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Original article

Inhibition of *Streptococcus pneumoniae* adenylate kinase by some 5-arylidene-thiazolidin-4-on-2-thione derivates

Efectul inhibitor al unor 5-ariliden-tiazolidin-4-on-2-tione asupra adenilat kinazei din *Streptococcus pneumoniae*

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Abstract

Adenylat kinase is an ubiquitous enzyme found in prokaryotes and eukaryotes. In the present study we examined the inhibition of Streptococcus pneumoniae adenylate kinase (AK_{SP}) by six 5-arylidene-thiazolidin-4-on-2-thione derivates using 2, 4-dinitrophenylhydrazine colorimetric assay. Inhibition of AK_{SP} activity with synthetic compounds was performed against recombinant enzyme over-expressed in E. coli. The compound $C_{10}H_6BrNOS_2$ with the bromine atom in –ortho position has shown the most efficient inhibitory activity; I_{50} value (the inhibitor concentration that leads to 50% activity inhibition) was 0.067mM.

Keywords: *adenylate kinase, 5-arylidene-thiazolidin-4-on-2-thione derivates, inhibitory activity*

Rezumat

Adenilat kinaza este o enzimă ubiquitară prezentă atât la procariote cât şi la eucariote. În această lucrare am testat acţiunea inhibitorie a şase 5-ariliden-tiazolidin-4-on-2-tione faţă de adenilat kinaza din Streptococcus pneumoniae (AK_{SP}) prin metoda colorimetrică cu 2,4-dinitrofenilhidrazonă. Efectul inhibitor al compusilor de sinteză a fost testat faţă de enzima recombinantă supra-exprimată în E. coli. Compusul $C_{10}H_6BrNOS_2$ cu atomul de brom în pozitia –orto a fost cel mai eficace inhibitor, valoarea I_{50} (concentraţia de inhibitor care determină inhibarea activităţii cu 50%) a fost 0.067mM.

Cuvinte cheie*: adenilat kinaza, 5-ariliden-tiazolidin-4-on-2-tione, acţiune inhibitorie*

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Introduction

Adenylate kinases (EC 2.7.4.3) belong to a subfamily of NMP kinases involved in the biosynthesis of nucleotides. They are phosphotransferases that catalyse an essential reaction for growth and division of all living cells. The reaction catalysed is: Mg^{2+}-ATP + NMP ↔ Mg^{2+}-ADP + NDP (where NMP is ATP). Adenylate kinases play a vital role in cellular energy metabolism and nucleic acid synthesis (1, 2). These enzymes share a common architecture but could have different biochemical and physico-chemical properties (3, 4). Finding new inhibitory compounds against bacterial pathogens is a challenging issue (5). This task is facilitated by existing data on a lot of enzymes as potential target, available on protein database (6). The aim of this study was to evaluate the inhibitory activity of a series of 5-arylidene-thiazolidin-4-on-2-thione derivates against *Streptococcus pneumoniae* adenylate kinase (AK_{SP}). This paper begins by describing procedures used to obtain a purified recombinant AK_{SP}. It will then go on to a brief overview of the synthesis of our derivates and their inhibition of AK_{SP}. Finally, we evaluate our recent results with previous data concerning *E.coli* adenylate kinase.

Material and methods

Sequence comparison of amino acid sequences

Protein sequence database searches were performed using the BL2SEQ program – BLAST version developed by NCBI – default parameters BLOSUM 62 with AK_{SP} (Q97SU1) as a query sequence (7). Sequence alignment was performed using multiple sequence alignment ClustalW program (SwissProt – EMBL database) (8).

Bacterial strains and growth conditions

The gene adk from Streptococcus pneumoniae was amplified by PCR and the PCR product was inserted into the expression vector pET-28a from Novagen. The recombinant plasmid was introduced into BL15 *E. coli* strain to overproduce the His-tag AK_{SP}.

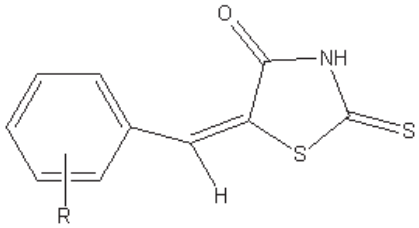
The recombinant strains were grown in 2YT medium supplemented with 30 μg/ml kanamycin and 34 μg/ml chloramphenicol with shaking at 37ºC until a value of 1.3 at OD_{600} was reached. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1mM and incubation was continued for 3 hours. Cells were harvested by centrifugation and stored at -80°C until purification.

