Phytochemistry 71 (2010) 1350–1359

Contents lists available at ScienceDirect

Phytochemistry

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

Anticancer activity of natural cytokinins: A structure–activity relationship study

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ARTICLE INFO

Article history:
Received 12 January 2010
Received in revised form 15 April 2010
Accepted 19 April 2010
Available online 1 June 2010

Keywords:
SAR
Cytokinins
Cancer
ortho-Topolin riboside
NCI_{60}

ABSTRACT

Cytokinin ribosides (N^6-substituted adenosine derivatives) have been shown to have anticancer activity both *in vitro* and *in vivo*. This study presents the first systematic analysis of the relationship between the chemical structure of cytokinins and their cytotoxic effects against a panel of human cancer cell lines with diverse histopathological origins. The results confirm the cytotoxic activity of N^6-isopentenyladenosine, kinetin riboside, and N^6-benzyladenosine and show that the spectrum of cell lines that are sensitive to these compounds and their tissues of origin are wider than previously reported. The first evidence that the hydroxylated aromatic cytokinins (*ortho*-, *meta*-, *para*-topolin riboside) and the isoprenoid cytokinin *cis*-zeatin riboside have cytotoxic activities is presented.

Most cell lines in the panel showed greatest sensitivity to *ortho*-topolin riboside (IC_{50} = 0.5–11.6 μM). Cytokinin nucleotides, some synthesized for the first time in this study, were usually active in a similar concentration range to the corresponding ribosides. However, cytokinin free bases, 2-methylthio derivatives and both *O*- and *N*-glucosides showed little or no toxicity. Overall the study shows that structural requirements for cytotoxic activity of cytokinins against human cancer cell lines differ from the requirements for their activity in plant bioassays. The potent anticancer activity of *ortho*-topolin riboside (GI_{50} = 0.07–84.60 μM, 1st quartile = 0.33 μM, median = 0.65 μM, 3rd quartile = 1.94 μM) was confirmed using NCI_{60}, a standard panel of 59 cell lines, originating from nine different tissues. Further, the activity pattern of oTR was distinctly different from those of standard anticancer drugs, suggesting that it has a unique mechanism of activity. In comparison with standard drugs, oTR showed exceptional cytotoxic activity against NCI_{60} cell lines with a mutated p53 tumour suppressor gene. oTR also exhibited significant anticancer activity against several tumour models in *in vivo* hollow fibre assays.

1. Introduction

Cytokinins are important plant hormones that are defined by their ability to promote cell division in plant tissue culture (Skoog et al., 1965). Cytokinins found in plants are adenine derivatives substituted at the N^6-position with either isoprenoid or an aromatic side chain (Table 1). Isoprenoid *trans*-zeatin (tZ) is the most abundant naturally occurring cytokinin. The abundance of other isoprenoid cytokinins (N^6-isopentenyladenine, iP, *cis*-zeatin, cZ) and derivatives with a saturated side chain, such as dihydrozeatin (DHZ), varies between plant species. While isoprenoid cytokinins are ubiquitous in plants, aromatic cytokinins (represented by N^6-benzyladenine, BA, and its hydroxylated derivatives, the topolins) have only been identified, as yet, in a limited group of plant taxa (Horgan et al., 1975; Strnad, 1997; Strnad et al., 1992, 1997). The most abundant appears to be *ortho*-topolin riboside, which is present at micro-molar concentrations in poplar leaves after daybreak (Hewett and Wareing, 1973). Another aromatic cytokinin, N^6-furfuryladenine (kinetin, K), first recognized as a synthetic compound, has been reported to occur naturally (for a review see Barciszewski et al. (2007)). Both families of cytokinin occur in several forms: free bases, ribosides, riboside-5′-phosphates, 3-, 7-, 9- and *O*-glucosides, and amino acid conjugates (Table 1). The isoprenoid cytokinin iP is an atypical base, present in the tRNA of all studied organisms, which plays a role in the precise control of protein synthesis. In mammals, iP is part of $tRNA^{[Ser]Sec}$ and the cognate tRNA-isopentenyltransferase is a putative tumour suppressor (Spinola et al., 2005). Molecules with a N^6-isopentenyladenine moiety are released into the cytosol and subsequently into the body fluids as a result of tRNA turnover (Chheda and Mittelman, 1972).

Abbreviation: PBS, phosphate buffer saline; DTP, developmental therapeutics program of National Cancer Institute (DTP, Bethesda, USA); SAR, structure–activity relationship.

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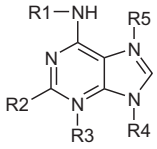
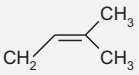
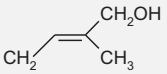
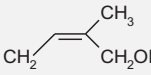
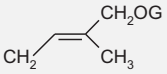
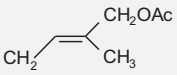
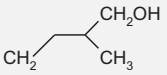
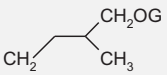
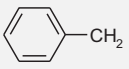
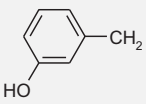
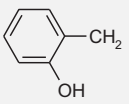
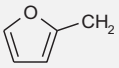
doi:10.1016/j.phytochem.2010.04.018

Table 1
Structures of the cytokinins used in this study.



