Cytotoxic and Antitumoral Activity of N-(9H-purin-6-yl) Benzamide Derivatives and Related Water-soluble Prodrugs

Emeline Cros-Perrial, Steve Saulnier, Muhammad Zawwad Raza, Rémi Charmelot, David Egron, Charles Dumontet, Laurent Chaloin, Suzanne Peyrottes, Lars Petter Jordheim

Cytotoxic and antitumoral activity of N-(9H-purin-6-yl) benzamide derivatives and related water-soluble prodrugs

Running title: Cytotoxic purine derivatives

Emeline Cros-Perrial[1], Steve Saulnier[2], Muhammad Zawwad Raza[1], Rémi Charmelot[1], David Egron[2], Charles Dumontet[1, 3], Laurent Chaloin[4], Suzanne Peyrottes[2], Lars Petter Jordheim[1]

[1]Univ Lyon, Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon Bérard, Centre de Recherche en Cancérologie de Lyon, Lyon, 69008, France

[2]Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS, Université de Montpellier, ENSCM, Campus Triolet, cc1705, Place Eugène Bataillon, 34095 Montpellier, France

[3]Hospices Civils de Lyon, F-69000 Lyon, France

[4]Institut de Recherche en Infectiologie de Montpellier (IRIM), Université Montpellier, CNRS, 34293 Montpellier, France

Acknowledgments : This study was supported by the Agence Nationale de la Recherche (ANR grant 11-BS07-032) and by Institut National du Cancer (INCA_11560). LPJ received funding from Olav Raagholt og Gerd Meidel Raagholts stiftelse for forskning.

Conflict of interest: Authors have no conflict of interest to declare.

Abstract

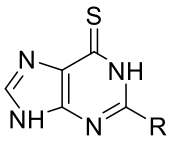
The development of small molecules as cancer treatments is still of both interest and importance. Having synthesized and identified initial cytotoxic activity of a series of chemically related N-(9H-purin-6-yl) benzamide derivatives, we continued their evaluation on cancer cell models. We also synthesized water-soluble prodrugs of the main compound and performed *in vivo* experiments. Our results show activities on cancer cell lines ranging from 3-39 µM for the best compounds, with both induction of apoptosis and decrease in cell proliferation. Two compounds evaluated in vivo showed weak antitumoral activity. In addition, the lead compound and its prodrug had synergistic activity with the nucleoside analogue fludarabine *in vitro* and *in vivo*.

Key-words: Cell death, cancer cells, prodrug, *in vivo*

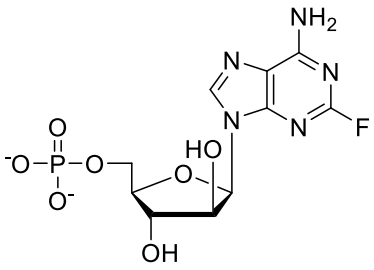
Introduction

Despite tremendous advances in the treatment options for cancer patients, in particular due to the development of immunotherapy and monoclonal antibodies, the search for new cytotoxic compounds is still of main importance in cancer research. Among the existing therapeutic arsenal, the purine scaffold is well represented [1, 2] (Figure 1). Purine nucleobases, such as 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are well known drugs used in the treatment of childhood ALL [3, 4], and fludarabine, cladribine and clofarabine among others are purine containing nucleoside analogues commonly used in clinic [5]. In addition, N-6-benzoyl adenine derivatives and some substituted 6-phenyl purine analogues (Figure 2) have recently been identified as potential anticancer agents, respectively as BRD4 inhibitors [6, 7] and as cytotoxic agents on a panel of hepatocellular cancer lines [8].

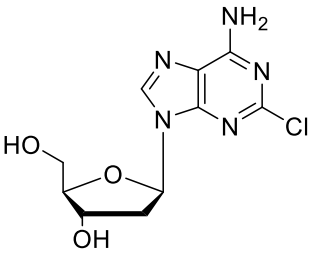
Within a research program aiming at discovering cytosolic 5'-nucleotidase II inhibitors using a fragment-based drug design approach, [9, 10] we identified a series of *N*-(9*H*-purin-6-yl)benzamide derivatives with cytotoxic effects in the micromolar range on human lymphoma cells. Some of these compounds showed moderate to good inhibition of the 5'-nucleotidase cN-II *in vitro* and synergy with nucleoside analogues on cancer cell lines. Based on the structural analogy of these series with the previously cited 6-substituted purine analogues (Figure 2) and the identified anticancer activity in the initial study, we continued herein the evaluation of the biological activity of these compounds. To this purpose, we included additional cell lines and performed various cell biology experiments on proliferation, cell cycle arrest and cell death induction. In order to perform *in vivo* experiments of the hit compound and due to its very low aqueous solubility, we also designed and synthesized water-soluble prodrugs that were evaluated both on cell models and in mice. These were obtained by the introduction of biolabile hydrophilic substituents on the N-9-position of the purine.



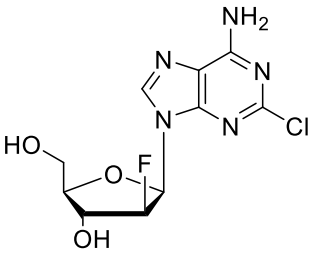
6-MP, R=H
6-TG, R=NH_2



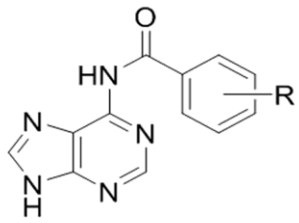
Fludarabine



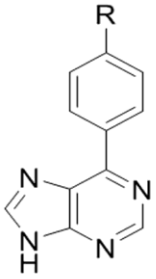
Cladribine



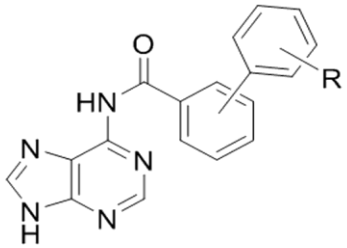
Clofarabine



N-6-Benzoyl adenines



6-Phenyl purines



N-(9H-purin-6-yl)benzamide derivatives

Materials and Methods

Synthesis of derivatives **1**-**9** and water-soluble prodrugs of compound **1** (derivatives **10**-**15**)

Synthesis of *N*-(9*H*-purin-6-yl)benzamide derivatives (**1**-**9**) has been previously reported [9]. Experimental details for the preparation of water-soluble prodrugs **10**-**15** and corresponding NMR spectra are available as SI.

Cell lines and culture

Human follicular lymphoma cells (RL), human acute promyelocytic leukemia cell (HL-60), human acute lymphoblastic leukemia (ALL) cells (CCRF-CEM), human acute T cell lymphoma cells (Jurkat), human B cell precursor leukemia cells (REH and RS4;11) and murine T cell lymphoma cells (L1210) were grown in RPMI media (Life technologies) containing L-glutamine, penicillin (200 IU/mL, Life technologies), streptomycin (200 µg/mL, Life technologies) and 10% feral bovine serum at 37 °C in a humidified atmosphere and in the presence of 5% of CO_2.

Cell survival assay and synergy experiments

The sensitivity to nucleoside analogues was assessed by cell survival after 72 hours' exposure to molecules using the MTT assay and 20,000 cells per well in 96 well plates. Inhibitory concentrations 50 (IC_{50}) and combination index 95 (CI_{95}) were calculated with CompuSyn software 1.0 (ComboSyn, Inc., USA) as indicated earlier [9, 10]. Synergy was defined as $CI_{95} < 0.9$, additivity as $0.9 < CI_{95} < 1.1$, and antagonism as $CI_{95} > 1.1$ [11].