Purification of adenylate kinase from Streptococcus pneumoniae

N-terminal His-tagged AK_{SP} was purified by affinity chromatography on Nickel-nitriloacetic acid (Ni-NTA) resin using QIA system (9). Bacterial pellets expressing recombinant AK_{SP} were disrupted by sonication in lysis buffer: 50 mM Tris/HCl (pH 8) 300 mM NaCl, 10 mM imidazole. The bacterial extract was applied onto 1 ml Ni-nitrilotriacetic acid (Ni-NTA) column pre-equilibrated with lysis buffer. The column was washed with 50 mM Tris/HCl pH 8, containing 300 mM NaCl, 50 mM imidazole. AK_{SP} was eluted with elution buffer (50 mM Tris/HCl pH 8, containing 300 mM NaCl, 250 mM imidazole).

Activity assay of recombinant enzymes

AK_{SP} activity was determined at 30°C using colorimetric assay at 460nm (1 ml final volume). The reaction medium contained 50 mM Tris/HCl (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate (PEP), 1-2 U of pyruvate kinase (PK), 1 mM ATP and 0.3 mM AMP. The reaction was started with supernatant or purified protein diluted in 50 mM Tris/HCl pH 7.4. The reaction was stopped with 0.08 ml solution 0.1% 2, 4-dinitrophenylhydrazine DNFH in HCl 2N after 10 min at 30°C. After another 10 min of incubation at 30°C, 0.5 ml NaOH 2.5N was added. The difference between standard (without enzyme) and "blank" (without AMP) represent ATP-asic activity of supernatant. The difference between sample and standard represents AK_{SP} activity.



1a. R=4F
1b. R=3OCH_3
1c. R=2OCH_3
1d. R=4Br
1e. R=2Br
1f. R=3Cl

Figure 1. 5-arylidene-thiazolidin-4-on-2-thione derivatives 1a-f structures

Analytical procedures

Protein concentration was measured according to Bradford (10). SDS-PAGE was performed as described by Laemmli (11) and gels were stained with Coomassie Blue. Aliquots of compound solution in dimethylformamide (DMF) were added in reaction tube before reaction start. I_{50} constant represents the inhibitor concentration that yields 50% enzymatic activity inhibition. I_{50} was calculated from the equation of plots enzyme inhibition (%) versus compound concentration. Curve-fit was performed using KaleidaGraph (Adelbeck Software) for a hyperbolic progress equation.

Synthesis of 5-arylidene-thiazolidin-4-on-2-thione derivatives

In a previous paper we reported the synthesis in good yields of a series of 5-arylidene-thiazolidin-4-on-2-thione derivatives **1a-f**, by Knoevenagel condensation between various substituted arylaldehydes and thiazolidin-4-on-2-thione, by refluxing in glacial acetic acid, in the presence of anhydrous sodium acetate (12).

We report that the newly obtained derivatives were capable of inhibiting the adenylate kinase wild-type from *E.coli*, a Gram-negative organism (13). In continuation of our work, we present here the testing of inhibitory efficiency against the same enzyme from a Gram-positive organism, *Streptococcus pneumoniae.*

Results

Sequence comparison of amino acid sequences

Bacterial adenylate kinases share significant sequence similarities (14). Among eight adenylate kinases examined (four from Gram-positive bacteria, three from Gram-negative bacteria and one from yeast), 48 strictly conserved residues were noticed. The N-terminal region is well conserved, mainly ATP binding site. In LID domain we noticed the motif Cys-X_2-Cys-X_{16}-Cys-X_2-Cys/Asp, present in most of the Gram-positive adenylate kinases, with cysteine residues being involved in zinc binding. Adenylate kinases with the zinc-binding motif shows enhanced thermal stability (15). In AK_{SP} cysteine residues we notice non-conservative replacement. The reason of this replacement is not known.

Purification and specific activity of recombinant AK_{SP}

Recombinant AK_{SP} overproduced as having N-terminal His-tag was purified as described in Material and methods. The protein of interest was eluted in the second fraction (6.23 mg/ml). The specific activity of AK_{SP} was 33.52 µmol/min.mg of protein.