R1	R2	R3	R4	R5	Trivial name	Abbreviation
	H	–	H	–	N^6-isopentenyladenine	iP
	H	–	Ribosyl	–	N^6-isopentenyladenosine	iPR
	CH_3S	–	Ribosyl	–	2-Methylthio-iPR	2MeSiPR
	H	–	–	Glucosyl	iP-7-glucoside	iP7G
	H	–	Glucosyl	–	iP-9-glucoside	iP9G
	H	–	Ribotide	–	iPR-5′-monophosphate	iPRMP
	H	–	Ribotide	–	iPR-5′-diphosphate	iPRDP
	H	–	Ribotide	–	iPR-5′-triphosphate	iPRTP
	H	–	H	–	*trans*-Zeatin	tZ
	H	–	Ribosyl	–	*t*-Zeatin riboside	tZR
	H	–	–	Glucosyl	*t*-Zeatin-7-glucoside	tZ7G
	H	–	Glucosyl	–	*t*-Zeatin-9-glucoside	tZ9G
	H	–	Ribotide	–	tZR-5′-monophosphate	tZRMP
	CH_3S	–	Ribosyl	–	2-Methylthio-*t*-zeatin riboside	2MeStZR
	H	–	H	–	*cis*-Zeatin	cZ
	H	–	Ribosyl	–	*cis*-Zeatin riboside	cZR
	H	–	H	–	*t*-Zeatin-*O*-glucoside	tZOG
	H	–	Ribosyl	–	*t*-Zeatin riboside-*O*-glucoside	tZROG
	H	–	H	–	t-Zeatin-O-acetyl	ActZ
	H	–	H	–	Dihydrozeatin	DHZ
	H	–	Ribosyl	–	Dihydrozeatin riboside	DHZR
	H	–	–	Glucosyl	Dihydrozeatin-7-glucoside	DHZ7G
	H	–	Glucosyl	–	Dihydrozeatin-9-glucoside	DHZ9G
	H	–	Ribotide	–	DHZR-5′-monophosphate	DHZRMP
	H	–	H	–	Dihydrozeatin-*O*-glucoside	DHZOG
	H	–	Ribosyl	–	Dihydrozeatin riboside-*O*-glucoside	DHZROG
	–	–	H	–	N^6-benzyladenine	BA
	–	–	Ribosyl	–	N^6-benzyladenosine	BAR
	–	Glucosyl	–	–	N^6-benzyladenine-3-glucoside	BA3G
	–	–	–	Glucosyl	N^6-benzyladenine-7-glucoside	BA7G
	–	–	Glucosyl	–	N^6-benzyladenine-9-glucoside	BA9G
	–	–	Ribotide	–	BAR-5′-monophosphate	BARMP
	–	–	Ribotide	–	BAR-5′-diphosphate	BARDP
	–	–	Ribotide	–	BAR-5′-triphosphate	BARTP
	–	–	H	–	*meta*-Topolin	mT
	–	–	Ribosyl	–	*meta*-Topolin riboside	mTR
	–	–	Glucosyl	–	*meta*-Topolin-9-glucoside	mT9G
	–	–	H	–	*ortho*-Topolin	oT
	–	–	Ribosyl	–	*ortho*-Topolin riboside	oTR
	–	–	Glucosyl	–	*ortho*-Topolin-9-glucoside	oT9G
	–	–	Ribotide	–	oTR -5′-monophosphate	oTRMP
	–	–	Ribotide	–	oTR -5′-diphosphate	oTRDP
	–	–	Ribotide	–	oTR -5′-triphosphate	oTRTP
	CH_3S	–	Ribosyl	–	2-Methylthio-oTR	2MeSoTR
	H	–	H		Kinetin	K
	H	–	Ribosyl		Kinetin riboside	KR
	H	–	Ribotide		KR -5′-monophosphate	KRMP

Knowledge that cytokinins play key roles in the regulation of plant growth and differentiation led to postulation that they could also affect growth and differentiation in animals, and have potential utility for treating human diseases that involve dysfunctional cell proliferation and/or differentiation. Abundant evidence supporting these hypotheses has subsequently been obtained. The ability of cytokinin bases to induce or promote the differentiation of human cells has been demonstrated in both keratinocytes (Berge et al., 2006) and several leukaemia cell lines, including HL-60 and K-562 (Ishii et al., 2003).

However, while cytokinin bases induce differentiation at relatively high concentrations (25–100 μM), their ribosides cause rapid

apoptosis of leukaemia cell lines at low micro-molar concentrations (Mlejnek, 2001). Cell death in HL-60 is preceded by depletion of adenosine triphosphate, activation of caspases and mitochondrial depolarization (Mlejnek, 2001; Ishii et al., 2002). Intracellular conversion of ribosides to their respective monophosphates is known to be required for their action (Mlejnek and Doležel, 2005). It has recently been demonstrated that kinetin riboside (KR) is a potential drug for the treatment of multiple myelomas (Tiedemann et al., 2008). In several models of multiple myeloma, KR has been found to induce rapid suppression of cyclin D1 and D2 transcription, followed by arrest of the cell-cycle and selective apoptosis in tumour cells (Tiedemann et al., 2008). Several authors have reported cytotoxic effects of N^6-isopentenyladenosine (iPR), KR and N^6-benzyladenosine (BAR) on human cell lines derived from solid tumours (Cabello et al., 2009; Choi et al., 2008; Griffaut et al., 2004; Laezza et al., 2009; Meisel et al., 1998; Spinola et al., 2007). Whether treatments resulted in cell cycle block and/or apoptosis was dependent on the cell line and the cytokinin used. The anticancer activity of iPR, KR and BAR has been demonstrated *in vivo* using several animal and xenograft models of cancer (Choi et al., 2008; Laezza et al., 2006; Tiedemann et al., 2008). iPR and BAR have also shown promising activity against a diverse range of cancers in a limited clinical trial (Mittelman et al., 1975).

