenosine uptake was observed in 5–10 mM range and nearly complete inhibition was seen at 50 mM concentrations of the compounds. As shown in the figure, ^{3}H-adenosine incorporation into macromolecules also decreased in a similar pattern, indicating the impaired ^{3}H-adenosine uptake is indeed due to lowered intracellular AK activity. The concentration range in which these compounds inhibited ^{3}H-adenosine uptake is similar to that at which they inhibited the purified enzyme, also suggesting that these two effects are related.

Cellular toxicity studies

We have also examined whether the different AK inhibitors at the concentrations at which they inhibit AK activity were toxic to the cells. To examine this aspect, the cells were treated with different concentrations of the AK inhibitors, in a similar manner as in the Ado uptake experiment, and the viability of the treated cells was evaluated by determining the cloning efficiency of the cells in normal growth medium. Results of the cloning or plating efficiency measurement of the treated

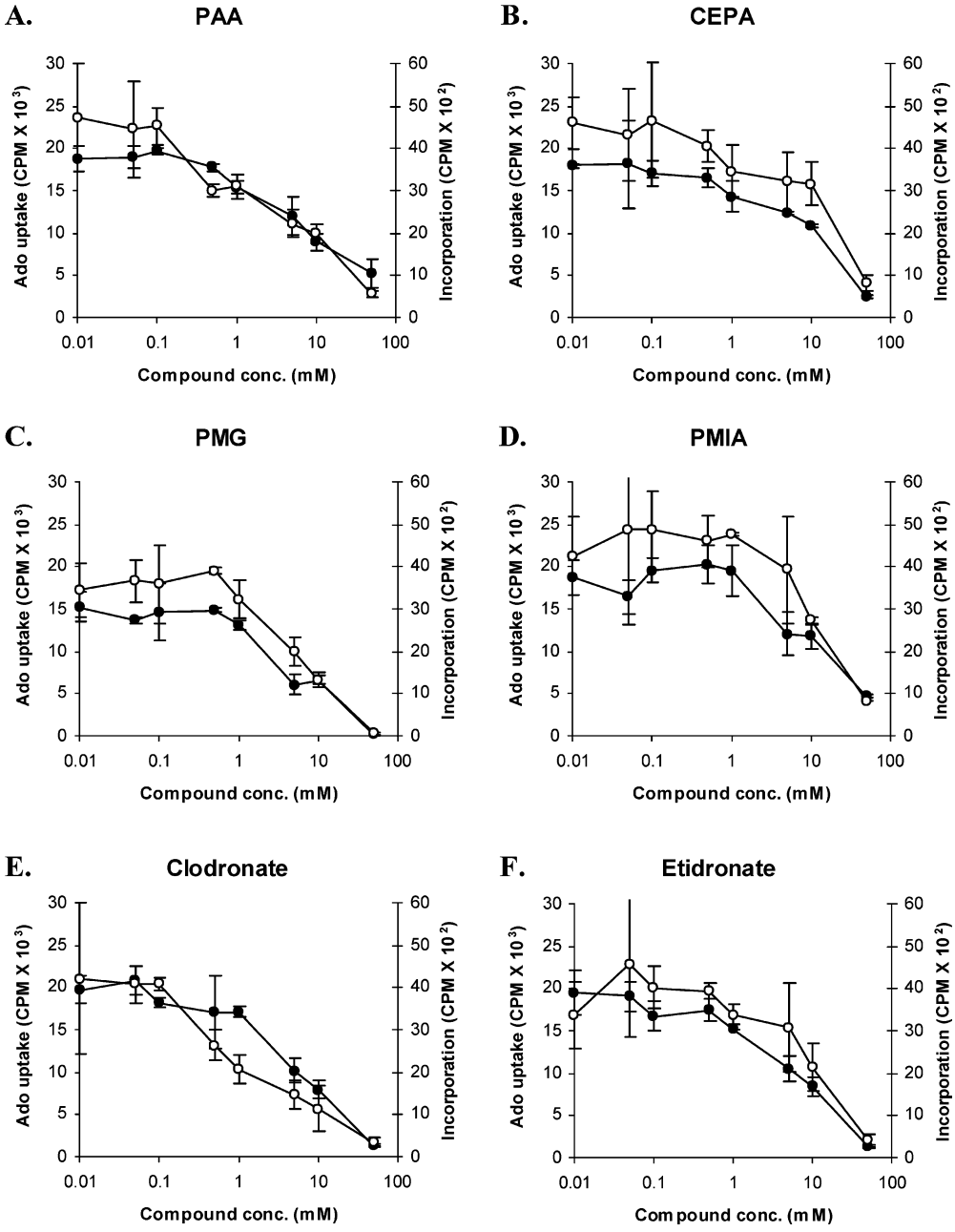


Fig. 4. Effect of AK inhibitors on the cellular uptake (•) and incorporation (○) of ^{3}H-adenosine by cultured CHO cells. Minimum of three independent experiments were carried out for each compound, and the resulting standard error values are indicated.

