ORIGINAL ARTICLE

Dedicated to the memory of Prof. PhD. Octavian BÂRZU

INHIBITION OF *STREPTOCOCCUS PNEUMONIAE* AND *ESCHERICHIA COLI* ADENYLATE KINASE BY 2-AMINO-4-METHYL-N'-ARYLIDENE-THIAZOLE-5-CARBOHYDRAZIDES

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Abstract

Adenylate kinase (AK) is involved in an essential step in nucleotide metabolism, catalysing the reversible transfer of the terminal phosphate group from ATP to AMP. The aim of this study was to describe 2-amino-4-methyl-N'-arylidene-thiazole-5-carbohydrazides effects on the activity of recombinant adenylate kinase from *S. pneumoniae* (AK_{SP}) and *E. coli* (AK_{EC}). Inhibition of AK_{SP} and AK_{EC} activity with synthetic compounds were performed against enzymes over-expressed in *E. coli*. The activity of recombinant enzymes was determined colorimetrically with 2,4-dinitrophenylhydrazine. 2-amino-4-methyl-N'-arylidene-thiazole-5-carbohydrazides inhibit AK_{SP} and AK_{EC} with different efficiencies. 2-amino-4-methyl 5-*o*-metoxi-phenyl-hydrazido thiazol ($C_{13}H_{15}N_4O_2S$) (compound I) binds tighter than 2-amino-4-methyl *p*-nitro-phenyl-hydrazido thiazol ($C_{12}H_{11}N_5O_3S$) (compound II). The I_{50} values (the inhibitor concentration that leads to 50% activity inhibition) for AK_{SP} was 34.8 μM for compound I and 178 μM for compound II. The I_{50} AK_{EC} was 38.63 μM for compound I and 170 μM for compound II.

Rezumat

Adenilat kinaza intervine într-o etapă importantă a metabolismului nucleotidelor purinice catalizând transferul reversibil al unei grupări fosfat terminale de pe ATP pe AMP. Obiectivul principal al acestui studiu a fost caracterizarea efectului a doi derivaţi 2-amino-4-metil-N'-ariliden-tiazolil-5-carbohidrazidici faţă de activitatea unor adenilat kinaze recombinante din *S. pneumoniae* (AK_{SP}) şi *E. coli* (AK_{EC}). Inhibarea activităţii AK_{SP} şi AK_{EC} a fost testată faţă de enzimele supra-exprimate în *E. coli*. Activitatea enzimelor recombinante a fost determinată colorimetric cu 2,4-dinitrofenilhidrazină. Derivaţii 2-amino-4-metil-5-carbonidrazol tiazol inhibă AK_{SP} şi AK_{EC} cu eficienţă diferită. 2-amino-4-metil-5 *o*-metoxi-fenil-hidrazido tiazol ($C_{13}H_{15}N_4O_2S$) (compus I) se leagă mai eficient decât 2-amino-4-metil *p*-nitro-fenil-hidrazido tiazol ($C_{12}H_{11}N_5O_3S$) (compus II). Valorile I_{50} (concentraţia de inhibitor care determină inhibarea activităţii cu 50%) pentru AK_{SP} a fost 34,80 μM pentru compusul I şi 178 μM pentru compusul II. The I_{50} AK_{EC} a fost 38,63 μM pentru compusul I şi 170 μM pentru compusul II.

Keywords: adenylate kinase, 2-amino-4-methyl-N'-arylidene-thiazole-5-carbohydrazides, inhibitory action

Introduction

Streptococcus pneumoniae is a major cause of life-threatening infections mainly in young children and elderly, in spite of development of polyvalent vaccines [9, 12]. Recently, the genome of *S. pneumoniae* was deciphered [8]. Compared analysis of genome of different kind of microorganisms offered premises for understanding some molecular mechanisms involved in emerging and spreading of pathogens. Furthermore, special Bioinformatics software programs used for sequences alignments allow the identification of conserved regions in phylogenetically related organisms. This is useful for identifying the efficient targets for new compounds with antibiotic potential.

Adenylate kinases (ATP:AMP phosphotransferase, EC 2.7.4.3) are present in prokaryotes and eukaryotes cells and participate in maintaining energy homeostasis in cells [6, 15]. Among the members of the adenylate kinase family, the structure of adenylate kinase from *E. coli* has been elucidated [4, 7, 13, 16]. We believe that in-depth understanding of metabolic pathways will contribute to the development of new therapeutic agents for the treatment of infections due to Gram positive bacteria. We have chosen *S. pneumoniae* as model of Gram positive extracellular human pathogens. Comparative analysis of the same enzyme from *S. pneumoniae* (SwissProt - Q97SU1) and *E. coli* (SwissProt - P69441) permit further approaches of inhibitory activity of new derivatives.