Micro-molar concentrations of both cytokinin ribosides and cytokinin bases can also induce cell death in plant cell cultures, with some identifiable characteristics of apoptosis (activation of caspase-like proteases and fragmentation of DNA) (Mlejnek and Procházka, 2002). This cell death is preceded by depletion of adenosine triphosphate and the production of reactive oxygen species. In contrast to their hormonal activity, which requires interaction with specific membrane-bound receptors, intracellular conversion of cytokinins to monophosphates is necessary for this cytotoxic effect. The concentrations of cytokinin required to produce cytotoxic effects are higher than those found endogenously in plant tissues, but they do fall within the range used in plant bioassays (Carimi et al., 2003; Mlejnek et al., 2003, 2005).

Although the cytotoxic activity of natural cytokinins and their analogues (Doležal et al., 2006, 2007) against mammalian cells has been repeatedly demonstrated, there have been no previously published systematic studies of the structure-cytotoxic activity relationship (SAR) of natural cytokinins. Available reports indicate that only KR, iPR, BAR, *trans*-zeatin riboside (tZR) and their free bases have been tested, to date. Therefore, the present study was undertaken to examine the cytotoxic effects of almost all known naturally occurring cytokinins against a panel of cancer cell lines of diverse histopathological origin and then determine the basic SAR in terms of their growth-inhibitory effects. Furthermore, the activity pattern of *ortho*-topolin riboside (oTR) against NCI_{60}, a thoroughly characterized panel of 59 human cancer cell lines (Shoemaker, 2006) was analysed. Finally, we report results of *in vivo* tests of the anticancer activity of oTR against models representative of human tumours in hollow fibre assays.

2. Results and discussion

In addition to their essential roles in the growth and development of plants, cytokinins have various effects in man and animals at both cellular and whole organism levels (Berge et al., 2006; Slaugenhaupt et al., 2004; Rattan and Sodagam, 2005; Wu et al., 2007). Hence, cytokinins and their derivatives have many potential therapeutic applications, including possible efficacy in the treatment of proliferative diseases such as cancers. The anticancerous activity of cytokinins in a variety of cultured cell lines, several xenografts and even a clinical trial has been documented (Ishii et al., 2002; Mittelman et al., 1975; Tiedemann et al., 2008). However, only a few cytokinins have been tested as yet for anticancer activity. Here, we report an analysis of the relationship between the structures of 47 cytokinins (Table 1) and their cytotoxic activity against a panel of cell lines derived from diverse malignancies (Table 2). The tested compounds include almost all known natural purine cytokinins, representing all structural variants. Several compounds were synthesized for the first time for this study, including 2-methylthio-*ortho*-topolin riboside (2MeSoTR), *ortho*-topolin riboside-5'-monophosphate (oTRMP) and selected cytokinin riboside di- and triphosphates. Cytotoxic activity was evaluated after 72-h treatments using a standard viability test, based on quantification of the fluorescent product of the enzymatic hydrolysis of Calcein AM. The determined activities were expressed as IC_{50} values (concentrations leading to a 50% decrease in cellular esterase activity).

Treatment with the ribosides, oTR, iPR, BAR and KR resulted in a dose-dependent reduction in the viability (IC_{50} = 0.5–13.6 μM) of the cell lines derived from both haematological malignancies (CEM, HL60, K562 and RPMI) and solid tumours (MCF7, HeLa, and HOS). The leukaemia cell lines, HL60 and CEM, were most sensitive, with determined IC_{50} values $\leqslant$1.7 μM. Median IC_{50} values across the whole panel of cancer cell lines were 2.27, 4.15, 5.10 and 5.95 μM for oTR, BAR, KR and iPR, respectively. While the cytotoxic activity of the cytokinin ribosides iPR, BAR and KR against cancer cell lines has already been reported (Ishii et al., 2002; Laezza et al., 2009; Meisel et al., 1998; Mlejnek and Doležel, 2005; Spinola et al., 2007) this is the first report of the activity of oTR. The findings that cytokinin ribosides are active at sub-micro-molar (some leukemias) or low micro-molar concentrations (other leukemias, adherent cells) are consistent with those of previous studies, despite variations in the design of the cytotoxicity assays used in terms of principle/mechanism, definition of the endpoint and length of treatment. In contrast to oTR, the *meta*- and *para*-isomers only exhibited inhibitory activity against HL60 (with IC_{50} values of 24 and 7.5 μM, respectively). Among the cytokinin ribosides with hydroxylated isoprenoid side chains, *cis*-zeatin riboside (cZR) but not tZR or dihydrozeatin riboside (DHZR), was active against the leukaemia cell lines CEM and HL60 (with IC_{50} values of 18.8 and 7.9 μM, respectively).

