The purine analogue fludarabine acts as a cytosolic 5'-nucleotidase II inhibitor

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ABSTRACT

For several years the IMP/GMP-preferring cytosolic 50-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 50-nucleotidases are a family of enzymes catalyzing the dephosphorylation of nucleoside monophosphates, thus regulating, along with nucleoside kinases or nucleobasephosphoribosyl transferases, the catabolism and salvage of purine and pyrimidine compounds [6]. Among the human 50-nucleotidases, the IMP-GMP specific cytosolic 50-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 (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 (9b-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 nontargeting 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 Pa¨r 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: cattctgaacacactagccaacctaccagagacc, F157A_R: ggtctctggtaggttggctagtgtgttcagaatg, F127A_F: gtgcacatggatttaacgctataa ggggaccag, F127A_R: ctggtccccttatagcgttaaatccatgtgcac. 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 QIAexpressionistTM 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, Mg2+ 20 mM, ATP 5 mM using 0.6 mg purified protein. Tris–Base and HCl were from J.T. Baker1 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 ml 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 x 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 8C. 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). Docking was achieved on the 2XCW structure of cN-II using 50 genetic algorithm runs for conformational poses searching with a radius of 10–12 Å around a target atom, Mg^{2+} ion for IMP binding site, CD atom of Gln453 for effector site 1 and CE atom of Met436 for effector site 2. The docking of adenosine was also compared to the crystal structure (2JC9) in which adenosine is bound to effector site 2. Water molecules present in the crystal structure were retained and allowed to contribute with a 2 Å cut-off of translational and rotational freedom. Docking poses were analyzed by the clustering method (complete linkage) from the RMSD matrix of ranking solutions. Solutions were classified according to their respective scores calculated by the Goldscore scoring function. Results were analyzed through the integrated graphical interface Hermes or using the PyMOL Molecular Graphics System (version 1.3, Schrödinger, LLC).

2.8. Statistical analysis

All statistical analyses were performed using InStat software (ver. 3.05, GraphPad Software, Inc., La Jolla, CA 92037, USA).

3. Results

3.1. Inhibition of cN-II activity by fludarabine

cN-II hydrolyzes IMP producing free inorganic phosphate and inosine. In the presence of a suitable nucleoside, the phosphate produced can be transferred on another nucleoside thus catalyzing a phosphotransferase reaction. Indeed, in the presence of millimolar concentrations of inosine, guanosine or some nucleoside analogs, the rate of phosphate produced during the hydrolysis of IMP is substantially decreased, while the rate of inosine produced in the same condition is unaffected [26]. We evaluated the effect of clinically used adenosine analogs (fludarabine, cladribine and clofarabine) at different concentrations on the rate of IMP hydrolysis catalyzed by recombinant cN-II in the presence of its preferred substrate IMP. Fig. 1 shows that while cladribine did not substantially affect

the rate of phosphate produced during the reaction, 1 mM clofarabine reduced this rate by approximately 20% and 1 mM fludarabine by approximately 55%. To ascertain if this decrease in release of the free inorganic phosphate registered in the presence of fludarabine could be due to a transfer of the phosphate to the nucleoside analog, we also measured inosine produced in the presence of the highest concentration of the three analogs. We found that in the case of cladribine and clofarabine, inosine concentration was either identical or almost similar to that of the control, while in the presence of fludarabine it was also reduced by approximately 55% (Fig. 1, see insert box). Therefore, fludarabine was demonstrated to be an inhibitor of cN-II and not a substrate for phosphotransferase. We then performed kinetic analysis of the inhibition using different concentrations of fludarabine (Fig. 2 and Table 1). Given the increased K_m and decreased V_{max} values observed for increasing concentrations of fludarabine, we concluded that fludarabine behaves as a mixed inhibitor. From the kinetic parameters we calculated K_i (0.47 ± 0.03 mM) and $K_{i'}$ (9.5 ± 1.2 mM) values indicating that fludarabine binds with higher affinity to the free enzyme than to the ES complex.

3.2. Identification of fludarabine binding site on cN-II by molecular docking

As fludarabine was shown to act as a mixed inhibitor, a prediction of the most likely binding site was investigated by molecular docking. Crystal analysis of cN-II in the presence of adenosine [27], indicated three potential binding sites: substrate site, effector site 1 and effector site 2. Docking poses obtained either for adenosine or fludarabine are reported in Fig. 3. The docking score was lower for fludarabine (94.2) as compared to adenosine (101) concerning the IMP binding site region (Fig. 3A). This suggests that fludarabine will not bind tightly to the IMP binding site (especially in presence of IMP as the docking score for IMP is approximately 155). Regarding site effector 1 (Fig. 3B), binding predictions for both compounds are highly similar and almost superimposed. The scores were similar for both fludarabine (70.9) and adenosine (70.5), suggesting that fludarabine may bind to the effector site 1 but with less affinity than ATP (docking score of 129 due to strong interactions between oxygen atoms of the phosphate and Mg2+ ion). Finally, in the effector site 2 (Fig. 3C) both compounds bind in a less ordered manner (as it was described in crystal structure for adenosine). The nucleobase is not fully inserted in the pocket. However, the score was slightly higher for fludarabine (53.15) compared to adenosine (51.6) suggesting that fludarabine may bind to the effector site 2.