Materials and Methods

Cloning and expression of the gene coding for adenylate kinase from E. coli and S. pneumoniae

The genes encoding adenylate kinase (AK) were amplified by polymerase chain reaction (PCR) and cloned into pET vectors from Novagen. AK from *E. coli* and *S. pneumoniae* were cloned into pET24a and pET28a vectors respectively. Recombinant adenylate kinase from *S. pneumoniae* had a 6xHis-tagg at the N-terminus. Vectors with encoding genes were introduced by heat shock treatment, in the presence of calcium ions, into *E. coli* cells strain BL15. For overexpression of recombinant proteins, the BL15 cells containing vectors with genes were grown at 37ºC in 2YT medium supplemented with kanamycin (30 μg/mL) and chloramphenicol (34 μg/mL). When OD_{600} (optical density at λ = 600 nm) value reached 1.3, the overexpression of proteins was induced with 1 mM β-D-thiogalactopyranoside (IPTG). After 3 hours bacterial cells were harvested by centrifugation and stored at -80ºC until purification.

Purification of recombinant enzymes

Purification of adenylate kinase from E. coli

Adenylate kinase from *E. coli* was purified by pseudo-affinity chromatography on Blue-Sepharose followed gel-permeation chromatography on Ultrogel AcA 54 [3]. Bacterial cells containing recombinant AK were defrosted in 50 mM Tris-HCl (pH 7.4) and disrupted by sonication. The bacterial extract was clarified by centrifugation for 30 min at 10 000*g* at 4ºC. The supernatant was applied onto a Blue-Sepharose column pre-equilibrated with Tris/HCl 50 mM (pH 7.4). AK_{EC} adsorbed onto the column was eluted with a mixture of 1 mM ATP and 1 mM AMP in the same buffer. Other proteins adsorbed onto the column were eluted with 0.5 M NaCl. The fractions were protein has been spectrophotometrically detected at 280 nm were further analysed for activity and concentration.

Purification of adenylate kinase from S. pneumoniae

Adenylate kinase from *S. pneumoniae* was purified by affinity chromatography on Ni-nitriloacetic acid (Ni-NTA) resin [11]. The bacteria containing AK_{SP} was 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. 6xHis-tagged 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

Enzyme activity was determined photocolorimetrically with 2,4-dinitrophenylhydrazine (DNPH). The reaction medium (1 mL final volume at 30ºC) containing 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate (PEP), 1-2 U 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-DNPH in HCl 2N after 10 min at 30ºC. After another 10 min of incubation at 30ºC, 0.5 mL NaOH 2.5 N was added. Orange/brown colored solution was measured at 460 nm. The difference between standard (without enzyme) and "blank" (without AMP) represented the level of the ATP-ase activity of supernatant. The difference between sample and standard corresponded to adenylate kinase activity.

Analytical procedures

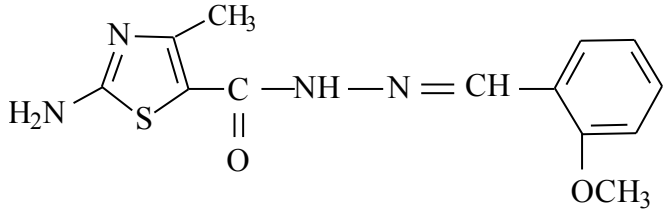
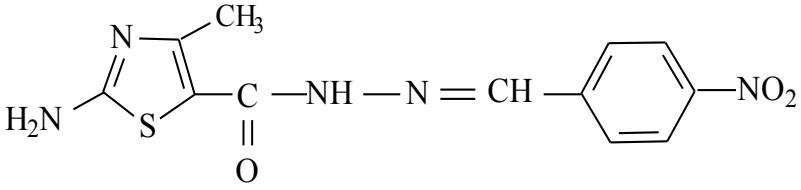
Protein concentration was determined according to Bradford [5]. SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) was performed as described by Laemmli [10] and gels stained with Coomassie Blue. Enzyme inhibition by compounds studied was determined photocolorimetrically with 2,4-dinitrophenylhydrazine (DNPH). Aliquots of compound solution in dimethylformamide (DMF) were added in reaction tube before reaction start. I_{50} constant represents the inhibitor concentration that gives 50% enzymatic activity inhibition. I_{50} was calculated from the equation of plots enzyme inhibition (%) *versus* compound concentration.

Synthesis of 2-amino-4-methyl-N'-arylidene-thiazole-5-carbohydrazides

The compounds were synthesized through the reaction of hydrazine hydrate with 4 methyl-5-carboxyethyl-thiazole. The resulting hydrazide was then condensed with 2 methoxy-benzoic aldehyde or with 4 nitro-benzoic aldehyde. The detailed method of synthesis and the physical analysis of the obtained compounds, together with some bacteriological testing against different microorganisms, were described in a previous work [14] (Table I).