Inhibition of AKSP activity by 5-arylidene-thiazolidin-4-on-2-thione derivatives

AK_{SP} was tested as potential target for six 5-arylidene-thiazolidin-4-on-2-thione derivatives **1a-f** at a single concentration (0.2 mM) as indicated in *Table 1*. As control, we have also tested the effect of dimethylformamide, the substance used as a solvent. The solvent did not reduce the enzymatic activity.

The most efficient compound **1e** was tested at different concentration between 0.005 and 1 mM. I_{50} constant for compound **1e** was 0.067mM calculated from equation of inhibition plot: $y = 106.09 * x^{0.28236}$.

Discussion

Bacterial adenylate kinases are monomeric enzymes members of NMP kinase family.

Figure 2. Sequence alignment of adenylate kinase from: *Streptococcus pneumoniae* (Q97SU1), *Streptococcus mutans* (Q8DS33), *Bacillus subtilis* (P16304), *Clostridium perfringens* (Q8XHU4), *Haemophilus influenzae* (P24323), *Saccharomyces cerevisiae* (P07170), *Neisseria meningitidis* (P49980) and *E.coli* (P05082). The C-terminal sequences are not shown (43 residues). The ATP binding-site is framed in grey and conserved residues are shown in red. Cysteine residues, belong to LID domain, are shown in yelow. At Gram-negative adenylate kinase Cys residues diplay a conservative replacement: His, Ser, Asp and Thr (labeled in blue). At AMP binding-site are labeled in pink residues Leu and Val, very well conserved at adenylate kinases. Consensus sequence (LIVMFYWCA) - (LIVMFYW) (2) – D – G - (FYI) – P – R – X (3) - (NQ) is framed in green and well conserved residues Asp and Arg are bolded.

Existing protein databases provide detailed description of the proteins whose structure/ sequence is known (http://www.rcsb.org/pdb/ home/home.do). Analysis of the relationships between sequence, structure and biochemical properties could reveal unexpected features that could be the premises for interesting correlations between well characterised proteins. A lot of new thiazolidinones tested *in vitro* for their antimicrobial activity were very efficient against Gram positive bacteria (16).

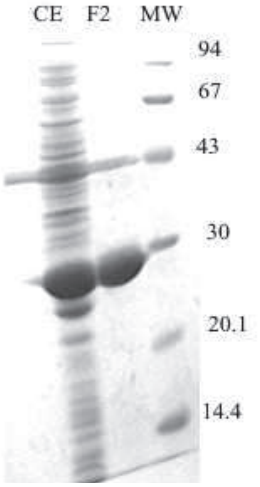


Figure 3. SDS-PAGE of fractions during purification of AK_{SP} : CE - crude extract, F2 - His-tag fraction 2, protein size marker (94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa, 14.4kDa)

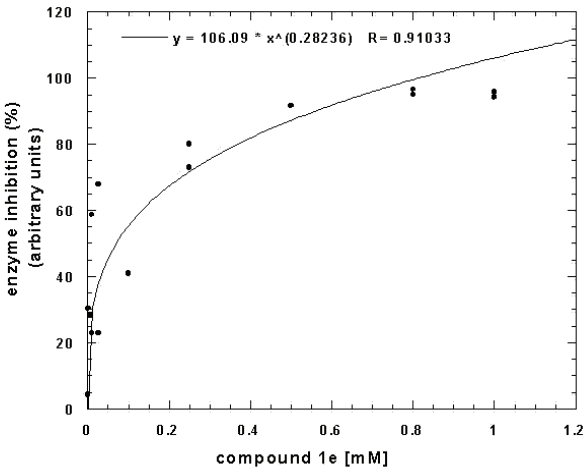


Figure 4. Inhibition of *Streptococcus pneumoniae* adenylate kinase by compound 1e