cells are shown in Fig. 5. As seen, treatment of the cells with CEPA and PMG had no observable effect on their viability. The other four compounds however reduced the cloning efficiency of the cells by 30–60%. At 50 mM, the highest concentration tested, PAA and PMIA reduced the plating efficiency by approximately 60%. Treatment with 50 mM clodronate and etidronate, on the other hand, resulted in a decrease in cell viability by 30–40%. Much of the decrease in cell viability for these two compounds was seen at 10 mM or lower concentration and treatment with higher concentrations did not lead to further reduction in cell viability. It is clear from these experiments that although some of these compounds exhibit limited toxicity, a large proportion of the cells remain viable after treatment with the higher concentration of these drugs. Thus, the inhibition of ^{3}H-adenosine uptake and incorporation by these compounds cannot be accounted for by their

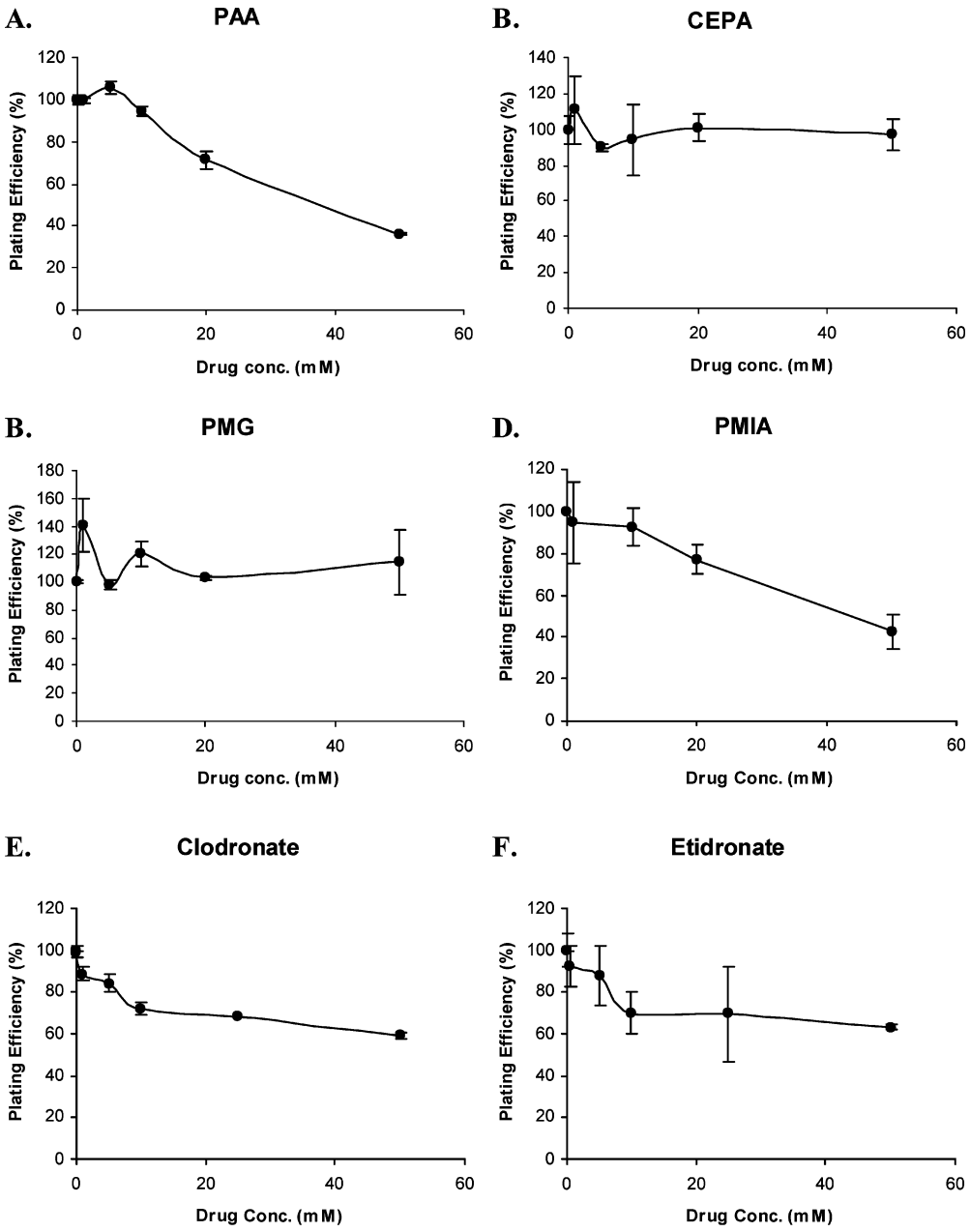


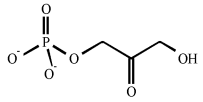
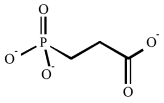
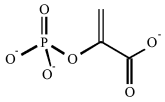
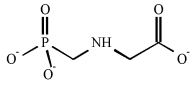
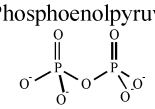
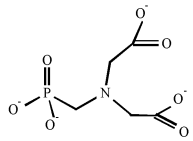
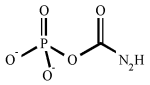
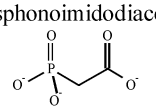
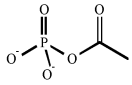
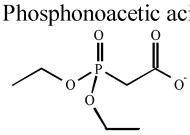
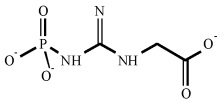
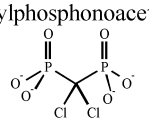
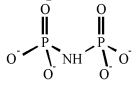
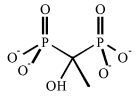
Fig. 5. Effect of AK inhibitors on the viability or relative plating efficiency of cultured CHO cells. The plating efficiency of the control cells not treated with any inhibitor has been assumed as 100%. Experiments were performed in duplicate

cellular toxicity, and their effect on inhibition of Ado uptake in CHO cells is likely due to inhibition of the cellular AK.

Structural comparisons of AK activators and inhibitors