Compared to the tested cytokinin ribosides, the free bases typically had much weaker effects on cell proliferation, with IC_{50} values either over the highest concentration used (>166 μM) or at least 50 times higher than the IC_{50} values determined for their respective ribosides. Similar differences between the cytotoxic activity of cytokinin bases (K, iP, and BA) and their corresponding ribosides have been reported by other authors (Ishii et al., 2002; Mlejnek and Doležel, 2005). In cultures of plant cells, the toxicities of cytokinin bases and the corresponding ribosides are comparable, because in contrast to human cells plant cells can convert both forms of cytokinin efficiently into riboside-5′-monophosphates (Mlejnek et al., 2003, 2005). Therefore, it was postulated that low toxicity of K, iP and BAP in human leukaemia HL-60 cell line is due to the low activity of human phosphoribosyltransferase towards cytokinin bases (Mlejnek and Doležel, 2005). The differences observed in the cytotoxic activities of *ortho*-topolin (oT), *para*-topolin (pT), *meta*-topolin (mT) and *cis*-zeatin (cZ) compared to their corresponding ribosides may have similar causes.

With the exception of *trans*-zeatin riboside-*O*-glucoside (tZROG), which exhibited some activity against the leukaemia cell lines CEM and HL-60 ($IC_{50} \sim 26$ μM), cytokinin *O*- and *N*-glucosides were inactive in the assay ($IC_{50} > 166$ μM). The observation that cytokinin bases and cytokinin glucosides showed limited activity, or none at all, supports the hypothesis that the presence of a ribose moiety at N9 of the purine ring is essential for potent anticancer activity in cytokinins. A decrease in the cytotoxic activity of two orders of magnitude was also observed after substitution of oTR and

Table 2
Antiproliferative activity of cytokinins expressed as IC_{50} values in a 3-day Calcein-AM assay. Presented values are averages of at least three independent experiments, where individual replicate values fell within 25% of the average.

	CEM	HL60	K562	RPMI 8226	HOS	MCF7	G361	HELA	BJ
BA	>166	>166	140	>166	>166	>166	>166	>166	>166
BA7G	>166	>166			>166				
BA9G	>166	>166			>166				
BA3G	>166	>166			>166				
BAR	1.3	0.93	5.9	4.6	13.6	3.7	15.0	1.9	1.7
BARMP	1.1	0.95	3.7	5.3	14.4	5.0	>166	2.3	2.6
BARDP	2.0	1.3	4.0	4.5		8.0			
BARTP	2.3	4	3.9	4.7		11			
K	155	>166	>166	>166	>166	>166	>166	>166	>166
K9G	>166	>166			>166				
KR	1.6	0.8	10.5	7.4	5.9	4.3	21.9	4.3	2.1
KRMP	1.8	1	13.6	16.5	11.9	4.3	38.8	12.2	
iP	92	>166	150	>166	>166	>166	>166	>166	>166
iP9G	>166	>166			>166				
iPR	1.7	0.71	5.2	6.4	11.7	6.9	>166	5.5	2.5
iPRMP	2.4	1.2	4.3	5.3	5.1	2.8	>166	4.2	2.1
iPRDP	2.5	1.4	5.3	5.4		3.5			
iPRTP	3.4	2.8	6.6	7		5.2			
2MeSiPR	119	85.5	>166	>166	>166	>166	>166	>166	>166
oT	118	78	>166	>166	>166	>166	103	120	>166
oT9G	>166	>166			>166				
oTR	0.5	0.6	2.4	1.5	3.1	7.7	11.6	2.13	1.5
oTRMP	1.3	0.48	4.0	3.0	2.5	10.5	13.5	4.5	1.2
oTRDP	0.8	1.9	2.1	1.9	1.9	12.1	11.9		
oTRTP	1.6	1.6	3.5	4.3	2.7	14.4	17.2		
2MeSoTR	>166	>166	>166	130	>166	>166	155	>166	>166
mT	>166	>166	140	>166	>166	>166	>166	>166	>166
mTR	>166	24	>166	>166	>166	>166	>166	>166	3.9
mT9G	>166	>166			>166				
pT	>166	123	>166	>166	>166	>166	>166	>166	>166
pTR	>166	7.5	>166		>166	>166	>166	>166	15.3
pT9G	>166	>166			>166				
tZ	>166	>166	>166	>166	>166	>166	>166	>166	>166
tZ7G	>166	>166			>166				
tZ9G	>166	>166			>166				
tZOG	>166	>166			>166				
tZROG	27.5	26.3	120		166	>166	>166		
tZR	>166	95.9	>166	>166	>166	>166	>166	>166	
tZRMP	>166	78.6	>166		>166	>166	>166	>166	
ActZ	>166	>166			>166				
2MeStZR	>166	>166	>166	>166	>166	>166	>166	>166	>166
cZ	>166	>166	>166	>166	>166	>166	>166	>166	>166
cZR	18.8	7.9	>166	>166	>166	>166	>166	42.3	55.2
cZRMP	61	26.4	>166	>166	>166	>166	>166	>166	>166
2MeScZR	>166	>166	>166	>166	>166	>166	>166	>166	>166
DHZR	>166	>166	>166	>166	>166	>166	>166	>166	>166
DHZROG	151	>166	>166		>166	>166	>166	>166	
DHZMP	>166	>166	>166		>166	>166	>166		>166

iPR at C2 with a 2-methylthio group. This, along with other substitutions that also led to attenuation of the cytotoxic effects of cytokinin ribosides could be useful in programmes to develop drugs to treat conditions other than cancers. It will be of interest to establish whether these observed decreases in cytotoxic activity are the result of reduced affinity of adenosine kinase for C2-substituted cytokinin ribosides.