Our research is focused in design and synthesis of new thiazolidinone derivates with potential antibacterial activity. In other study (13) inhibitory activity of seven 5- aryliden-2-thioxo-thiazolydin-4-one derivatives against wild – type *E.coli* adenylate kinase was tested. Inhibitory compounds show enzyme inhibition between 11.88 – 72.33 % when tested at 0.2 mM. The I_{50} (0.065mM) was determined for the most efficient compound, that with the bromine atom in –ortho position. Analyzing these results, another bacterial adenylate kinase from a human pathogen was cloned and purified - AK_{SP}. Since there is no significant difference in specific activities of wild-type and His-tagged forms of bacterial adenylate kinase the recombinant AK_{SP} was overproduced as having N-terminal His-tag (V_m = 33.52 µmol/min.mg of protein). Six 5-arylidene-thiazolidin-4-on-2-thione derivatives were tested as inhibitory agents at a single concentration (0.2mM). The I_{50} (0.067mM) was determined for the compound with the bromine atom in –ortho position **(1e)**. Comparative testing of the same compound against adenylate kinase from different microorganisms reveal no striking differences suggesting the same binding-site for both recombinant enzymes. This finding, while preliminary, suggests that the same residues are involved in binding compound $C_{10}H_6BrNOS_2$. However, these results were not very encouraging because do not explain the mechanism of inhibitor-enzyme interaction.

Table 1. Inhibition of AK_{SP} by 0.2 mM 5-arylidene-thiazolidin-4-on-2-thione derivatives 1a-f

compound	enzyme inhibition (%)
1a	30.56
1b	25.31
1c	22.80
1d	39.96
1e	72.15
1f	39.12

Our study had some limitations. First, performing an activity assay of inhibitors against a purified enzyme is an exhaustive method when large panels of inhibitors are considered. Therefore we tested just six new inhibitory compounds. Another source of weakness in this study is related with the physico-chemical properties of our inhibitors. Due to their low solubility in Tris buffer, we chose the colorimetric method instead of a more reliable enzyme activity assay (3). Important limit-

ations of this study include the lack of three-dimensional structure of AK_{SP} which hampers advances in understanding structure-function relationship. While *Bacillus subtilis* adenylate kinase structure (17) is deposed in Protein Data Bank (PDB), we analyse sequence alignment of eight adenylate kinases. Interestingly, cysteine residues conserved at majority Gram-positive adenylate kinase (18), as we noticed at *Bacillus subtilis* and *Clostridium perfringens* adenylate kinase, are replaced at *Streptococcus pneumoniae* and *Streptococcus mutans* counterpart. It can therefore be assumed that these residues have different functions in Gram-positive bacteria. In future investigations it might be possible to use a different approach in finding new biologically active compounds, not only inhibition studies but molecular docking studies of new compounds into active site of adenylate kinase (19-21).

Conclusions

In conclusion, adenylate kinase represents an interesting target for new inhibitory compounds as its inactivation is not compatible with cell survival (22, 23). Six 5-arylidene-thiazolidin-4-on-2-thione derivatives were screened for their inhibitory activity against recombinant adenylate kinase from *Streptococcus pneumoniae* (this study) and recombinant adenylate kinase from *E.coli* (previous study) (13). The results of this study indicate that the compound $C_{10}H_6BrNOS_2$ with the bromine atom in –ortho position has shown the most efficient inhibitory activity with no differences between *Streptococcus pneumoniae* and *E.coli* adenylate kinases. The present results are significant in at least one major respect: we designed and synthesised a series of thiazolidinone derivates, one of them with a good inhibitory activity of two bacterial adenilate kinases. It would be interesting to assess the inhibitory activity of other new compounds, without solubility problems, against well characterised adenylate kinases.

Abbreviations

adk = adenylate kinase gene,
ADP = Adenosine diphosphate,
AK_{SP} = *Streptococcus pneumoniae* adenylate kinase,
AMP = Adenosine monophosphate,
ATP = Adenosine-5'-triphosphate,
CE = crude extract protein,
DMF = dimethylformamide,
EMBL = European Molecular Biology Laboratory,
F2 = *Streptococcus pneumoniae* adenylate kinase fraction 2,
His-tag = hexa histidine-tag,
I_{50} = inhibitor concentration that gives 50% enzymatic activity inhibition,
IPTG = isopropyl-β-D-thiogalactoside,
MW = molecular weight,
NDP = nucleoside diphosphate,
Ni-NTA = Nickel-nitriloacetic acid,
NMP = nucleoside monophosphate,
OD_{600} = Optical Density at 600 nm,
PCR = polymerase chain reaction,
PEP = phosphoenolpyruvate,
PK = pyruvate kinase,
SDS-PAGE = sodium dodecyl sulphate - polyacrylamide gel electrophoresis,
Tris/HCl = Tris (hydroxymethyl) aminomethane hydrochloride,
V_m = maximal reaction rate

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