Apoptosis

Cell death was determined on cells incubated for 48 hours in absence or presence of compounds, by AnnexinV-positivity assessment and propidium iodide (PI) incorporation into DNA by flow cytometry using a FACS Calibur in conditions earlier described [12] using 5×10^5 cells per well in

24-well plates and a final volume of 0.5 mL. Dead or dying cells were identified as AnnexinV and/or PI positive cells.

Cell proliferation

Cell proliferation in absence or presence of selected compounds was determined by quantification of CellTrace CFSE in dividing cells by flow cytometry on a FACScalibur as indicated earlier [13], using 0.5 x 10^6 cells per well in 6-well plates and a final volume of 3 mL. Results were analyzed with FlowJo and expressed as percentage of MFI of CFSE as compared to the signal on day 1.

Cell cycle assessment

Cell cycle distribution was assessed in cells incubated for 24 hours in absence or presence of selected compounds using an LSR-II and conditions described earlier [13], using 5 x 10^5 cells per well in 24-well plates and a final volume of 0.5 mL. Results were analyzed using FlowJo and expressed as percentage of cells in each phase of the cell cycle.

Animal studies

For *in vivo* studies, 4 weeks old and acclimated female scid CB17 mice (Charles River) were injected subcutaneously with 5 x 10^6 RL cells. When tumors reached approximately 100 mm^3 (calculated by the formula $4/3 \times \pi \times [(\text{length} + \text{width})/2]^3$), mice received two intraperitoneal treatments with seven days interval. Body weight and tumor volume were determined 2-3 times per week and mice sacrificed when it reached 1500 mm^3. All mice were raised in SPF environment with free access to standard food and water. This study was approved by the Animal Ethics Committee of the University Claude Bernard of Lyon.

Results

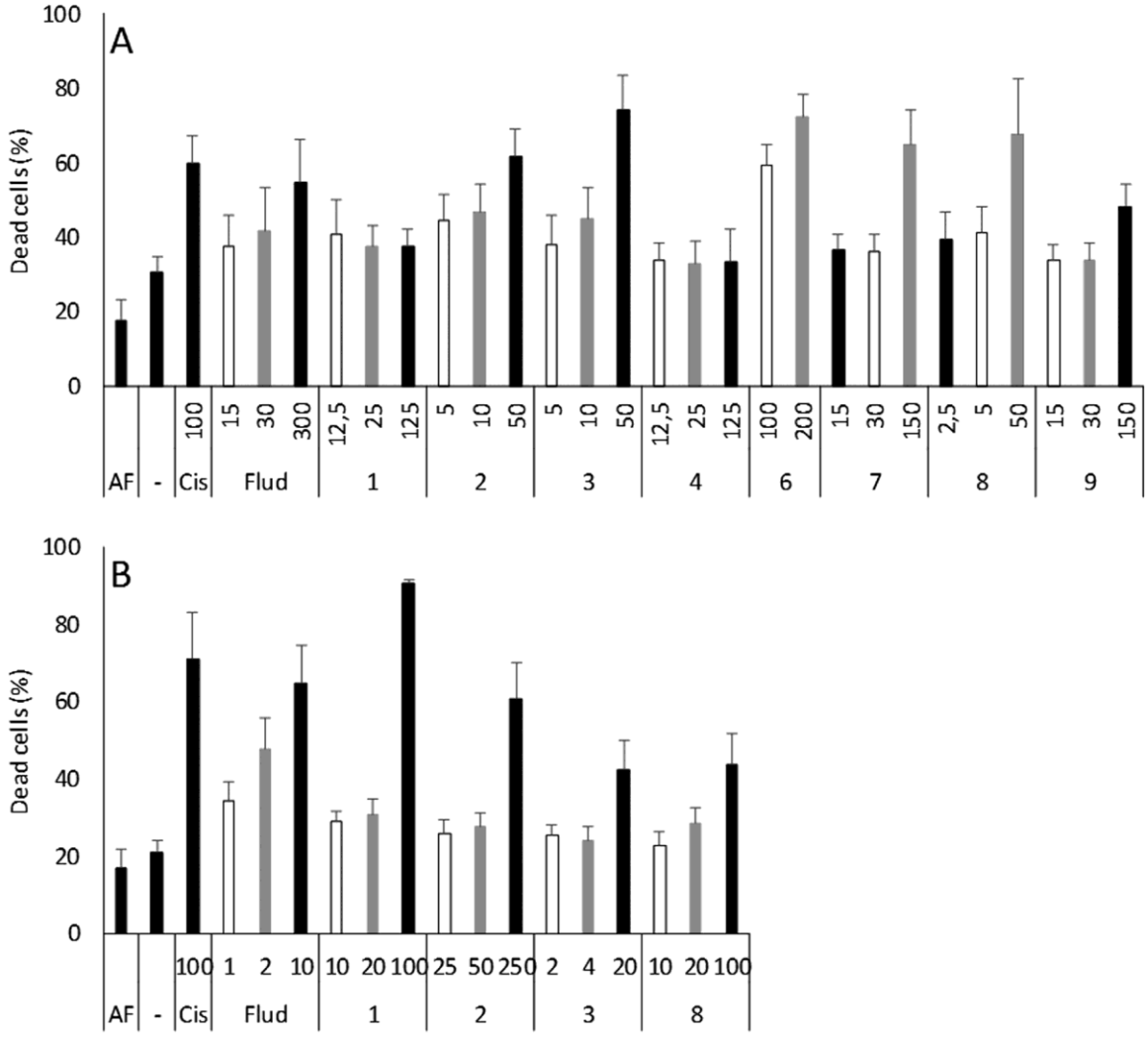
Studied compounds are cytotoxic

Compounds **1**-**9** were first evaluated for their intrinsic cytotoxic activity on a panel of three cancer cell lines (RL, HL-60, L1210) using the MTT assay (Table 1). We obtained IC_{50}-values from 3.4 µM (**8** on L1210 cells) to more than 300 µM (in particular on L1210 cells). Comparable toxicity was observed on all tested cell lines for several compounds (**1**, **2**, **3**, **6** and **8**), whereas others had cell line specific toxicity with a better sensitivity for RL cells (**4**, **7** and **9**).

In an initial work, and linked to its inhibitory effect on the 5'-nucleotidase cN-II [9], **1** was found to act synergistically with cladribine and clofarabine but not with fludarabine, in RL cells. Thus, we extended this work to other cell lines (CCRF-CEM, HL-60, Jurkat, REH, RS4;11 and L1210) as well as to the purine nucleobase 6-mercaptopurine (Table 2). No synergy was observed between **1** and the purine nucleoside analogues cladribine, clofarabine and fludarabine in human or murine cells of haematological origin, whereas there was a very good synergy with 6-mercaptopurine in REH and Jurkat cells, and a weak synergy in HL-60 and L1210 cells.