The present SAR study illustrates that the structural requirements for cytokinins to show potent cytotoxic activity against human cancer cell lines are different from those needed for their activity in plant bioassays. While the ribose moiety appears to be important for cytotoxic effects against a diverse range of cancer cells, conjugation of cytokinin bases with sugars, including ribose, serves to limit cytokinin signalling in plant cells. The hydroxyl position of the side chain of cytokinins had a marked effect on anticancer activity, in both aromatic (oTR >> *meta*-topolin riboside – mTR, *para*-topolin riboside – pTR) and isoprenoid (cZR >> tZR) cytokinins. In contrast, tZ and mT cytokinins are more active than their positional isomers (cZR, oTR, pTR) in plant bioassays (Holub et al., 1998; Letham and Palni, 1983; Spíchal et al., 2004).

Extracellular adenosine nucleotides play important roles in a diverse range of physiological processes, including cell death in mammals (reviewed by Burnstock (2007), for example). In addition, information is available on: the release of intracellular adenine nucleotides; their inter-conversion (cycling) in the blood and intercellular space by both membrane-bound and soluble enzymes; and specific signalling pathways (Yegutkin, 2008; Yegutkin et al., 2003). Given their importance we synthesized 5′-nucleotides of selected cytokinin ribosides and examined their activity in various tests, including the Calcein-AM viability assay.

Phosphorylation of purine ribosides also increases the solubility of nucleosides, which can lead to improvements in both drug formulation and bioavailability. The practicality of this approach has been demonstrated by the development of fludarabine (9-β-D-arabinosyl-2-fluoroadenine-5'-monophosphate), the active ingredient in the drug Fludara®, which has been approved for the treatment of certain haematological malignancies (Anderson and Perry, 2007).

Cytokinin riboside-5′-phosphates derived from oTR, iPR, KR, BAR and cZR markedly reduced the growth of cell lines that were sensitive to the parent compounds. In most cases the activity of

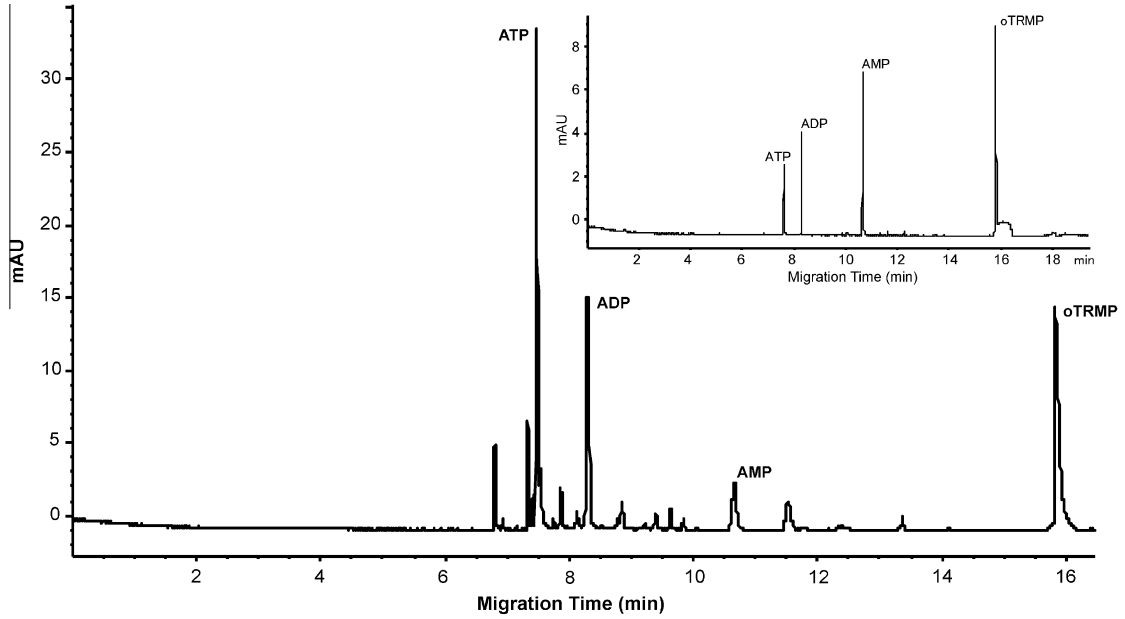


Fig. 1. Identification of intracellular oTRMP by capillary electrophoresis with UV detection (268 nm). CEM cells were treated with 25 μM oTRMP for 3 h. The inset window illustrates separation of the standards.

the ribosides and the respective riboside-5′-phosphates was either similar, or phosphorylation led to decreased activity. Simultaneous treatment with an adenosine kinase inhibitor, A-134974 (5 μM), protected CEM and K562 cells (IC_{50} > 100 μM) not only against oTR and iPR, but also against their respective mono-, di- and triphosphates. Therefore, intracellular phoshorylation appears to be an important step in the sequence of events leading to cytotoxicity after the application of not only cytokinin ribosides (Cabello et al., 2009; Mlejnek and Doležel, 2005) but also their respective riboside-5′-phosphates. These observations suggest that, similarly to other nucleotide drugs, such as fludarabine monophosphate (Malspeis et al., 1990) and triciribine monophosphate (Wotring et al., 1986), cytokinin riboside-5′-phosphates are dephosphorylated extracellularly and the resulting nucleosides are transported across the membrane and then re-phosphorylated in the cell. Intracellular accumulation of monophosphorylated oTR after the treatment of CEM cells with oTRMP (25 μM, 3 h) was demonstrated by capillary electrophoresis (see Fig. 1).