3.3. Identification of fludarabine binding site using cN-II mutants

With the aim of confirming the effector site 2 as the preferred region for fludarabine interaction, enzymatic activity evaluation with cN-II mutants was conducted. cN-II variants previously described

and carrying the mutation F157A in the active site [28,29], R144E in the effector site 1 and F127A in the effector site 2 [23,27] were used. The purified F127A and R144E had a specific activity of approximately 1 U/mg of protein, while the active site F127A mutant activity was 0.12 U/mg in the standard assay. As compared to wild type enzyme both mutants in the effector sites showed poor activation by all the enzyme activators tested, including ATP, ADP and BPG. The relative enzymatic activity was plotted as a function of increasing concentrations of fludarabine for each variant (Fig. 4). EC50 for the wild type cN-II was estimated to be 800 µM, with comparable values for F157A and R144E. On the contrary, no appreciable inhibition of the F127A cN-II mutant by fludarabine was observed up to a concentration of 1 mM. These data confirm the results obtained by molecular docking suggesting that inhibition of enzymatic activity occurs through binding of fludarabine to the effector site 2.

3.4. Co-exposure of RL and HL-60 cells to adenosine analogs and 6MP

Since fludarabine is a commonly used drug in hematological malignances, we hypothesized that, acting as cN-II inhibitor, it could enhance the effect of 6MP in co-exposure conditions. In fact increased cN-II activity resulted in resistance to 6MP [16,18], so globally, the modification of cN-II activity should modify the activity of 6MP. On the contrary, this enhancement should not be appreciable in cells with low cN-II expression where 6MP activity would be independent of cN-II. To demonstrate our hypothesis, we used two cancer cell lines (RL and HL-60) stably transfected with shRNA-coding plasmids (pScont and pScN-II). Initial sensitivity assays with 6MP, fludarabine, cladribine and clofarabine, showed a sensitization to these compounds when cN-II is down-regulated (Table 2). Data are in line with those obtained earlier using the same cell lines (Jordheim et al., unpublished). A remarkable sensitization was obtained for 6MP in HL-60 (31-fold sensitization) compared to RL (2-fold sensitization). Moreover, HL-60-pScN-II and RL-pScN-II cells displayed increased sensitivity also for fludarabine (2.7- and 1.4-fold, respectively) and clofarabine (2.2- and 2.6-fold, respectively). On the contrary, a resistance (ratios below 1) was detected for cladribine in both cell lines (0.3 in HL-60 and 0.6 in RL). Finally, synergy experiments were performed to confirm this hypothesis (Fig. 5). A synergistic effect between 6MP and fludarabine was detected in both pScont cell lines (CI95 of 0.6 in HL-60 and 0.7 in RL cells). In contrast, an antagonistic effect was reported in both pScN-II cell lines (CI95 of 1.3 in HL-60 and 2.5 in RL cells). Similarly, a synergistic combination index was observed for HL-60-pScont (CI95 of 0.5 for 6MP with cladribine and 0.8 for 6MP with clofarabine) and an antagonistic for HL-60-pScNII (CI95 of 1.2 for 6MP and cladribine and 1.3 for 6MP and clofarabine). On the contrary, an antagonistic effect was found both in RL-

pScont and RL-pScN-II cells for the same combinations (CI95 of 1.5 and 3.4 in pScont; 6.4 and 11.9 in pScN-II cells).

4. Discussion

The bi-functional cN-II nucleotidase hydrolyzes purine nucleoside monophosphates through the formation of a covalent enzyme-phosphate intermediate. The covalent intermediate can either transfer the phosphate to a suitable nucleoside through a phosphotransferase reaction or be hydrolyzed producing free phosphate and nucleoside [30]. Indeed, a number of nucleosides and nucleoside analogs can be phosphorylated by cN-II in vitro and in vivo [7,31–33]. We studied three adenosine analogs (fludarabine, clofarabine and cladribine) as phosphate acceptors in a cN-II catalyzed reaction, in the presence of IMP as donor. The production of free inorganic phosphate was significantly inhibited only in the presence of fludarabine and this was associated with inhibition of inosine production to the same degree, suggesting an inhibitory role. The inhibition was a mixed type, indicating that fludarabine may bind both the free enzyme and the enzyme–substrate complex, with higher affinity toward free enzyme. The previously described crystal structure of cN-II was obtained in the presence of adenosine, showing that adenosine can bind three different sites, the active site and putative regulatory sites 1 and 2 [27]. Our molecular docking indicated that fludarabine can also bind in these three sites but the best score relative to the one of adenosine was obtained for regulatory site 2. This result was confirmed by experiments using cN-II with single mutations in each site. The mutation in regulatory site 2 completely abolished the inhibitory effect promoted by fludarabine. The same mutant displayed a very low activation in presence of the best-known activators (ATP, ADP and BPG) of wild type enzyme. Further crystal analysis and characterization of single mutants cast some doubts about the role of putative regulatory site 2 since it appears to bind adenosine in a not ordered manner [29], and mutants in that site either had no effect on enzyme activity and regulation or decreased the effect induced by all the activators of the enzyme [23]. Our results indicate that more structural and functional studies are needed to understand all the aspects of the regulation of this complex enzyme. Since cN-II is considered as a therapeutic target in oncology, the finding that fludarabine can act as cN-II inhibitor open new perspective of application for this drug in association with other analogs in anticancer therapy. The biological relevance of the in vitro observations in cancer cells with decreased cN-II expression and activity may be questionable but may reflect the pathological context. We have already shown that these cells are more sensitive to several cytotoxic compounds, including nucleoside analogs, and this was confirmed in this series of experiments. Further, we showed that fludarabine acts in synergy with 6MP in control cells. This is in line with the