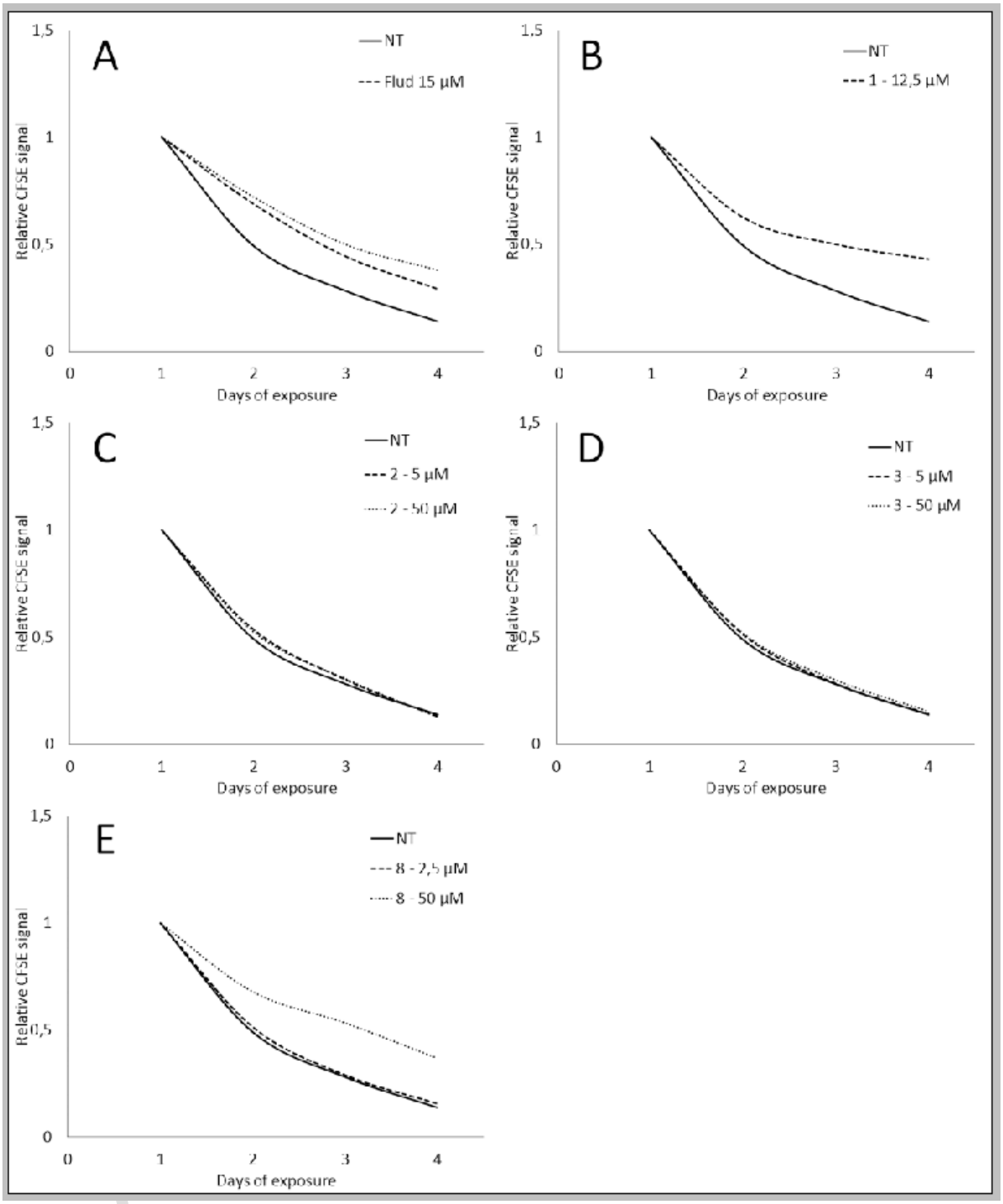
Different induction of apoptosis by compounds **1**-**9**

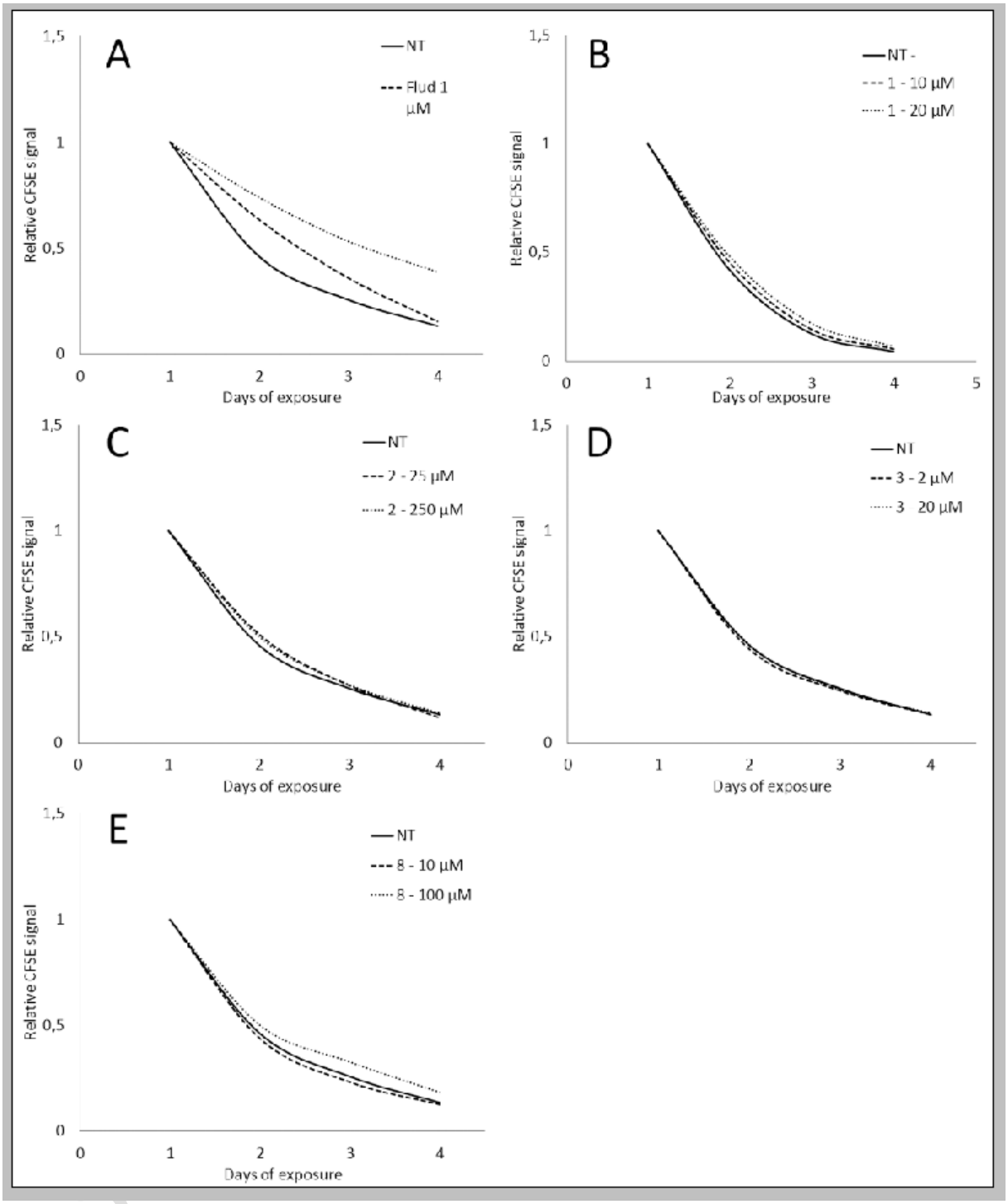
In order to better describe the biological activities of these *N*-(9*H*-purin-6-yl)benzamide derivatives, we evaluated their capacity to induce apoptosis in RL and HL-60. We used high concentration of cisplatin as a positive control for induction of cell death, and two or three concentrations for fludarabine (control compound) and our compounds. These concentrations were selected based on MTT results as approximatively $IC_{50}/2$, IC_{50} and IC_{50}x5. As shown in Figure 3A, a dose-dependent induction of apoptosis was observed for fludarabine, **2**, **3**, **6**, **7**, **8** and **9**, but not for **1** and **4** on RL cells. For HL-60 cells (Figure 3B), we observed a dose-dependent induction of cell death with all tested compounds (fludarabine, **1**, **2**, **3** and **8**).