Table I

Structures of 2-amino-4-methyl- N'-arylidene-thiazole-5-carbohydrazides

	Structure	MW
Compound I		291 g/mol
Compound II		305 g/mol

Results and Discussion

Sequence comparison of amino acid sequences

The amino acid sequences of AK_{EC} (SwissProt - P69441) and AK_{SP} (SwissProt - Q97SU1) were aligned using the BL2SEQ program – BLAST version developed by NCBI (Altschul, Madden, Schaeffer, Zhang, Miller, Lipman) [2]. We used BLOSUM 62, the default matrix in BLAST.

The percentage of identity (38%) is defined as the number of identical amino acids observed after alignment of the two sequences divided by the number of residues in the shorter sequence. In the middle row, identical amino acids are connected by the letter of identical residue. Conservative replacements are marked by a plus. Program inserted gaps to improve the alignment (Figure 1).

```
AK_STRPN     1   MNLLIMGLPGAGKGTQAAKIVEQFHVAHISTGDMFRAAMANQTEMGVLAKSYIDKGELVP 60
                 M ++++G PGAGKGTQA  I+E++ +  ISTGDM RAA+ + +E+G  AK  +D G+LV
AK_ECOLI     1   MRIILLGAPGAGKGTQAQFIMEKYGIPQISTGDMLRAAVKSGSELGKQAKDIMDAGKLVT 60

                                           *   *
AK_STRPN    61   DEVTNGIVKERLSQDDIKETGFLLDGYPRTIEQAHALDKTLAELGIELEGVINIEVNPDS 120
                 DE+   +VKER++Q+D +  GFLLDG+PRTI QA A    + E GI ++ V+  +V  +
AK_ECOLI    61   DELVIALVKERIAQEDCR-NGFLLDGFPRTIPQADA----MKEAGINVDYVLEFDVPDEL 115

AK_STRPN    121  LLERLSGRIIHRVTGETFHKVFNPPV-----DYKEEDYYQREDDKPETVKRRLDVNIAQG 175
                 +++R+ GR +H  +G  +H  FNPP      D   E+   R+DD+ ETV++RL
AK_ECOLI    116  IVDRIVGRRVHAPSGRVYHVKFNPPKVEGKDDVTGEELTTRKDDQEETVRKRLVEYHQMT 175

AK_STRPN    176  EPIIAHYRAKGLVHD-----IEGNQDINDVFSDIEKVL 208
                  P+I +Y  +    +     ++G + + +V +D+EK+L
AK_ECOLI    176  APLIGYYSKEAEAGNTKYAKVDGTKPVAEVRADLEKIL 213
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Figure 1.

Analysis of sequence alignment of *S.pneumoniae* and *E.coli* adenylate kinase

ATP - binding domain is bolded. Consensus pattern [LIVMFYWCA]-[LIVMFYW] (2)-D-G-[FYI]-P-R-X (3)-[NQ] is marked with italic fonts. Inside consensus region, aspartic acid (D) and arginine (R) residues are marked with *. Sequence was aligned by bl2seq-NCBI

The structure of the complex between *E.coli* adenylate kinase and the inhibitor AP5A is already solved and is deposited in RCSB Protein Data Bank [1]. Therefore, we have chosen adenylate kinase from *E.coli* for sequence comparison. Sequence alignment revealed two well conserved domains: ATP binding domain (amino acids 7 to 15) and consensus pattern which is a region detected in all sequences belonging to adenylate kinase family (amino acids 82 to 93).

Purification and specific activity of recombinant adenylate kinases

When AK_{EC} elution curve has been analysed by SDS-PAGE, the electropherogram indicated the fraction number 8, where the protein of interest was eluted at highest concentration (0.23 mg/mL) (Figure 2). Specific activity found was 1839 U/mg. AK_{SP} was purified as a His-tagged protein eluted in the second fraction (6.23 mg/mL). Specific activity of AK_{SP} was 38.7 U/mg (Figure 3).

