Biochemical Pharmacology 94 (2015) 63–68

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The purine analog fludarabine acts as a cytosolic 5′-nucleotidase II inhibitor

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ARTICLE INFO

Article history:
Received 26 November 2014
Accepted 26 January 2015
Available online 2 February 2015

Keywords:
Cytosolic 5′-nucleotidase II
Fludarabine
Adenosine analogs
Chemoresistance
Cancer pharmacology

ABSTRACT

For several years the IMP/GMP-preferring cytosolic 5′-nucleotidase II (cN-II) has been considered as a therapeutic target in oncology. Indeed, various reports have indicated associations between cN-II expression level and resistance to anticancer agents in several cancer cell lines and in patients affected with neoplasia, mainly by hematologic malignancies. In this paper we present evidence showing that, among the commonly used cytotoxic nucleoside analogs, fludarabine can act as a cN-II inhibitor. *In vitro* studies using the wild type recombinant cN-II demonstrated that fludarabine inhibited enzymatic activity in a mixed manner (K_i 0.5 mM and K_i' 9 mM), whereas no inhibition was observed with clofarabine and cladribine. Additional experiments with mutant recombinant proteins and an *in silico* molecular docking indicated that this inhibition is due to an interaction with a regulatory site of cN-II known to interact with adenylic compounds. Moreover, synergy experiments between fludarabine and 6-mercaptopurine in human follicular lymphoma (RL) and human acute promyelocytic leukemia (HL-60) cells transfected with control or cN-II-targeting shRNA-encoding plasmids, showed synergy in control cells and antagonism in cells with decreased cN-II expression. This is in line with the hypothesis that fludarabine acts as a cN-II inhibitor and supports the idea of using cN-II inhibitors in association with other drugs to increase their therapeutic effect and decrease their resistance.

1. Introduction

Cytotoxic nucleoside analogs, an important class of drugs used in the treatment of both hematologic malignancies and solid tumors, are continuously evolving with novel agents in clinical trials or recently approved [1–3]. The cytotoxicity of these agents against target cells depends on the intracellular active drug concentration that can be attained during treatment. Activation and inactivation of the nucleoside analog depend on the enzymatic pattern, in particular intracellular deaminating, phosphorylating and dephosphorylating enzymes, as well as on the efficiency of the transport system [4]. Consequently, cell resistance is commonly attributed to the inability to transport or to phosphorylate the analog and/or to increased of dephosphorylating activities [5]. The cytosolic 5′-nucleotidases are a family of enzymes catalyzing the dephosphorylation of nucleoside monophosphates, thus regulating, along with nucleoside kinases or nucleobase-phosphoribosyl transferases, the catabolism and salvage of purine and pyrimidine compounds [6]. Among the human 5′-nucleotidases, the IMP-GMP specific cytosolic 5′-nucleotidase (cN-II) has attracted the attention of a number of research groups as a potential drug inactivating enzyme [7,8]. Several reports have suggested involvement of cN-II in drug resistance in hematological malignancies and shown its influence on clinical outcome of patients treated with nucleoside analogs [9–11]. Interestingly, high cN-II expression strongly correlates with poor outcome of therapies based on analogs whose monophosphates were poor

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http://dx.doi.org/10.1016/j.bcp.2015.01.010
0006-2952/

(fludarabine monophosphate, cladribine monophosphate) or not at all (cytarabine monophosphate and gemcitabine monophosphate) substrates in *in vitro* tests [12,13]. To explain these results, it was postulated that cN-II can be a marker of tumor aggressiveness independently of its catalytic activity on nucleoside analogs [14,15]. Therefore, the enzyme can be considered a biological marker of poor prognosis rather than a predictive marker of resistance to prodrugs.

More recently, the expression of hyperactive variants of cN-II was found to drive chemo-resistance both in ALL and in AML [15–18]. Such variants resulted in a higher cN-II expression in terms of mRNA and/or in terms of higher activity. Although the implication of cN-II expression and/or activity appears to be well correlated with drug resistance and poor prognosis in different hematological malignancies, no data have been produced so far demonstrating the molecular mechanisms by which cN-II mediates drug resistance. Recently, we found that transient cN-II overexpression in HEK-293 cells induced a significant decrease of all nucleotide triphosphates as well as of adenylate content and of energy charge (Cividini et al., unpublished). cN-II-overexpressing cells were resistant to fludarabine, cytarabine and, to a lesser extent to gemcitabine, indicating that the increase of cN-II activity was sufficient to cause resistance and suggesting that the unbalance of nucleotide pools could be involved in this mechanism. This last finding indicates that a variation in enzyme activity could impact on the metabolism of analogs both in a direct and/or indirect way. Conversely it is likely that a nucleoside analog, structurally similar to substrates or regulators of key enzymes in nucleoside metabolism such as cN-II, could act as an inhibitor of such enzymes, by binding on their active or regulatory sites. Since variations in cN-II activity affect a number of cell mechanisms, such as energy charge, AMPK activity, apoptosis and cell proliferation [19], we hypothesize that the cytotoxicity of analogs may thus be at least partly due to inhibition of cN-II.