Antiproliferative activity of the studied compounds

As the decreased MTT reducing activity was not always associated with increased cell death as shown by apoptosis, we evaluated whether some of the compounds had antiproliferative effects using CFSE staining on RL and HL-60 cells (Figures 4 and 5). For fludarabine, even low concentrations not related to cell death induction, were associated with decreased cell proliferation. Compound **1** decreased proliferation of RL cells at a low concentration that was not associated with cell death, but not on HL-60 cells. Higher concentrations (125 µM for RL and 100 µM for HL-60, data not shown) were also evaluated but cells did not survive, in line with the results on apoptosis. Compounds **2** and **3** did not decrease cell proliferation even at high doses associated with cell death induction. Finally, **8** at IC_{50}x5 slowed down proliferation of RL cells but not HL-60 cells even though this concentration induced cell death in both cell lines.

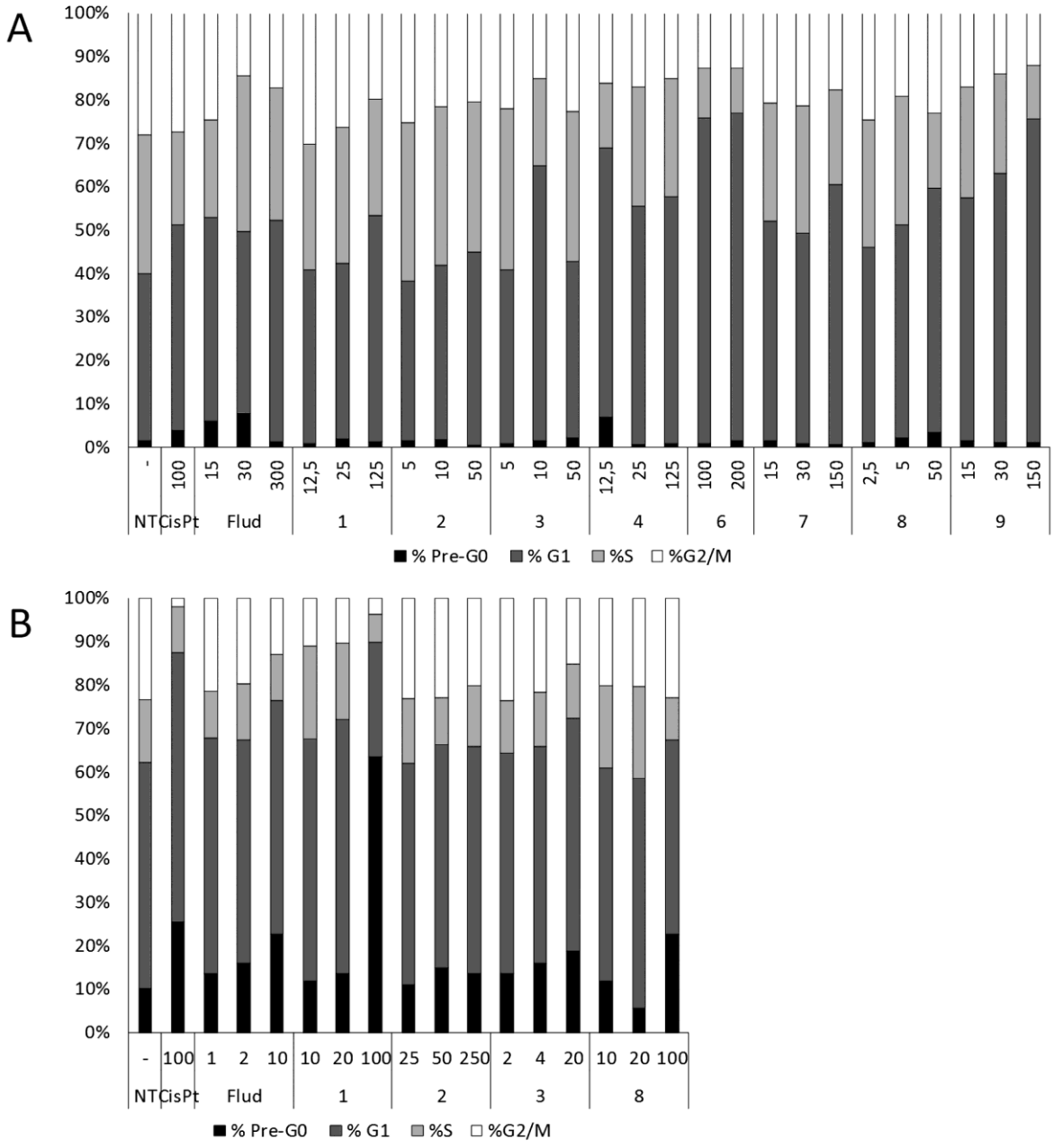

A
NT
Flud 15 µM
Relative CFSE signal
Days of exposure
B
NT
1 - 12,5 µM
Relative CFSE signal
Days of exposure
C
NT
2 - 5 µM
2 - 50 µM
Relative CFSE signal
Days of exposure
D
NT
3 - 5 µM
3 - 50 µM
Relative CFSE signal
Days of exposure
E
NT
8 - 2,5 µM
8 - 50 µM
Relative CFSE signal
Days of exposure



Cell cycle arrest of compounds **1**-**9**

We completed the *in vitro* evaluation of our *N*-(9*H*-purin-6-yl)benzamide derivatives with their effect on cell cycle distribution. For RL cells (Figure 6A), we observed a dose-dependent increase in G1-phase for **1**, **8** and **9**, as well as an important increase at all concentrations tested for **4**, **6** and **7**. For **3**, the increase was only observed at the concentration corresponding to IC_{50}, whereas no modification

was seen with the three concentrations of **2**. For HL-60 cells (Figure 6B), we did not observe any main modification in cell cycle distribution, except for with the highest concentration of **1** inducing an important increased in pre-G0 phase, consistent with AnnexinV results.



Synthesis of water-soluble prodrugs of compound **1**

Due to the low water solubility of the studied compounds, that is an issue commonly encountered for large aromatic and heterocyclic derivatives, we envisaged to obtain prodrugs [14-16] of our best characterized compound, **1**. Thus, synthesis of acyl-oxymethyl (**11a** and **11b**) and phosphor-

oxymethyl (**13**, **15a**, **15b** and **15c**) derivatives was performed and they also included different type of salts (Table 3 and Supplemental Information).