hypothesis that fludarabine can act as an inhibitor of cN-II also in cells as this confirms results obtained with other cN-II inhibitors on such cells [20]. Finally, in absence of cN-II, there was no synergy between the compounds clearly indicating that the synergistic effect was dependent on this protein. We therefore strongly believe that fludarabine can act as a cN-II inhibitor in cancer cells, and that its cytotoxic effect is partially due to this inhibition. In fact, we demonstrated that cN-II activity is required for the maintenance of the intracellular nucleotide pool and in some instances for cell survival [34,35] (Cividini et al., unpublished). Another argument in support of this hypothesis is the lack of synergy between 6MP and clofarabine in RL cells as well as the lack of synergy with cladribine in both RL and HL60 cells. These two compounds have similar, but not identical, metabolisms and mechanisms of action to fludarabine, but are much weaker (clofarabine) or not (cladribine) cN-II inhibitors. The stronger synergistic effect between 6MP and purine nucleoside analogs in HL-60 cells than in RL cells, could be due to the higher dependency of HL-60 cells upon cN-II activity. Indeed, it has been shown that HL-60 cells have 2.4-fold higher cN-II activity than RL cells (5.5 vs 2.3 mU/mg), whereas both cell lines transfected with pScN-II had similar and low residual cN-II activity (0.9 vs 1.3 mU/mg) (Jordheim et al., unpublished). In addition, this biological inhibition of cN-II was associated with a much better sensitization to nucleoside analogs in HL-60 cells than in RL cells confirming the differences in the importance of cN-II in the two cell types. Additionally, it may be hypothesized that intracellular fludarabine metabolite such as Fara-MP, which was demonstrated to be a poor cN-II substrate [13], may also act as cN-II inhibitor thus contributing with fludarabine in synergizing other drugs effect. Finally, the antagonism between fludarabine and 6MP in cells with low cN-II expression could be explained by the most widely reported mechanism of action of fludarabine involving phosphorylation to triphosphate and incorporation into nucleic acids that is dependent on the same cellular metabolism as 6MP. In conclusion, our results indicate an important role of cN-II in the metabolism of purine nucleoside analogs. Furthermore, we underline the improvement that the association of 6-MP, and potentially other drugs, with fludarabine or potentially over more potent cN-II inhibitor, will bring to enhance the efficacy of chemotherapy. Finally, our results support the hypothesis that the determination of cN-II activity in tumor cells in patients could be of use for the design of a suitable chemotherapeutic protocol.

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REFERENCES

[1] Van den Berghe G, Van Pottelsberghe C, Hers HG. A kinetic study of the soluble 5'-nucleotidase of rat liver. Biochem J 1997;162:611–6.

[2] Cheson BD. New antimetabolites in the treatment of human malignancies. Semin Oncol 1992;19:12.

[3] Jordheim LP, Durantel D, Zoulim F, Dumontet C. Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. Nat Rev Drug Discov 2013;12:447–64.

[4] Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. Leukemia 2001;15:875–90.

[5] Galmarini CM, Graham K, Thomas X, Calvo F, Rousselot P, El Jafaari A, et al. Expression of high Km 5'-nucleotidase in leukemic blasts is an independent prognostic factor in adults with acute myeloid leukemia. Blood 2001;98:1922–6.

[6] Bianchi V, Spychala J. Mammalian 5'-nucleotidases. J Biol Chem 2003;278:46195–98.

[7] Banditelli S, Baiocchi C, Pesi R, Allegrini S, Turriani M, Ipata PL, et al. The phosphotransferase activity of cytosolic 5'-nucleotidase; a purine analog phosphorylating enzyme. Int J Biochem Cell Biol 1996;28:711–20.

[8] Galmarini CM, Jordheim L, Dumontet C. Role of IMP-selective 5'-nucleotidase (cN-II) in hematological malignancies. Leuk Lymphoma 2003;44:1105–11.

[9] Giovannetti E, Mey V, Loni L, Nannizzi S, Barsanti G, Savarino G, et al. Cytotoxic activity of gemcitabine and correlation with expression profile of drug-related genes in human lymphoid cells. Pharmacol Res 2007;55:343–9.

[10] Galmarini CM, Cros E, Thomas X, Jordheim L, Dumontet C. The prognostic value of cN-II and cN-III enzymes in adult acute myeloid leukemia. Haematologica 2005;90:1699–701.

[11] Galmarini CM, Thomas X, Graham K, El Jafaari A, Cros E, Jordheim L, et al. Deoxycytidine kinase and cN-II nucleotidase expression in blast cells predict survival in acute myeloid leukaemia patients treated with cytarabine. Br J Haematol 2003;122:53–60.

[12] Mazzon C, Rampazzo C, Scaini MC, Gallinaro L, Karlsson A, Meier C, et al. Cytosolic and mitochondrial deoxyribonucleotidases: activity with substrate analogs, inhibitors and implications for therapy. Biochem Pharmacol 2003;66: 471–9.

[13] Jordheim LP, Cross E, Galmarini CM, Dumontet C, Bretonnet AS, Krimm I, et al. F-ara-AMP is a substrate of cytoplasmic 5'-nucleotidase II (cN-II): HPLC and NMR studies of enzymatic dephosphorylation. Nucleosides Nucleotides Nucleic Acids 2006;25:289–97.