The different activators and inhibitors of AK that have been identified in our work are structurally related. Hence, it is important to determine what structural feature(s) distinguishes an activator from an inhibitor. The simplest of the activating compounds (i.e. PO_4^{3-}, AsO_4^{3-} and VO_4^{3-}) contain a pentavalent core atom, connected to four oxygen atoms. Due to the higher electronegativity of oxygen in comparison to phosphorus, arsenic or vanadium, a partial positive charge (δ^+) is present on the central pentavalent atom of these compounds. All of the other identified AK activators have the general structure $PO_3^{2-}XR$, in which the X atom generally consists of an electronegative oxygen or nitrogen (Table 1). The X atom in various activators is connected to either a carbon or a phosphorus atom, which in turn is double-bonded to a

Table 1. Structures and partial charges on the pentavalent atoms of the AK activators and inhibitors

Activators		Inhibitors	
Compound	Charge	Compound	Charge
 Inorganic phosphate	1.29 ± 0.03	 Methylphosphonic acid	0.95 ± 0.01
 Dihydroxyacetone phosphate	1.37 ± 0.01	 2-Carboxyethylphosphonic acid	1.11 ± 0.01
 Phosphoenolpyruvate	1.35 ± 0.01	 N-Phosphonomethylglycine	0.99 ± 0.01
 Inorganic pyrophosphate	1.35 ± 0.01	 N-Phosphonoimidodiacetic acid	0.95 ± 0.01
 Acetyl phosphate	1.35 ± 0.05	 Phosphonoacetic acid	1.12 ± 0.01
 Carbamoyl phosphate	1.29 ± 0.01	 Diethylphosphonoacetic acid	1.17 ± 0.01
 Phosphocreatine	1.34 ± 0.02	 Clodronate	N/D*
 Imidodiphosphate	1.33 ± 0.01	 Etidronate	N/D*

The partial charge values were calculated as described in the Section Materials and methods.
*The 3D structures of some of the compounds were computationally unstable, and as a result, the partial charges on their phosphorous atoms could not be determined.

carbon, oxygen or nitrogen (C=C, C=O, C=N or P=O) in the R group (Table 1). These carbon and phosphorous atoms have a partial positive charge due to the contributing structures (e.g. C^{+}—C^{-}, C^{+}—O^{-}, C^{+}—N^{-} or P^{+}—O^{-}), and this reinforces the electron-withdrawing ability of the X atom by the inductive effect. Thus, the X atoms in various activating compounds aid in maintaining or enhancing the partial positive (δ^{+}) charge on the core pentavalent atom, which we have previously hypothesized as a key feature of AK activating molecules [32].