Here, we demonstrate that fludarabine (9β-D-arabinofuranosyl-2-fluoroadenine) acts as inhibitor of cN-II and we determine the site of binding. Furthermore, we obtained evidence that in tumor cell lines expressing normal levels of cN-II, the combination of fludarabine and 6-mercaptopurine exerts a synergistic cytotoxic effect, while if cN-II is silenced the combination results in an antagonistic effect underlining a biological relevance of our findings.

2. Materials and methods

2.1. Plasmids and cell models

Plasmid constructions and cell models are described elsewhere (Jordheim et al., unpublished). Briefly, we stably transfected RL and HL-60 cells with pSuperior.neo plasmids containing either a non-targeting sequence (pScont) or a sequence targeting the position 347 of the coding section of cN-II mRNA (pScN-II).

2.2. Synergy experiments

Stably transfected HL-60- and RL-pScont and -pScN-II cells were used for the evaluation of synergy between 6-mercaptopurine (6MP) and fludarabine, clofarabine or cladribine. Cells were seeded (10,000 per well) in 96-well plates containing different concentrations of drugs alone or in combination with 6-MP for 72 h before living cells were quantified with the MTT assay. Ratios of the concentrations of the two compounds used were approximately equal to the ratio of their effect concentrations 50 (EC50) values. EC50 and 95% combination index (CI95) were calculated using CompuSyn software 1.0 (ComboSyn, Inc., Paramus, NJ). Combinations were considered synergistic for CI95 values below 0.8, additive for CI95 values between 0.8 and 1.2 and antagonistic for CI95 values over 1.2 [21,22].

2.3. Site directed mutagenesis

Plasmid pET28a-cN-II was kindly provided by Pär Nordlund (KI, Stockholm, Sweden) and used for site-directed mutagenesis with "QuikChange II site Directed Mutagenesis" kit (Agilent Technologies, Santa Clara, CA, USA) as indicated by the manufacturer to introduce F127A and F157A mutations. Primers for PCR mutagenesis were (modified nucleotides in bold): F157A_F: cattctgaacacacta**gcc**aacctaccagagacc, F157A_R: ggtctctggtaggtt**ggc**tagtgtgttcagaatg, F127A_F: gtgcacatggatttaac**gct**ataa ggggaccag, F127A_R: ctggtccccttat**agc**gttaaatccatgtgcac. Plasmid carrying the mutation R144E was obtained as described previously [23].

2.4. Recombinant protein expression and purification

The expression of the recombinant cN-II and its point mutants R144E, F127A, F157A was performed as previously reported [23]. The 6× His-tagged proteins were purified using the Ni-NTA Agarose (Qiagen, Milan, Italy) method as described by the QIAexpressionist™ Handbook. The wild type cN-II and its point mutant R144E are bovine proteins, whereas mutants F127A and F157A are human proteins. Human and bovine cN-II sequences share 99% homology [19].

2.5. Enzyme assays

Phosphatase activity of wild-type and mutant cN-II was assayed according to Chifflet [24] in Tris–HCl 100 mM pH 7.4, in the presence of IMP 2 mM, Mg^{2+} 20 mM, ATP 5 mM using 0.6 μg purified protein. Tris–Base and HCl were from J.T. Baker® Chemicals (Avantor Performance Materials, Center Valley, PA, USA), IMP and ATP were from Sigma–Aldrich (Milan, Italy), MgCl was from BDH Laboratory Supplies (Poole, UK).

2.6. High performance capillary electrophoresis analysis of phosphatase reaction analytes

Fifty μl of *in vitro* phosphatase reaction mixture from the enzyme assay described above, were recovered, boiled for 5 min at 95 °C and centrifuged (15,000 × *g*, 1 min, at room temperature). The supernatant was stored at −20 °C until analysis. Runs were performed using a Beckman P/ACE MDQ Capillary Electrophoresis System equipped with an UV detector. The best resolution of the nucleoside and nucleobase contents was obtained with the following capillary electrophoresis conditions: uncoated silica capillary (75 μm id × 375 μm od; Polymicro Technologies, Phoenix, AZ, USA), effective length was 52 cm (60 cm total length), BGE was boric acid 125 mM, pH 8.5, samples were run at a constant voltage of 15 kV using a ramp time of 0.5 min, temperature was kept at 25 °C. Solutions were syringe filtered (0.45 μm, Millipore Corporation, Billerica, MA, USA) before use. All samples were loaded by a low-pressure injection (0.5 psi, 6 s); these conditions ensured that the amount loaded was lower than 1% of the total capillary volume. The detection was performed either at 254 nm or 280 nm. Data were reported as peak area quantification.

2.7. Molecular docking

Molecular docking was carried out with the GOLD 5.2 program (Genetic Optimization for Ligand Docking) from CCDC Software Limited [25]. Prior to docking, the potential energy of ligands was minimized using 500 steps of steepest descent followed by 5000 steps of conjugate gradient (tolerance of 0.01 kcal/mol Å).