[14] Galmarini CM. What does over-expression of cN-II enzyme signify in haematological malignancies? Leuk Res 2007;31:1325–6.

[15] Mitra AK, Crews KR, Pounds S, Cao X, Feldberg T, Ghodke Y, et al. Genetic variants in cytosolic 5'-nucleotidase II are associated with its expression and cytarabine sensitivity in HapMap cell lines and in patients with acute myeloid leukemia. J Pharmacol Exp Ther 2011;339:9–23.

[16] Meyer JA, Wang J, Hogan LE, Yang JJ, Dandekar S, Patel JP, et al. Relapse-specific mutations in NT5C2 in childhood acute lymphoblastic leukemia. Nat Gen 2013;4:290–4.

[17] Meyer JA, Carroll WL, Bhatla T. Screening for gene mutations: will identification of NT5C2 mutations help predict the chance of relapse in acute lymphoblastic leukemia? Expert Rev Hematol 2013;6:223–4.

[18] Tzoneva G, Perez-Garcia A, Carpenter Z, Khiabanian H, Tosello V, Allegretta M, et al. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. Nat Med 2013;19:368–71.

[19] Tozzi MG, Pesi R, Allegrini S. On the physiological role of cytosolic 5'-nucleotidase II (Cn-II): pathological and therapeutical implications. Curr Med Chem 2013;34:4285–91.

[20] Jordheim LP, Marton Z, Rhimi M, Cros-Perrial E, Lionne C, Peyrottes S, et al. Identification and characterization of inhibitors of cytoplasmic 5'-nucleotidase cN-II issued from virtual screening. Biochem Pharmacol 2013;85:497–506.

[21] Peters GJ, van der Wilt CL, van Moorsel CJA, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther 2000;87:227–53.

[22] Bijnsdorp IV, Giovannetti E, Peters GJ. Analysis of drug interactions. Methods Mol Biol 2011;731:421–34.

[23] Pesi R, Allegrini S, Careddu MG, Filoni DN, Camici M, Tozzi MG. Active and regulatory sites of cytosolic 5'-nucleotidase. FEBS J 2010;277:4863–72.

[24] Chifflet STA, Chiesa R, Tolosa S. A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases. Anal Biochem 1988;168:4.

[25] Jones G, Willett P, Glen RC, Leach AR, Taylor R. Development and validation of a genetic algorithm for flexible docking. J Mol Biol 1997;267:22.

[26] Turriani M, Pesi R, Nardone A, Turchi G, Sgarrella F, Ipata PL, et al. Cytosolic 5'-nucleotidase/nucleoside phosphotransferase: a nucleoside analog activating enzyme? J Biochem Toxicol 1994;9:51–7.

[27] Wallden K, Stenmark P, Nyman T, Flodin S, Graslund S, Loppnau P, et al. Crystal structure of human cytosolic 5'-nucleotidase II: insights into allosteric regulation and substrate recognition. J Biol Chem 2007;282:17828–36.

[28] Gallier F, Lallemand P, Meurillon M, Jordheim LP, Perigaud C, Lionne C, et al. Structural insights into the inhibition of cytosolic 5'-nucleotidase II (cN-II) by ribonucleoside 5'-monophosphate analogues. PLoS Comp Biol 2011;7: e1002295. http://dx.doi.org/10.1371/journal.pcbi.1002295.

[29] Wallden K, Nordlund P. Structural basis for the allosteric regulation and substrate recognition of human cytosolic 5'-nucleotidase II. J Mol Biol 2011;408: 684–96.

[30] Baiocchi C, Pesi R, Camici M, Itoh R, Tozzi MG. Mechanism of the reaction catalysed by cytosolic 5'-nucleotidase/phosphotransferase: formation of a phosphorylated intermediate. Biochem J 1996;317:797–801.

[31] Johnson MA, Fridland A. Phosphorylation of 20,30-dideoxyinosine by cytosolic 5'-nucleotidase of human lymphoid cells. Mol Pharmacol 1989;36:291–5.

[32] Ahluwalia G, Cooney DA, Bondoc LL, Currens MJ, Ford H, Johns D, et al. Inhibitors of IMP dehydrogenase stimulate the phosphorylation of the antiviral nucleoside 2',3'-dideoxyguanosine. Biochem Biophys Res Commun 1990;171:1297–303.

[33] Tozzi MG, Camici M, Allegrini S, Pesi R, Turriani M, Del Corso A, et al. Cytosolic 5'-nucleotidase/phosphotransferase of human colon carcinoma. Adv Exp Med Biol 1992;309B:173–6.

[34] Allegrini S, Filoni DN, Galli A, Collavoli A, Pesi R, Camici M, et al. Expression of bovine cytosolic 5'-nucleotidase (cN-II) in yeast: nucleotide pools disturbance and its consequences on growth and homologous recombination. PLOS ONE 2013. http://dx.doi.org/10.1371/journal.pone.0063914.

[35] Careddu MG, Allegrini S, Pesi R, Camici M, Garcia-Gil M, Tozzi MG. Knockdown of cytosolic 5'-nucleotidase II (cN-II) reveals that its activity is essential for survival in astrocytoma cells. Biochim Biophys Acta 2008;1783:1529–35.