The intracellular concentration was determined to be between 0.67 and 0.92 mM (in three biological replicates), exceeding the concentration applied to cells more than 25-fold. The identity of oTRMP was confirmed by spiking the sample with a standard solution of the compound. If adenosine kinase was inhibited by A-134974 or the experiment was carried out in serum-free medium, concentrations of oTRMP were found to be below the limit of detection. The peak of oTRMP was missing when the cells were treated with DMSO vehiculum (data not shown).

In order to obtain preliminary information about the selectivity of cytokinin nucleosides and nucleotides, their cytotoxic effects against human BJ fibroblasts (as a model primary cell line) were also examined. Most of the compounds tested, including those previously used in mouse xenograft experiments (iPR, BAR, KR) and clinical trials (iPR, BAR) showed significant cytotoxicity, with IC_{50} values frequently in the low micro-molar range (Table 2). Future *in vivo* experiments will be required to demonstrate whether there is a therapeutic window for cytotoxic cytokinins. The results of the mouse hollow fibre assay described below, demonstrating the *in vivo* anticancer activity of oTR against implanted human tumours at concentrations causing no acute toxicity, indicate that this is a possibility.

Due to its drug-like properties and the promising biological activity it demonstrated, oTR was selected for further testing against the NCI_{60} cancer cell line panel at the developmental therapeutics program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (DTP, Bethesda, USA). NCI_{60} is a collection of 59 human cancer cell lines that have been extensively characterized at the DNA, RNA and protein levels and are used for routine drug screening at DTP. Comparisons of patterns of activity (GI_{50} values of a compound for individual NCI_{60} cell lines) by, for example, Pearson correlation, may be useful for identifying compounds that could have common mechanisms of action. Similarly, comparison of activity patterns and expression patterns of molecular targets provides a means to detect molecular markers that influence the cells' sensitivity to a compound (Shoemaker, 2006).

The cytotoxic activity of oTR against the NCI_{60} panel is shown in Fig. 2, in terms of GI_{50} values (concentrations causing a 50% reduction in cell growth). oTR was potently active against most of the cell lines, with a median GI_{50} value of 0.65 μM and, for all except two lines, GI_{50} values <10 μM. Similarities in the median GI_{50} values (range 0.34–1.32 μM) together with generally high degrees of variability within tissue sub-groups (max/min ratio >15 in all sub-panels except the ovarian and prostate sub-groups) suggest that factors other than the tissue origin determine sensitivity of cancer cells to oTR. Below we analyse the effects of p53 status of the cells and adenosine kinase expression on the activity of oTR and compare its activity pattern with those of 214 antineoplastics ("clinical agents") that have already been clinically evaluated.

Analysis of the influence of the mutational status of the NCI_{60} cell lines (Ikediobi et al., 2006) on their sensitivity to oTR led to the important observation that cell lines carrying the mutant p53 tumour suppressor gene (median GI_{50} = 0.60 μM) are generally more sensitive than cells with the wild type variant (median GI_{50} = 1.59 μM). TP53, the protein product of this gene, is a vital component of the regulatory system that responds to various cell stressors, including DNA damage, oncogene activation, hypoxia, and disruption of cell adhesion. Activation of TP53 can result in cell cycle arrest, senescence and apoptosis. The p53 gene is known to be either mutated or deleted in over 50% of all human cancers (Vazquez et al., 2008). Dysfunction of TP53 has also been implicated in chemo- and radio-resistance (Bossi and Sacchi, 2007;

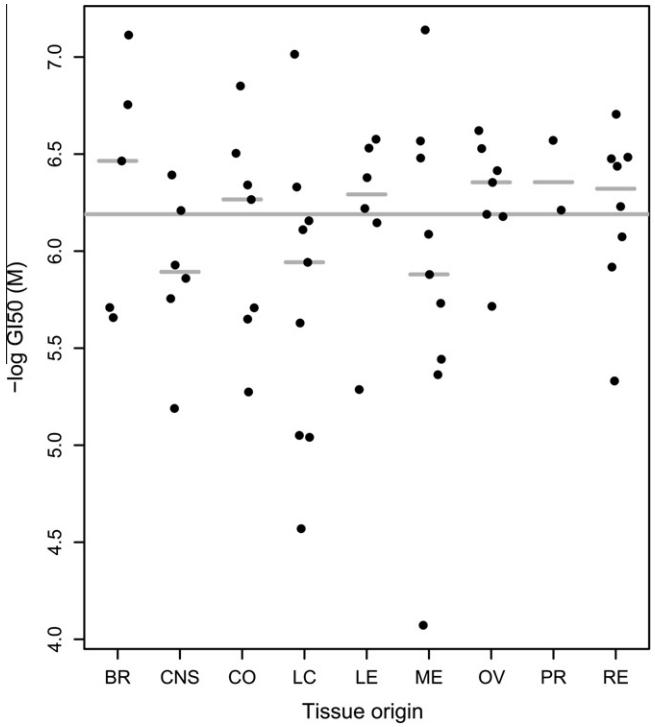


Fig. 2. Negative log GI_{50} values (M) for individual tissue types in NCI_{60}. Grey lines indicate the global and tissue-specific median. BR – breast cancer, CNS – central nervous system cancer, CO – colon cancer, LC – non-small cell lung cancer, LE – leukaemia, ME – melanoma, OV – ovarian cancer, PR – prostate cancer and RE – renal cancer.