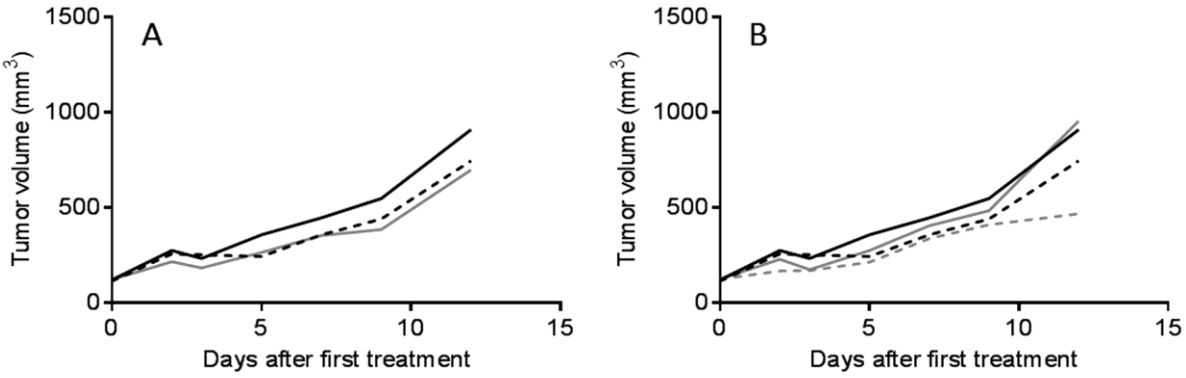
The aqueous solubilities of **11a** and **15c** have been determined by HPLC to be respectively 47 mg and 83 mg per mL, which is an increase of more than 1000-fold compared to the parent compound **1** (22.6 µg per mL) [10].

In vitro activity of soluble prodrugs

In vitro assays with RL and L1210 cells (Table 3) showed that four out of six compounds (**15c**, **15a**, **11a** and **11b**) had similar cytotoxicity as the parent compound **1** (IC_{50} = 16-28 µM), whereas the two others had weaker (IC_{50} = 72 µM for **15b**) or no toxicity (IC_{50} > 300 µM for **13**). When assessed together with nucleoside analogues and 6-mercaptopurine, we observed only synergy for **1** with fludarabine on RL cells and with 6-mercaptopurine on L1210 cells, for **15a** with cladribine on RL cells, and for **11b** with fludarabine and 6-mercaptopurine on RL cells (Table 4).

In vivo antitumoral activity of soluble prodrugs

Based on availability, we designed an *in vivo* experiment with **15c** and **11a** alone and in combination with fludarabine using immunodeficient mice with tumors from RL cells. We used fludarabine as all prodrugs are supposed to deliver compound **1** *in vivo*, and that this compound shows synergy with fludarabine in RL cell model. As shown in Figure 7, treatment with 100 mg/kg fludarabine and 15 mg/kg **15c** delayed tumor growth. For 21 mg/kg **11a**, only the combination with 100 mg/kg fludarabine resulted in important tumor growth inhibition. None of these treatments was associated with weight loss or any other macroscopic sign of toxicity. The combination of 100 mg/kg fludarabine with 15 mg/kg **15c** was however lethal with 2 of 5 mice dying three days after the first treatment.



Discussion

The first part of our study concerned the additional biological evaluation of selected *N*-(9*H*-purin-6-yl)benzamide derivatives on cancer cells. We used various techniques to determine the activity of the compounds on metabolic activity (MTT assay), cell death induction (AnnexinV/PI staining), cell proliferation (CFSE staining) and cell cycle arrest (PI staining).

Three compounds (**2**, **3**, and **8**) out of nine clearly showed the best cytotoxicity in MTT assay with IC_{50} values below 15 µM, and compounds **3** and **8** exhibited significant cytotoxic activity against all three cell lines tested. For comparison, IC_{50} values on RL cells are approximately 40 nM for clofarabine, 100 nM for cladribine, 7 µM for fludarabine, 0.8 µM for 6-mercaptopurine and 1.2 µM for 6-thioguanine (raw data from [13]), whereas other chemically related compounds had IC_{50} of around 5 µM on liver cancer cells [8]. In addition, these compounds were also able to induce apoptosis in RL cells at comparable levels as with fludarabine (more than 50% dead cells at 50 µM). These results suggest that the second phenyl group should rather be in the meta position (R_1=H) and a pyrrolidine substituent (compound **3**) for a better activity. When, the imidazolyl substituent is attached through the nitrogen atoms (compounds **6** and **7**), derivatives are lacking biological activity as determined by our MTT assay, whereas if it is linked through the C-4 atom and in the para orientation (compound **8**) some cytotoxic activity is recovered. Finally, compounds **1** and **8** slowed down cell proliferation, in particular in RL cells.

Our goal was not to determine the mechanism of action of these compounds, but to better describe their activity. Therefore, we can only hypothesize on how they induce cell death in our models. Other compounds belonging to similar chemical series have been reported to be inhibitors of BRD4, an enzyme of the bromodomain and extra-terminal domain protein family involved in gene regulations and cancer cell survival, but whether our compounds do the same is unknown [6, 7]. The initial target for these compounds, the 5'-nucleotidase cN-II is expressed in both RL and HL-60 cell lines, with an approximately 2-fold higher expression and activity in HL-60 cells [13]. If the mechanism of action is based upon cN-II inhibition, we would expect a higher sensitivity in RL cells. This is the case

for compounds **2**, **4**, **7**, **8** and **9** inhibiting cN-II activity *in vitro* by 28, 73, 39 and 26% respectively but not for compounds **1** and **3** inhibiting by 68 and 56% respectively [9]. This could therefore suggest that this is not the main mechanism, or that the cN-II inhibition in cells is different from and not proportional to our previously used *in vitro* conditions. Indeed, we do not know whether RL and HL-60 cells express wild-type or hyperactive mutants [17, 18] or how cN-II is regulated in these cells by allosteric regulators or by energy status [19, 20].