Table 1

Kinetic parameters for the hydrolysis of IMP catalyzed by cN-II in the absence or in the presence of fludarabine. Values represent the mean ± SEM of three independent assays.

Fludarabine (µM)	**K_m (µM)**	**V_{max} (U/ml)**
0	106 ± 0.9	22.2 ± 0.3
400	175 ± 3.9	20.7 ± 0.3
800	268 ± 11.9	20.1 ± 0.19
1200	330 ± 21.0	19.6 ± 0.05

Table 2

Sensitivity of human follicular lymphoma RL cells and human acute promyelocytic leukemia HL-60 cells to 6-mercaptopurine (6MP), fludarabine, clofarabine and cladribine. Values represent the mean EC50 (mM) ± SEM of three independent experiments.

	pScont	**pScN-II**	**Ratio**
HL-60			
6MP	67.00 ± 15	2.00 ± 1***	31.0
Fludarabine	0.80 ± 0.1	0.30 ± 0.01**	2.7
Cladribine	0.02 ± 0.01	0.07 ± 0.01**	0.3
Clofarabine	0.05 ± 0.02	0.03 ± 0.01*	2.2
RL			
6MP	2.50 ± 0.40	1.30 ± 0.70*	2.0
Fludarabine	25.60 ± 2.50	18.40 ± 3.40*	1.4
Cladribine	0.50 ± 0.06	0.90 ± 0.05**	0.6
Clofarabine	0.40 ± 0.08	0.13 ± 0.08*	2.6

* $P<0.05$.

** $P<0.01$.

*** $P<0.001$.

When comparing to corresponding pScont data using unpaired t test

Figure Legends

Figure 1
cN-II activity measured as rate of phosphate production from IMP in presence of increasing µM concentrations of fludarabine (◆), clofarabine (■) and cladribine (▲). Curves represent the mean ±SEM of three independent assays. In the insert box is reported the rate of production of inosine obtained in presence of 1 mM analogs in the same assay conditions

Figure 2
Lineweaver–Burk plot showing the dependence of the steady rate constant for IMP hydrolysis by cN-II upon IMP concentrations, in the absence (●) or in the presence of increasing concentrations of fludarabine: (■ 400 µM), (▲ 800 µM) and (◆ 1200 µM). Curves are representative of three independent assays.

Figure 3
Molecular docking of fludarabine (ball and stick model, Fluor atom indicated by the arrow) either in the IMP binding site (A), the effector site 1 (B) or the effector site 2 (C) of cN-II. The score was lower for fludarabine (94.2) compared to adenosine (101) and far lower from the favorite substrate, IMP (155), comparable concerning the effector site 1 (fludarabine: 70.9, adenosine: 70.5) and higher concerning effector site 2 (fludarabine: 53.15, adenosine: 51.6). In panel C, adenosine docking pose (ball and stick model) is compared to adenosine binding in 2JC9 structure (stick model).

Figure 4
Recombinant purified wild type and mutant cN-II activities measured as rate of phosphate production from IMP in presence of increasing concentrations of fludarabine. (●) wt cN-II, (■) effector site 2 mutant F127A, (▲) active site mutant F157A, (**X**) effector site 1 mutant R144E. Curves represent the mean ±SEM of three independent assays.

Figure 5
Synergy evaluation on cell viability upon 6MP exposure in association with fludarabine, clofarabine and cladribine in HL60- (A) and RL- (B) -pScont (white bars) or -pScN-II (black bars). Ratios of 6MP/nucleoside analog concentrations were 52 and 4.7 for 6MP/fludarabine in HL-60-pScont and HL-60-pScN-II, 0.0667 and 0.05 for 6MP/fludarabine in RL-pScont and RL-pScN-II, 3250 and 450 for 6MP/clofarabine in HL-60-pScont and HL-60-pScN-II, 3 for 6MP/clofarabine in both RL-pScont and RL-pScN-II, 6500 and 450 for 6MP/cladribine in HL-60-pScont and HL-60-pScN-II and 3 for 6MP/cladribine in both RL-pScont and RL-pScN-II. Bars represent the mean SEM of three independent assays.