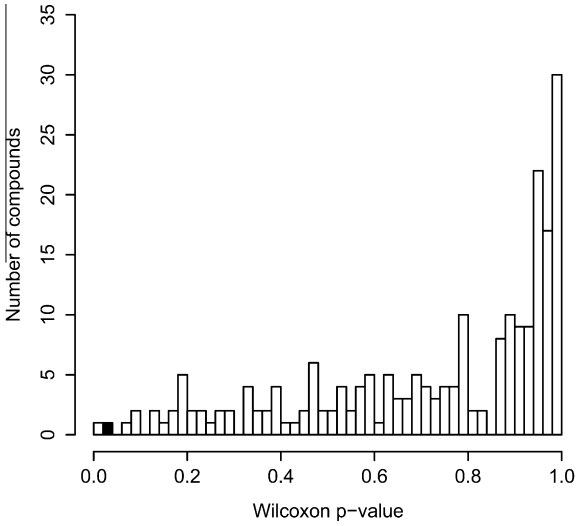


Fig. 3. Histogram showing the relationship between *p53* status and activity of oTR (black bar) and 214 clinically evaluated compounds. The strength of each relationship is expressed as a *p*-value derived from the Wilcoxon rank sum test. The alternative hypothesis was that the individual compounds are more active in cell lines with mutated p53.

Weller, 1998). The potential significance of the strong activity of oTR towards mutant p53 lines is highlighted by the report that most of nearly 90 clinically evaluated anticancer agents showed greater activity against NCI_{60} cell lines carrying wild-type p53 (Weinstein et al., 1997). We used the same approach as that applied by Weinstein et al. to compare the effect of p53 status on the growth inhibitory activity (GI_{50}) of oTR and 214 standard antineoplastics. This set of "clinical agents" was created by pooling antineoplastics from the "Approved Oncology Drugs" and "Standard Agents" DTP datasets (see Section 4 for details). *p*-Values obtained from one-sided Wilcoxon rank sum tests comparing the GI_{50} values of individual compounds on the cell lines with mutated and wild-type p53 were used as a metrics of the dependence of the activity on p53 status. The resulting distribution of *p*-values is shown in Fig. 3. While most of the compounds tested showed more activity against the cells with wild-type p53 (indicated by *p*-values > 0.5) oTR (*p*-value = 0.035, rank 2) was exceptionally active against the cells with the mutant p53 gene.

Previous studies on cytokinin ribosides have demonstrated that intracellular phosphorylation by adenosine kinase is a requirement for a cytotoxic effect (Mlejnek and Doležel, 2005) and, as mentioned above, the cytotoxic effect of oTR can be prevented by treatment with an adenosine kinase inhibitor. Therefore, it was of interest to determine whether a relationship between the activity of oTR and expression of adenosine kinase exists. The GI_{50} values of oTR were found to be negatively correlated ($r < -0.43$, $p < 0.0006$) with signals of both adenosine kinase probes on U133A Affymetrix expression arrays in the Genelogic dataset (Shankavaram et al., 2007). Significant negative correlations between the GI_{50} values of oTR and adenosine kinase expression ($r < -0.69$, $p < 0.019$) were also observed in the melanoma subgroup. Therefore, it is possible that adenosine kinase expression in cells could be used as a biomarker of the sensitivity and/or resistance of certain malignancies (for example, melanoma) to oTR. Further studies with an independent cancer cell panel are required to test this hypothesis.

In order to identify compounds that have a similar pattern of activity (and possibly, therefore, a similar mechanism of action) we calculated Pearson correlation coefficients for patterns of activity of oTR and "clinical agents". Compounds were ranked using respective *p*-values. Only an inhibitor of ribonucleotidase, caracemide (Moore and Loo, 1984) had a correlation coefficient that was higher than 0.4 ($r = 0.42$, $p = 0.0013$). Further positive correlations ($r > 0.35$, $p < 0.005$) were obtained for the purine anti-metabolites (3-deazaguanine and diglycoaldehyde), the cAMP analogue 8Cl-cAMP, and the tricyclic ribotide Akt inhibitor triciribine monophosphate (Cory et al., 1976; Kim et al., 2005; Pieper et al., 1988). None of these compounds is currently approved for use in humans. Although statistical significances (defined as $p < 0.05$) of these correlations did not survive Bonferroni correction for multiple testing, the relations reported here may have biological meaning. Correlations between the patterns of activity of oTR and the purine analogues may reflect the ability of oTR to interact with the human purinome and, more specifically, with enzymes involved in purine and nucleic acid metabolism. Triciribine monophosphate, a prodrug of triciribine, is known to be dephosphorylated extracellularly, transported into cells and then re-phosphorylated by adenosine kinase (Wotring et al., 1986). Here, we propose a similar mechanism of internalisation and metabolic activation for the cytokinin nucleotides. The correlation between the GI_{50} patterns of oTR and triciribine monophosphate might, therefore, reflect the importance of adenosine kinase in the metabolism of both drugs. We conclude that since the proportion of variance shared by the activity patterns of oTR and each individual "clinical agent" was always lower than 18% ($r^2 < 0.18$), a unique combination of biological factors is probably underlying the sensitivity of the NCI_{60} cell lines to oTR.