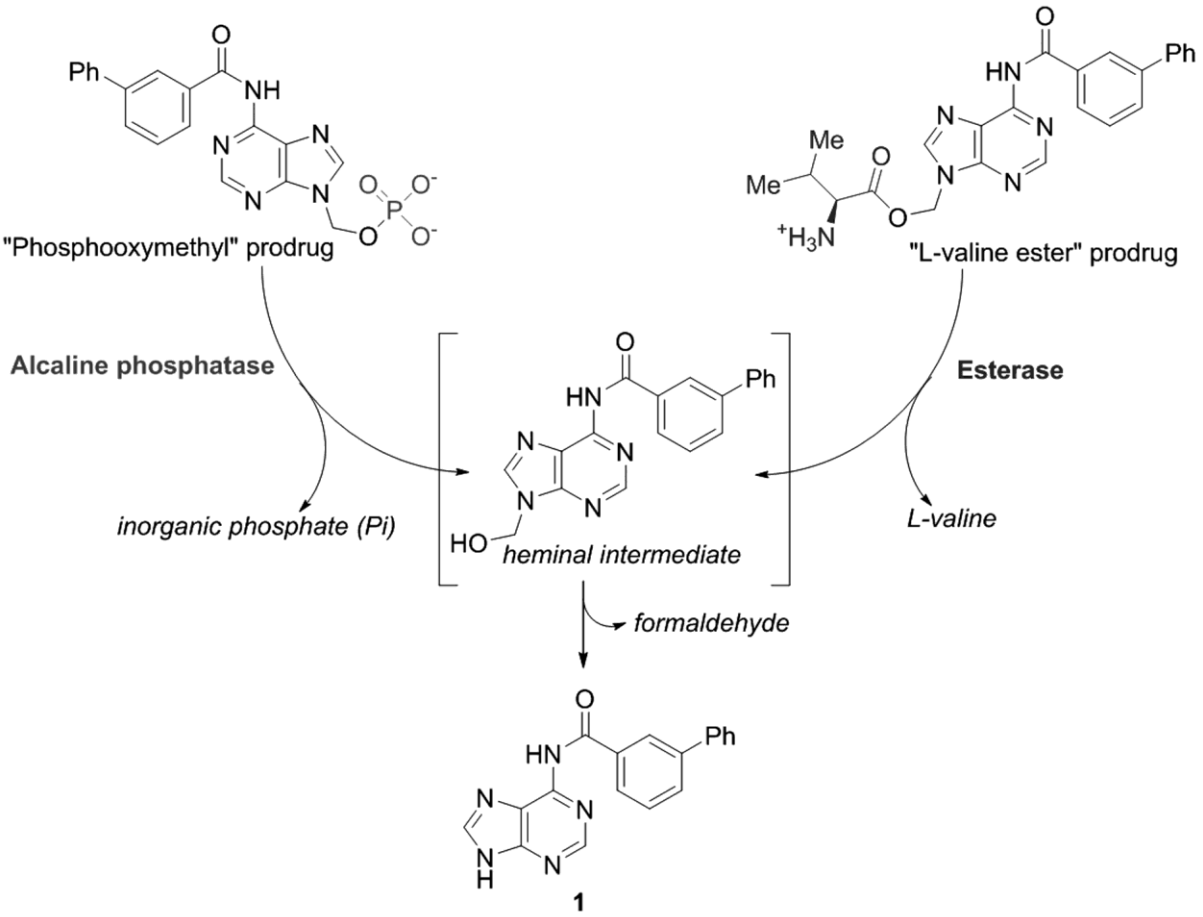
The extensive study of compound **1** revealed that its moderate cytotoxic activity (25-50 µM in RL, HL-60 and L1210) is neither associated with the induction of cell death in RL cells or significant antiproliferative effect in HL-60. However, this compound clearly showed synergic effect with 6-mercaptopurine in four out of six of the studied cell lines. Taking into account the mechanism of action of 6-mercaptopurine and its cellular metabolization (involving hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and its catabolic conversion into xanthine-5'-monophoshpate (from 6-thioguanosine-5'-monophosphate) and that compound **1** is one of the best cN-II inhibitors within the series of *N*-(9*H*-purin-6-yl)benzamide derivatives, we could hypothesize that the observed synergy may be associated with the inhibition of cN-II, thus limiting the catabolism of xanthine-5'-monophosphate [21, 22].

The second part of the study concerned the *in vivo* evaluation of our compounds. Common features of *N*-(9*H*-purin-6-yl)benzamide derivatives are their polycyclic aromatic structures, and as a consequence thereof, many of them have poor aqueous solubility. Despite its promising *in vitro* cytotoxic activity and synergistic effects with nucleoside analogues, **1** shows very low solubility in water (22.6 µg/mL), and this represents a major obstacle for its further development and study in experimental animal models. As initial attempts to formulate parenteral **1** using co-solvents (PEG…) or cyclodextrin-based agents were not successful, we designed prodrugs to improve physicochemical properties (such as water solubility) of our bioactive derivative [14]. Recent data from the literature [23, 24] demonstrate that the incorporation of a phosphate or amino group, either directly attached to the compound or introduced via a linker, enhance the solubility of compounds to be administered

intravenously or orally. We explored two series of water-soluble prodrugs of **1** which are expected to release the parent drug through different two-step enzyme-mediated hydrolytic activation (Figure 8). The first series involved the introduction of L-valine through an oxymethyl linker. According to literature data, valyl ester prodrugs are expected to be substrates of plasma esterase leading to the labile heminal intermediate that would rapidly degraded into the parent drug and formaldehyde. The second approach involved the introduction of an oxymethylphosphate moiety that is sensitive to an initial alkaline phosphatase hydrolysis leading to the same heminal intermediate as before and *in fine* to **1**.

Due to the potential effect of the nature of the salts on the physicochemical properties (hygroscopicity, physical form, solubility, stability…) of the resulting prodrugs, we studied five different salts (sodium, lysine and tromethamine for phosphate derivatives, trifluoroacetate and mesylate for valyl ester prodrugs) in order to select the best candidate for *in vivo* investigations. The water solubility, as determined for **11a** and **15c**, was 1000-fold increased as compared to **1**. MTT-based *in vitro* assay showed similar activity (22-28 µM and 72 µM for **15b**) of all compounds except for **13**, strongly suggesting intracellular decomposition into compound **1** as shown in Figure 8. The *in vivo* evaluation of these drugs validated the prodrug approach, and showed weak antitumoral activity for **15c** and a weak potentiation of fludarabine with compound **11a**.

In conclusion, our study offers extended biological evaluation of selected *N*-(9*H*-purin-6-yl)benzamide derivatives on cancer cell lines, and we also describe water-soluble prodrugs for such compounds that allow *in vivo* experiments. For compound **1**, we can conclude on the induction of a decreased proliferation without cell cycle modifications or induction of AnnexinV-related cell death RL cells, and the induction of apoptosis in HL-60 cells. For the other compounds, the cellular modifications responsible for the initially observed cytotoxicity is less clear. Further studies would be warranted to determine the mechanism of action as well as the potential toxicity on healthy cells as this would be of main importance in an eventual further development of these compounds as anticancer drugs.


"Phosphooxymethyl" prodrug
"L-valine ester" prodrug
Alcaline phosphatase
Esterase
inorganic phosphate (Pi)
L-valine
heminal intermediate
formaldehyde
1

References

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

[2] Savic, D, T Stankovic, I Lavrnja, A Podolski-Renic, J Bankovic, E Pekovic, M Stojiljkovic, L Takic, S Ruzdijic, M Pesic. Purine nucleoside analogs in the therapy of cancer and neuroinflammation. Motth. 2015. 1: 3-14.

[3] Bhatia, S, W Landier, L Hageman, Y Chen, H Kim, CL Sun, N Kornegay, WE Evans, AL Angiolillo, B Bostrom, J Casillas, G Lew, KW Maloney, L Mascarenhas, AK Ritchey, AM Termuhlen, WL Carroll, FL Wong, MV Relling. Systemic Exposure to Thiopurines and Risk of Relapse in Children With Acute Lymphoblastic Leukemia: A Children's Oncology Group Study. JAMA Oncol. 2015. 1: 287-95.

[4] Bostrom, B, G Erdmann. Cellular pharmacology of 6-mercaptopurine in acute lymphoblastic leukemia. Am J Pediatr Hematol Oncol. 1993. 15: 80-6.

[5] Robak, P, T Robak. Older and new purine nucleoside analogs for patients with acute leukemias. Cancer Treat Rev. 2013. 39: 851-61.

[6] Amemiya, S, T Yamaguchi, T Sakai, Y Hashimoto, T Noguchi-Yachide. Structure-Activity Relationship Study of N(6)-Benzoyladenine-Type BRD4 Inhibitors and Their Effects on Cell Differentiation and TNF-alpha Production. Chem Pharm Bull (Tokyo). 2016. 64: 1378-83.