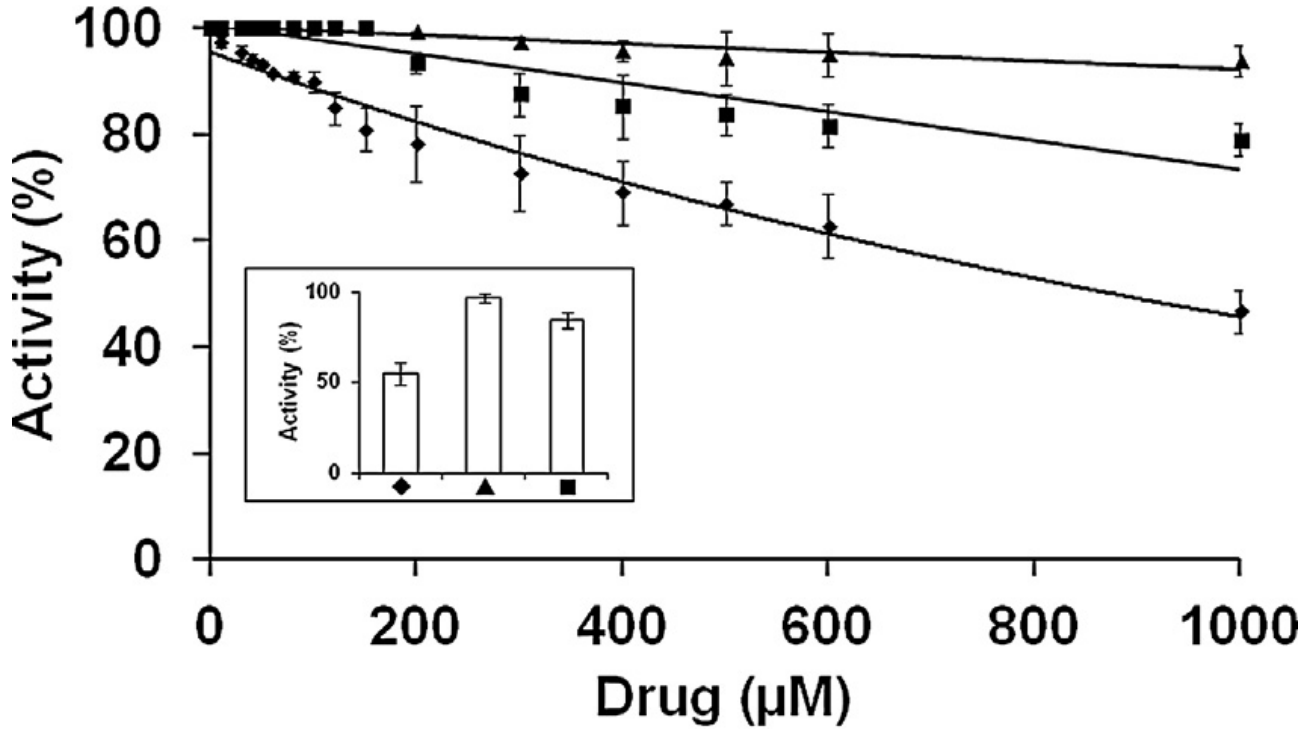
Activity (%)
100
80
60
40
20
0
0
200
400
600
800
1000
Drug (μM)
Activity (%)
100
50
0