Although the mechanism of AK activation by these compounds is not fully understood, we have suggested that the core pentavalent atom with a partial positive charge serves as an acceptor for a nucleophilic group in AK catalysis. In contrast to the AK activators, the various AK inhibitors that we have identified have a methylene (—CH_2—) group adjacent to the core phosphorous atoms (Table 1). Because of the electron-donating characteristic of —CH_2— group, the partial charges on the core pentavalent atoms in such compounds is expected to be weaker, which likely affects their ability to function as an effective electrophile (Table 1). This structural distinction between AK activating and inhibiting compounds however, was made based only on the empirical examination of their chemical structures.

To test whether the AK activators and inhibitors indeed differ in this regard, we have attempted to estimate the net partial positive (δ^+) charge on the core phosphorous atoms of different activators and inhibitors. Since partial charges are not measurable quantities, we employed MOPAC [36] to determine these values semi-empirically. MNDO wave function [37] was used in the calculation, which had been shown to produce more reliable and accurate atomic point charges [38, 39]. Calculations were carried out as described in the Section Materials and methods, and the resulting values for different compounds are given in Table 1. As seen, the net partial positive (δ^+) charge on various AK activators is in the range of 1.29–1.37, whereas for the different inhibitors the corresponding values are in the range of 0.95–1.17. These results reveal that the net partial positive (δ^+) charge on the central phosphorus atom is indeed higher for various activators in comparison to different inhibitors. For example, inorganic phosphate is an activator with the net charge of 1.29 on the phosphorous atom, whereas methylphosphonic acid, which is structurally closely related to inorganic phosphate, but has a net charge of 0.95, functions as an inhibitor of the enzyme. Several other pairs of structurally similar compounds differing in terms of net charges on the central phosphorous atom (*viz*. dihydroxyacetone phosphate, net charge 1.37 and 2-carboxyethylphosphonic acid, net charge 1.11) (Table 1), were also found to behave in a similar manner. The result of these charge calculation studies thus supports our hypothesis that the magnitude of the positive charge on the central phosphorus atom is an important determining factor in their acting as either AK activators or inhibitors. The value of the net positive charge for various AK activators is found to be in a close range (i.e. 1.29–1.37), indicating that a charge value above 1.25 is likely required for a compound to act as an AK activator.

Discussion

The purine nucleoside Ado exhibits potent pharmacological effect on cardiovascular, nervous, respiratory and immune systems. In view of the beneficial effects of increased Ado concentration in protecting these tissues from damage induced by ischemia and other stresses, there is much interest in developing inhibitors of AK. A number of AK inhibitors such as 5′-amino,5′-deoxyadenosine, 5-iodotubercidin (5IT) and 5′-deoxy,5-iodotubercidin (5′d-5IT) have shown positive effects in various animal models of pain, seizure and ischemia [40–43], but they also exhibited significant toxicity which limit their therapeutic potential [43, 44]. In an effort to find more potent AK inhibitors with better *in vivo* properties, other groups have synthesized a series of other Ado and tubercidin analogues and tested them [45, 46]. In spite of extensive studies, they were not able to identify compounds with higher AK inhibiting potency and fewer side effects than those of 5′d-5IT and 5IT. The apparent toxicity of nucleoside analogues and derivatives in part results from their close structural similarity to Ado, due to which these compounds can either bind to Ado receptors themselves or be metabolized by other Ado-recognizing enzymes, resulting in various side effects.

In this context, our observation that a number of phosphate derivatives can either activate or inhibit AK via a novel mechanism is of much interest. Because of their distinct chemical structures, these compounds are not expected to interact with the Ado receptors or be metabolized by AK, thus minimizing the possibility of side effects seen with Ado analogues. Our work has identified a number of AK inhibitors, which consist of phosphonate derivatives. Of particular significance is the observation that the two bisphosphonate compounds studied in our work (*viz*. clodronate and etidronate) were both found to inhibit the activity of purified AK, as well as AK activity in whole cells as determined by inhibition of ^{3}H-adenosine uptake in cells. Both these effects of the bisphosphonates as well as other AK inhibitors were observed at comparable concentrations indicating that they are related. Further, at the concentrations at which these effects on AK were observed, the cellular toxicity was limited. These observations are of great interest because bisphosphonates are an important group of drugs that are currently used to treat a wide spectrum of clinical disorders related to bone resorption [47, 48].