[7] Noguchi-Yachide, T, T Sakai, Y Hashimoto, T Yamaguchi. Discovery and structure-activity relationship studies of N6-benzoyladenine derivatives as novel BRD4 inhibitors. Bioorg Med Chem. 2015. 23: 953-9.

[8] Kucukdumlu, A, M Tuncbilek, E Bilget Guven, R Cetin Atalay. Synthesis of Some Substituted 6-Phenyl Purine Analogues and Their Biological Evaluation as Cytotoxic Agents. Acta Chim Slov. 2017. 64: 621-632.

[9] Marton, Z, R Guillon, I Krimm, Preeti, R Rahimova, D Egron, LP Jordheim, N Aghajari, C Dumontet, C Perigaud, C Lionne, S Peyrottes, L Chaloin. Identification of Noncompetitive Inhibitors of Cytosolic 5'-Nucleotidase II Using a Fragment-Based Approach. J Med Chem. 2015. 58: 9680-96.

[10] Guillon, R, R Rahimova, Preeti, D Egron, S Rouanet, C Dumontet, N Aghajari, LP Jordheim, L Chaloin, S Peyrottes. Lead optimization and biological evaluation of fragment-based cN-II inhibitors. Eur J Med Chem. 2019. 168: 28-44.

[11] Chou, TC, P Talalay. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul. 1984. 22: 27-55.

[12] Jordheim, LP, Z Marton, M Rhimi, E Cros-Perrial, C Lionne, S Peyrottes, C Dumontet, N Aghajari, L Chaloin. Identification and characterization of inhibitors of cytoplasmic 5'-nucleotidase cN-II issued from virtual screening. Biochem Pharmacol. 2013. 85: 497-506.

[13] Jordheim, LP, JY Puy, E Cros-Perrial, S Peyrottes, I Lefebvre, C Perigaud, C Dumontet. Determination of the enzymatic activity of cytosolic 5'-nucleotidase cN-II in cancer cells: development of a simple analytical method and related cell line models. Anal Bioanal Chem. 2015. 407: 5747-58.

[14] Jornada, DH, GF dos Santos Fernandes, DE Chiba, TR de Melo, JL dos Santos, MC Chung. The Prodrug Approach: A Successful Tool for Improving Drug Solubility. Molecules. 2015. 21: 42.

[15] Rautio, J, H Kumpulainen, T Heimbach, R Oliyai, D Oh, T Jarvinen, J Savolainen. Prodrugs: design and clinical applications. Nat Rev Drug Discov. 2008. 7: 255-70.

[16] Rautio, J, NA Meanwell, L Di, MJ Hageman. The expanding role of prodrugs in contemporary drug design and development. Nat Rev Drug Discov. 2018. 17: 559-587.

[17] Meyer, JA, J Wang, LE Hogan, JJ Yang, S Dandekar, JP Patel, Z Tang, P Zumbo, S Li, J Zavadil, RL Levine, T Cardozo, SP Hunger, EA Raetz, WE Evans, DJ Morrison, CE Mason, WL Carroll. Relapse-specific mutations in NT5C2 in childhood acute lymphoblastic leukemia. Nat Genet. 2013. 45: 290-4.

[18] Tzoneva, G, A Perez-Garcia, Z Carpenter, H Khiabanian, V Tosello, M Allegretta, E Paietta, J Racevskis, JM Rowe, MS Tallman, M Paganin, G Basso, J Hof, R Kirschner-Schwabe, T Palomero, R Rabadan, A Ferrando. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. Nat Med. 2013. 19: 368-71.

[19] Itoh, R. Enzymatic properties and physiological roles of cytosolic 5'-nucleotidase II. Curr Med Chem. 2013. 20: 4260-84.

[20] Pesi, R, S Allegrini, F Balestri, M Garcia-Gil, F Cividini, L Colombaioni, LP Jordheim, M Camici, MG Tozzi. Cytosolic 5'-Nucleotidase II Is a Sensor of Energy Charge and Oxidative Stress: A Possible Function as Metabolic Regulator. Cells. 2021. 10.

[21] Brouwer, C, TM Vogels-Mentink, JJ Keizer-Garritsen, FJ Trijbels, JP Bokkerink, PM Hoogerbrugge, ER van Wering, AJ Veerman, RA De Abreu. Role of 5'-nucleotidase in thiopurine metabolism: enzyme kinetic profile and association with thio-GMP levels in patients with acute lymphoblastic leukemia during 6-mercaptopurine treatment. Clin Chim Acta. 2005. 361: 95-103.

[22] Tzoneva, G, CL Dieck, K Oshima, A Ambesi-Impiombato, M Sanchez-Martin, CJ Madubata, H Khiabanian, J Yu, E Waanders, I Iacobucci, ML Sulis, M Kato, K Koh, M Paganin, G Basso, JM Gastier-Foster, ML Loh, R Kirschner-Schwabe, CG Mullighan, R Rabadan, AA Ferrando. Clonal evolution mechanisms in NT5C2 mutant-relapsed acute lymphoblastic leukaemia. Nature. 2018. 553: 511-514.

[23] Meanwell, NA, MR Krystal, B Nowicka-Sans, DR Langley, DA Conlon, MD Eastgate, DM Grasela, P Timmins, T Wang, JF Kadow. Inhibitors of HIV-1 Attachment: The Discovery and Development of Temsavir and its Prodrug Fostemsavir. J Med Chem. 2018. 61: 62-80.

[24] Oslob, JD, SA Heumann, CH Yu, DA Allen, S Baskaran, M Bui, E Delarosa, AD Fung, A Hashash, J Hau, S Ivy, JW Jacobs, W Lew, J Maung, RS McDowell, S Ritchie, MJ Romanowski, JA Silverman, W Yang, M Zhong, T Fuchs-Knotts. Water-soluble prodrugs of an Aurora kinase inhibitor. Bioorg Med Chem Lett. 2009. 19: 1409-12.

Table 1. Cytotoxic activity of compounds on cancer cell lines. IC_{50} values (μM) are mean values of at least three independent experiments ± SEM. Results on RL cells are from [9].