Figure 1

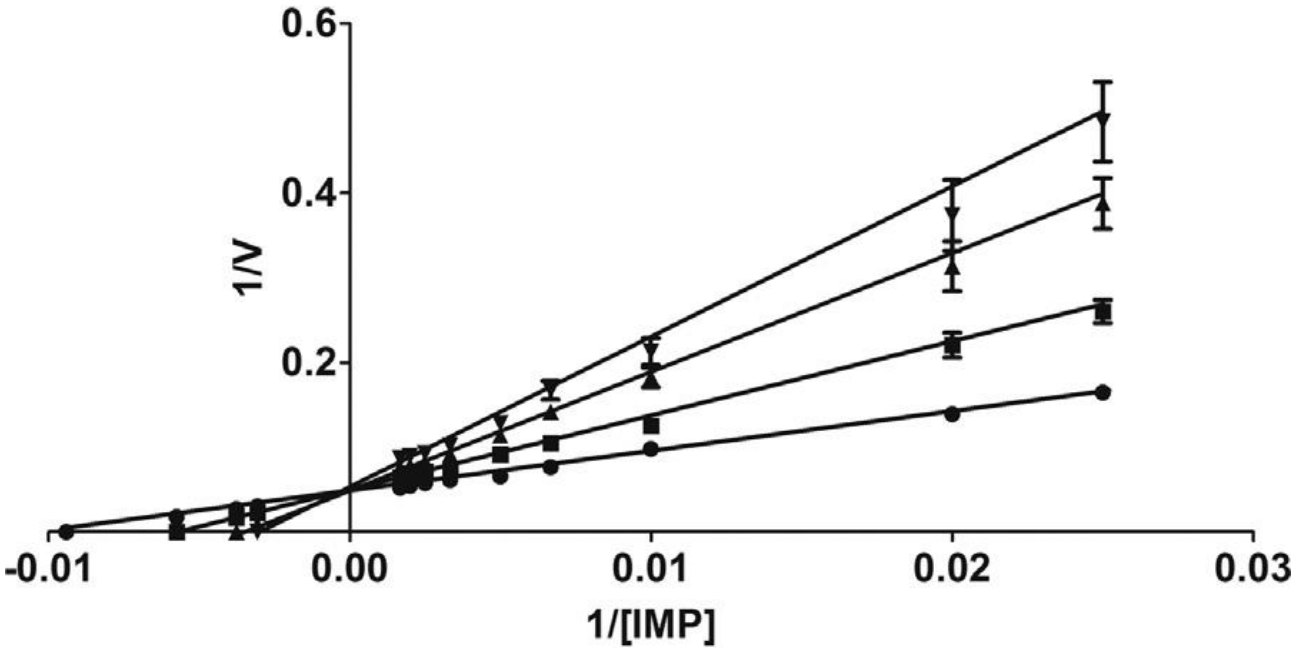

Figure 2

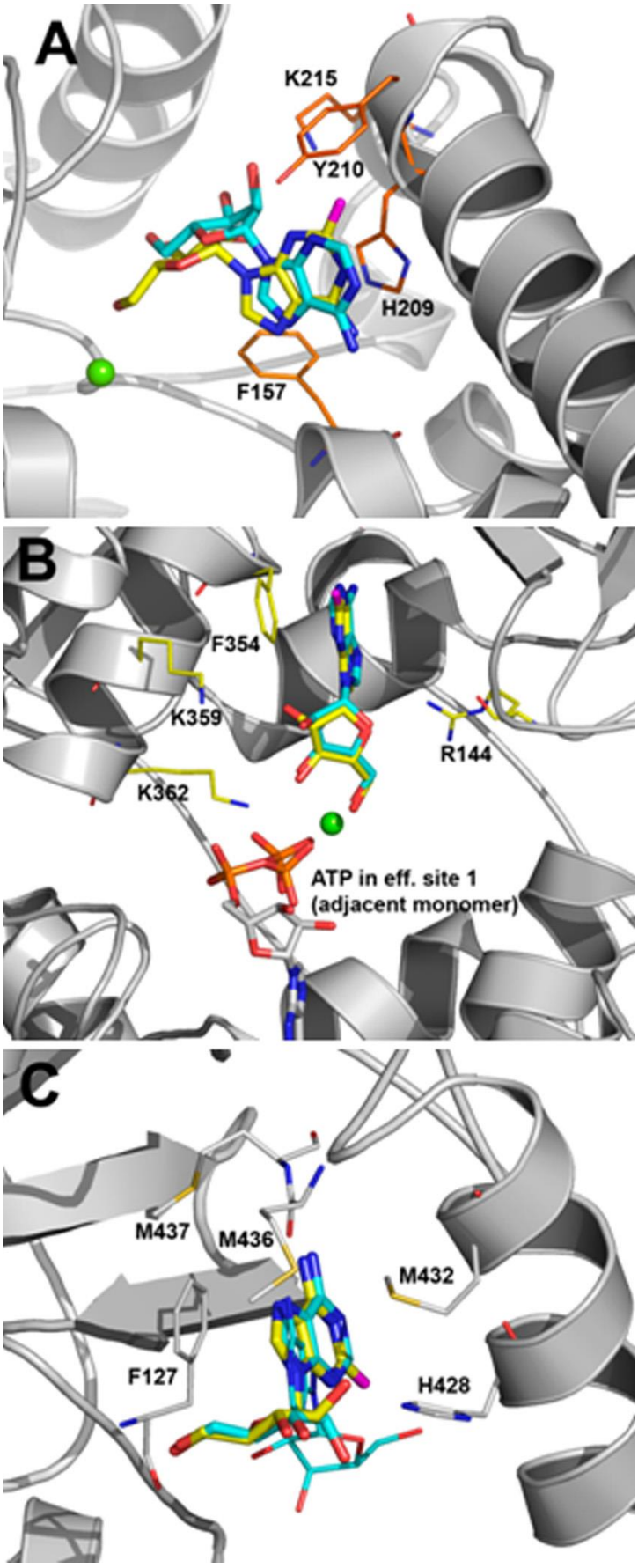
A
K215
Y210
H209
F157
B
F354
K359
R144
K362
ATP in eff. site 1
(adjacent monomer)
C
M437
M436
M432
F127
H428

Figure 3

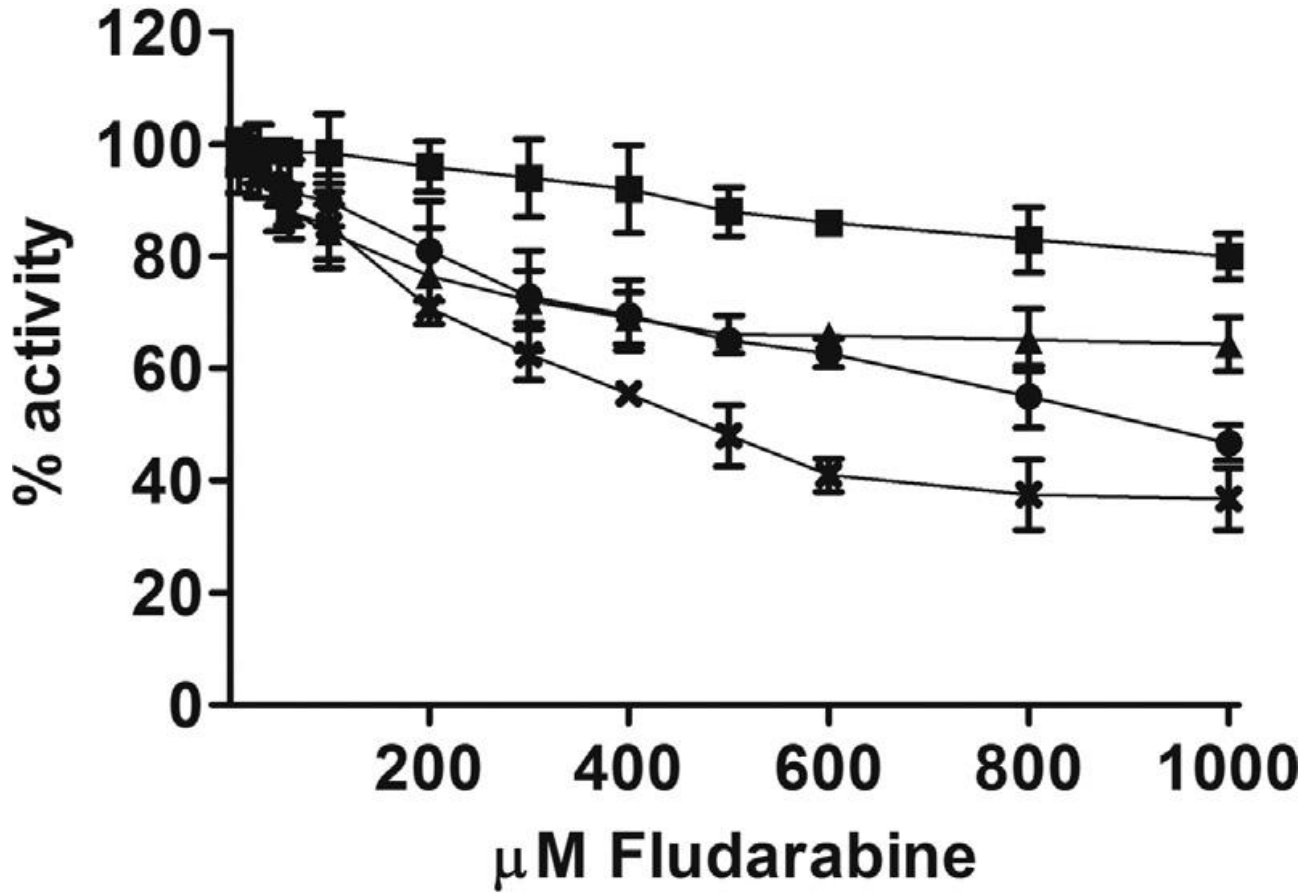
120
100
80
60
40
20
0
% activity
200
400
600
800
1000
μM Fludarabine