In view of the clinical importance of bisphosphonates, a large number of derivatives have been synthesized and subsequent structure–activity studies have been carried out. This has identified many compounds which are much more potent in comparison to the first generation bisphosphonates such as clodronate and etidronate. This new generation of compounds, referred to as nitrogen-containing bisphosphonates, seems to target the intracellular mevalonate pathway [33]. A number of enzymes such as squalene synthase and farnesyl transferase in this biosynthetic pathway, which is responsible for the production of cholesterol and isoprenoid lipids, are inhibited by these nitrogen-containing bisphosphonates [33]. Our results presented here indicate that inhibition of AK may provide an additional cellular target for bisphosphonates. Since K_i values in the several millimolar range (7.2 mM for clodronate and 5.4 mM for etidronate) are difficult to achieve in the clinical setting, it is of obvious interest to examine the effect of various other bisphosphonates on AK to identify more potent inhibitors of the enzyme. The pharmacodynamic and pharmacokinetic properties of bisphosphonate derivatives have also been extensively studied, and this should prove very helpful, if some of these compounds turn out to be more potent inhibitors of AK which could be examined for the AR-mediated tissue protection *in vivo*.

Another potential area for the clinical application of AK inhibitors is the treatment against ubiquitous parasitic

protozoans such as *Leishmania donovani* and *Toxoplasma gondii* [49, 50]. Infection by these parasites constitutes a serious health threat, and thus, the need for therapeutic agents active against these organisms is acute. For instance, leishmaniosis (also known as kala-azar) caused by *L. donovani* affects about 500,000 people per year worldwide, and proves fatal if no treatment is given. *T. gondii*, on the other hand, infects about one-third of the North American population, and causes toxoplasmosis which can have devastating consequences in neonates and immuno-compromised individuals. Like all parasitic protozoans, these organisms cannot synthesize purine bases de novo, and must rely on the host purines acquired through the salvage pathway. Enzymes in this pathway, such as AK, are absolutely essential for parasitic survival, and thus represent attractive drug targets [49, 50]. Use of AK inhibitors exploiting the biochemical differences between the host and the parasite enzymes may prove useful in the treatment of diseases caused by these organisms.

Our studies also provide important insights concerning the structural features of AK activators and inhibitors, which should be helpful in the design and discovery of other novel and more potent inhibitors of AK. We have shown that AK activating compounds have a higher partial positive charge (δ^+) on the central phosphorous atom in comparison to the inhibitors. The core phosphorous atom in the activating compounds is generally attached to electron withdrawing groups, which serve to enhance the net partial positive charge (δ^+) on this atom. In contrast, the phosphorus atom in various AK inhibitors is attached to a methylene ($-CH_2-$) group, which because of its electron-donating characteristic reduces the net positive charge on the central phosphorous atom. Based on these observations and the information regarding the potential phosphate-binding site in AK structure [35, 51–53], we have suggested a model to account for the activation and inhibition of AK by various phosphorylated derivatives [32]. According to this model, when an activating molecule is bound to a regulatory site near the active site on the enzyme, the positive charge on the central phosphorus atom serves as an electrophile and attracts the non-bridging oxygen of the β-phosphate in ATP. As a transient bond forms between the activating compound and β-phosphate of ATP, the bond between the β- and γ-phosphate in ATP weakens, until eventually, the γ-phosphate transfers to Ado. AK inhibitors are structurally very similar to the activators, and this suggests that they bind to the common site where the activators bind. Binding of activators and inhibitors at the same site is also supported by the competition studies [32]. Inhibition of AK by the inhibitory compounds then is likely due to antagonism towards the activators. With a similar or perhaps better binding affinity for AK, an inhibitor may compete or displace the activators from the AK regulatory site. Due to the lower partial positive charge on the central phosphorous atom however, such compounds are not able to function as an effective electrophile, thus leading to an impediment and inhibition of AK catalysis. Further studies aimed at testing this model should prove very useful in understanding how the biological activity of this key enzyme may be regulated *in vivo* and in the design of more potent mechanism-based inhibitors of AK.

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