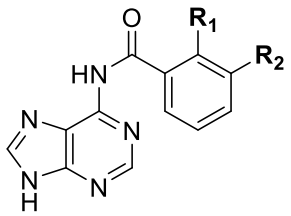
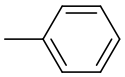
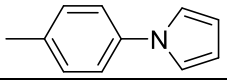
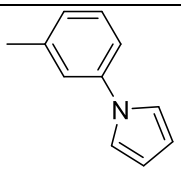
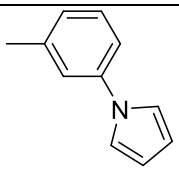
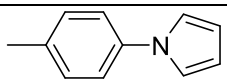
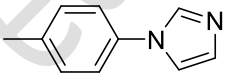
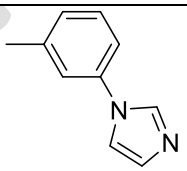
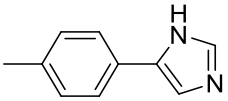
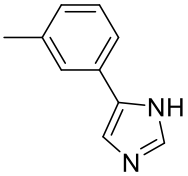
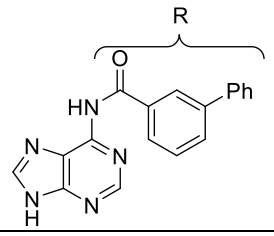
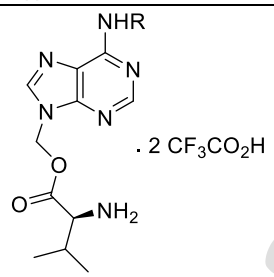
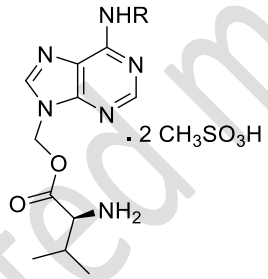
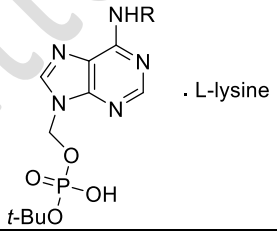
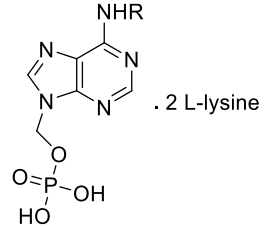
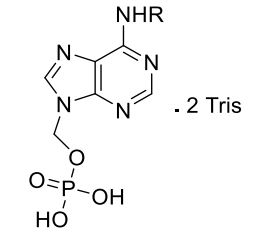
	 R1=	R2=	RL[a]	HL-60	L1210
1	H		25 ± 4	18 ± 3	39 ± 5
2	H		**8.0 ± 2.5**	49 ± 11	n.d.
3	H		**11 ± 2**	**4.1 ± 0.4**	**8.3 ± 0.2**
4		H	23 ± 9	>300	>300
5		H	35 ± 3	n.d.	n.d.
6	H		203 ± 20	91 ± 21	>300
7	H		34 ± 5	285 ± 8	>300
8	H		**4.7 ± 0.7**	**12.9 ± 0.6**	**3.4 ± 0.2**
9	H		30 ± 3	204 ± 7	>300

Table 2. Antiproliferative activity and synergy with nucleoside analogues of compound 1 on additional cell lines. IC_{50} values (µM) are mean values of at least three independent experiments ± SEM. CI95 values were calculated with the CompuSyn software and expressed as a range from 10^(mean(logCI95))-SEM to 10^(mean(logCI95))+SEM from at least three independent experiments. Synergy and additivity are indicated in bold.

	CCRF-CEM	**HL-60**	**Jurkat**	**REH**	**RS4,11**	**L1210**
IC50	20 ± 3	18 ± 3	20 ± 3	25 ± 7	28 ± 4	39 ± 5
CI95 fludarabine	1.0-1.4	1.5-2.9	0.8-2.5	1.0-4.8	1.1-1.9	2.2-4.5
CI95 cladribine	1.2-1.6	1.6-2.0	n.d.	n.d.	n.d.	n.d.
CI95 clofarabine	1.1-1.9	0.9-2.2	n.d.	n.d.	n.d.	n.d.
CI95 6-MP	0.9-1.8	**0.6-1.0**	**0.4-0.6**	**0.1-0.4**	3.4-11.9	**0.8-1.0**

Table 3. Cytotoxic activity on RL and L1210 cancer cell lines of compounds for *in vivo* administration.

IC_{50} values (µM) are mean values of at least three independent experiments ± SEM. n.d: not determined.

	Structure	RL	L1210
1		22 ± 5	16 ± 3
11a		28 ± 5	26 ± 3
11b		26 ± 3	24 ± 11
13		>300	>300
15a		26 ± 5	20 ± 3
15b		72 ± 28	n.d.

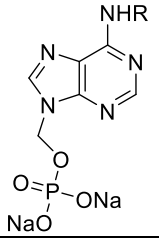
15c		26 ± 8	21 ± 4

Table 4. Synergy between nucleoside analogues and studied compounds on RL and L1210 cells. CI95 values were calculated with the CompuSyn software and expressed as a range from 10^(mean(logCI95))-SEM to 10^(mean(logCI95))+SEM from at least three independent experiments. Synergy and additivity are indicated in bold.

		1	11a	11b	15a	15c
RL	**CI95 fludarabine**	**0.49-0.74**	0.77-1.47	**0.78-0.95**	2.18-7.22	0.91-1.13
	CI95 cladribine	0.89-1.58	0.77-1.45	0.99-1.14	**0.15-0.83**	0.64-1.44
	CI95 clofarabine	0.63-1.20	0.92-1.38	1.09-1.43	0.52-1.05	1.05-1.69
	CI95 6MP	1.02-1.43	1.24-2.72	**0.95-0.95**	1.76-4.16	0.54-1.01
L1210	**CI95 fludarabine**	1.54-2.38	1.67-2.12	1.41-1.74	1.56-2.91	1.90-2.87
	CI95 cladribine	0.94-1.24	1.26-1.51	1.44-1.65	1.35-1.94	1.19-2.01
	CI95 clofarabine	1.52-3.46	1.64-2.20	0.91-1.19	1.29-2.22	2.22-2.96
	CI95 6MP	**0.47-0.53**	2.01-4.44	1.47-2.32	1.17-2.57	0.99-2.92

Figure legends

Figure 1. Examples of purine and purine nucleoside analogues used as anti-cancer drugs. 6-MP: 6-mercaptopurine; 6-TG: 6-thioguanine.

Figure 2. Generic structures of purine derivatives investigated in the literature as anticancer agents [6-8] and the studied compounds.

Figure 3. Induction of cell death by studied compounds in RL (A) and HL-60 (B) cells. Cell death was assessed by AnnexinV/IP staining by flow cytometry after 48 hours of exposure to indicated concentrations of compounds corresponding to approximately $IC_{50}/2$ (white bars), IC_{50} (grey bars) and $IC_{50}x5$ (black bars). Dead cells are AnnexinV and/or IP positive cells. Results are means from three independent experiments and error bars are SEM. AF: autofluorescence; - : unexposed cells; Cis: cisplatin; Flud: fludarabine.

Figure 4. Proliferation of RL cells in absence (NT) or presence of molecules at indicated concentrations. Proliferation was assessed by determination of CFSE staining at after indicated days of exposure with indicated concentrations of fludarabine (A), **1** (B), **2** (C), **3** (D) and **8** (E). Relative CFSE signal was calculated as compared to the signal of cells one day after CFSE staining, and graphs show mean results of three independent experiments. NT: not treated; Flud: fludarabine.

Figure 5. Proliferation of HL-60 cells in absence (NT) or presence of molecules at indicated concentrations. Proliferation was assessed by determination of CFSE staining at after indicated days of exposure with indicated concentrations of fludarabine (A), **1** (B), **2** (C), **3** (D) and **8** (E). Relative CFSE signal was calculated as compared to the signal of cells one day after CFSE staining, and graphs show mean results of three independent experiments. NT: not treated; Flud: fludarabine.

Figure 6. Cell cycle distribution of RL (A) and HL-60 (B) cells exposed 24 hours to indicated concentrations of studied molecules. The graph shows the mean results of three independent experiments.

Figure 7. Tumor growth of RL cells in mice treated with PBC prodrugs. Mice with RL-tumors were treated with fludarabine (100 mg/kg, dotted lines in A and B), **15c** (15 mg/kg, grey line in A) and **11a** (21 mg/kg, grey lines in B) alone or in combination as indicated in Material and Methods. Graphs show median tumor volumes of 5 mice per group. Individual tumor growths are shown in supplemental information (Figure S1).

Figure 8. Structures and expected bioactivation of the two series of water-soluble prodrugs of **1**.