Figure 4

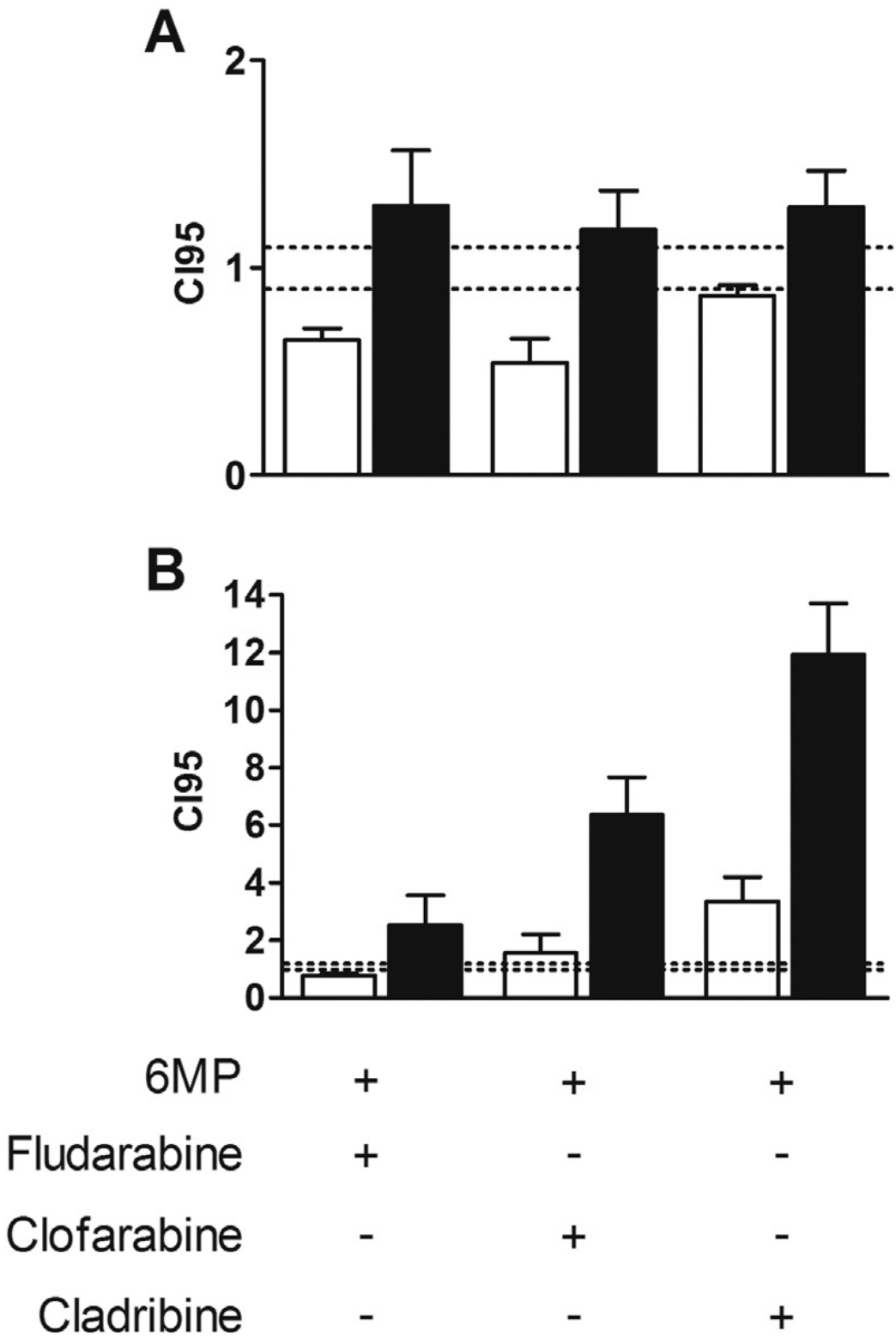
A
CI95
2
1
0
B
CI95
14
12
10
8
6
4
2
0
6MP + + +
Fludarabine + - -
Clofarabine - + -
Cladribine - - +

Figure 5