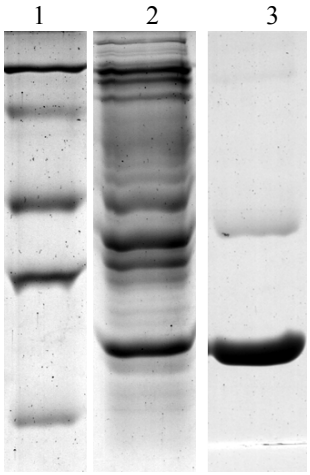

Figure 2.
SDS-PAGE of fractions during purification of AK_{EC}: Lane 1, protein size marker (94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa) lane 2, crude extract (18.6 µg protein), lane 3, Blue-Sepharose fraction (5.6 µg protein)

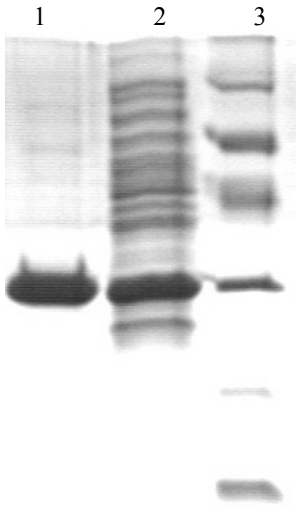

Figure 3.
SDS-PAGE of fractions during purification of AK_{SP}: Lane 1, 6xHis-tagged fraction (20 µg protein) lane 2, crude extract (20 µg protein), lane 3, protein size marker (97 kDa, 66 kDa, 45 kDa, 30 kDa, 20.1 kDa, 14.1 kDa)

Inhibition of adenylate kinases activity by 2-amino-4-methyl-N'-arylidene-thiazole-5-carbohydrazides

Recombinant AK_{SP} and AK_{EC} were tested as potential target for two 2-amino-4-methyl-N'-arylidene-thiazole-5-carbohydrazides. 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 concentration of compound I needed for 50 % inhibition (I_{50}) was 34.8 µM for AK_{SP} and 38.63 µM for AK_{EC} (Figure 4). The concentration of compound II needed for 50 % inhibition was 178 µM for AK_{SP} and 170 µM for AK_{EC} (Figure 5).

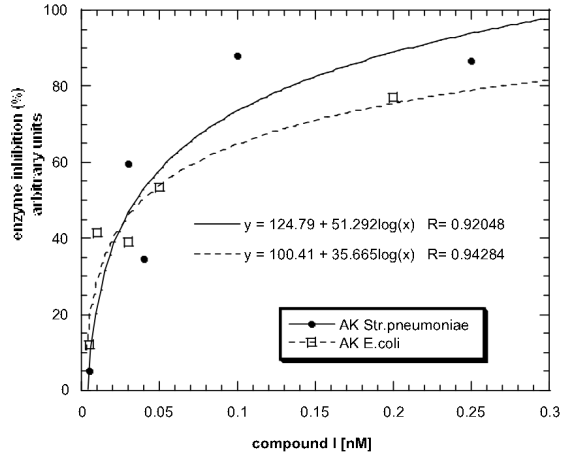

Figure 4.
Inhibition of *S. pneumoniae* and *E.coli* adenylate kinase by compound I (2-amino-4-methyl-5 o-metoxi-phenyl-hydrazido thiazol)

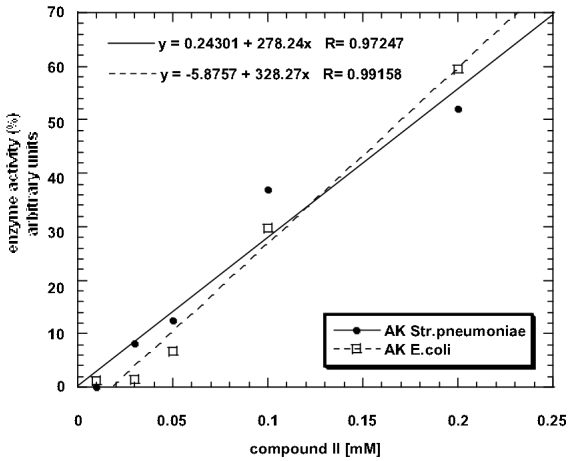

Figure 5.
Inhibition of *S. pneumoniae* and *E.coli* adenylate kinase by compound II (2-amino-4-methyl p-nitro-phenyl-hydrazido thiazol)

Conclusions

Because *S. pneumoniae* is of major importance for public health, we have evaluated the ability of two 2-amino-4-methyl-N'-arylidene-thiazole-5-carbohydrazides to inhibit adenylate kinase, enzyme involved in an essential step in nucleotides metabolism.

We have determined the inhibition efficiency of AK_{SP} and AK_{EC} activity with synthetic compounds performed against recombinant enzymes over-expressed in *E. coli*. SDS-PAGE analysis indicated that both enzymes eluted as symmetrical peaks with specific activity of 1839 U/mg of protein for *E.coli* enzyme. AK_{SP} exhibited a specific activity of 38.7 U/mg. Results indicate that the effectors have different inhibitory actions. 2-amino-4-methyl-5 *o*-metoxi-phenyl-hydrazido thiazol is more efficient than 2-amino-4-methyl *p*-nitro-phenyl-hydrazido

thiazol. But, there are not significant difference between AK_{SP} and $AK_{EC.}$ This could suggest that compounds interact at the same enzymatic sites in various bacterial species.

Cloning and purification of recombinant enzymes offer premises for choosing more specific methods for understanding subtle mechanisms involved in enzymes inhibition. Biochemical characterization of complex enzyme with synthetic compounds is an important step in pharmaceutical research.

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