Finally, the activity of oTR against tumours derived from 12 NCI_{60} cell lines was tested *in vivo* in hollow fibre assays. oTR was administered by intraperitoneal injection for four consecutive days at two dose levels, 100 and 150 mg/kg/day, which were found to be safe in a preliminary acute toxicity study (data not shown). oTR caused a 50% or greater reduction (as measured by a standard MTT assay) of tumour mass in 16 out of 24 intraperitoneal implants, resulting in an ip score of 32 out of 48. No tumour

reduction exceeding threshold assay values was achieved when the drug was injected subcutaneously (sc score 0). Ip score (but not sc score) was shown to be positively correlated with the likelihood of activity in xenograft models. Notably, the relation was stronger when both intra- and extraperitoneal grafts were considered (Johnson et al., 2001; Decker et al., 2004). According to DTP methodology, compounds with an ip score greater than 7 or total (ip + sc) score greater than 19 are considered as candidates for follow-up xenograft experiments.

While the high ip score shows promising anticancer activity of oTR at the site of application, the absence of the effect on the subcutaneous implants points to the limited systemic availability of the drug. Other routes of application might be more appropriate for the relatively polar cytokinin ribosides. In this context, it would be interesting to compare the ip and sc scores of other nucleoside analogs. In humans, significant differences in peritoneal and plasma exposure were observed after intraperitoneal application of cytarabine and gemcitabine. Both the drugs were successfully used in high dose regional therapy of intra-abdominal cancers (King et al., 1984; Kamath et al., 2009). Follow-up mouse xenograft experiments (testing various sites of implantation and modes of application) are necessary to further evaluate potential utility of oTR in cancer therapy.

3. Concluding remarks

The ability of the plant hormones cytokinins to induce apoptosis and/or block cell cycling in a wide range of cancer cells makes them potential candidates as drugs for treating a variety of cancers. This study represents the first systematic analysis of the relationship between the structure of cytokinins and their cytotoxic effects, assessed using a diverse panel of human cancer cell lines. The results confirm the anticancer activity of iPR, KR and BAR, and demonstrate that the range of cell lines sensitive to cytokinins and the tissue origin of these cells is wider than previously reported. In addition, the anticancer activity of the hydroxylated aromatic (oTR, pTR, mTR) and isoprenoid cytokinins (cZR) is reported for the first time. Against most cell lines tested, *ortho*-topolin riboside (IC_{50} = 0.5–11.6 μM) was the most active cytokinin. Cytokinin nucleotides (some synthesized for the first time in this study) were active against the same cell lines as the parent ribosides. Cytokinin free bases, including 2-methylthio derivatives as well as *O*- and *N*-glucosides, exhibited limited toxicity or none at all. It can be concluded from this study that cytokinins have different structural requirements for cytotoxic activity against human cancer cell lines than for activity in plant bioassays. The potent anticancer activity of oTR was confirmed through further testing of this compound on NCI_{60} (median GI_{50} = 0.65 μM), a standard panel of 59 cell lines originating from nine different tissues. The activity pattern determined for oTR against NCI_{60} could be clearly distinguished from the patterns of a set of standard, established anticancer drugs, suggesting that a unique combination of factors underlie its activity. Another significant finding was the high sensitivity of the NCI_{60} cell lines with mutated p53 tumour suppressor gene to oTR and significant differential toxicity of oTR in tissue origin sub-panels. oTR is toxic to rapidly dividing normal diploid fibroblasts *in vitro*, but was found to have significant anticancer activity in several tumour models *in vivo* at concentrations causing no acute toxicity.

4. Experimental

4.1. Materials

The cytokinins iP, iPR, tZ, tZR, DHZ, BA, BAR, K, and KR were purchased from Sigma (St. Louis, MO). ActZ, BA3G, BA7G, BA9G, BARMP, K9G, KRMP, iP9G, iPRMP, 2MeSiPR, cZ, tZ7G, tZ9G, tZRMP, tZOG, tZROG, 2MeStZR, cZR, cZRMP, 2MeScZR, (±)DZR, DZROG and DZMP were generous gifts from Olchemim Ltd. (Olomouc, Czech Republic). The aromatic cytokinins oT, oTR, oT9G, mT, mTR, mT9G, pT, pTR and pT9G were synthesized, according to procedures described by Holub et al. (1998). Before they were used the purity of all cytokinins was tested by HPLC (Strnad et al., 1997). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and A-134974 were purchased from Sigma (MO, USA) and Calcein AM from PAA Laboratories GmbH (Pasching, Austria). All reagents used were either of analytical grade or the highest grade available from commercial suppliers.

4.2. Cytokinin nucleotide synthesis

All N^6-substituted adenosine-5′-*O*-di- and tri-phosphates used in the present study were synthesized by treating the triethylammonium salt of the corresponding 6-chloropurine-9β-D-riboside-5′-*O*-di- or triphosphate precursors with appropriate side chain amines under aqueous, alkaline conditions. The reactions were monitored by analytical chromatography using 250 × 4.6 mm stainless steel column packed with Gemini™ C18–110, 5 μm particles (Phenomenex, Aschaffenburg, Germany) as solid phase. The mobile phase was 25% CH_3CN, 25 mM Na_2HPO_4, 4 mM tributylammonium sulphate (pH 7). The flow rate was 1.25 ml/min. The products were partially purified by preparative MPLC with DEAE-Sepharose (125 × 25 mm glass column of Q Sepharose FF, 90 μm, Amersham Biosciences, Freiburg, Germany) as the stationary phase and 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. The flow rate was 10 ml/min. The fractions containing the required product were collected, concentrated under reduced pressure, and then desalted using preparative HPLC equipped with 220 × 50 mm stainless steel column packed with ODS-AQ™ C18–100, 16 μm (YMC Europe/Sinsheim/Germany) at the flow rate of 10 ml/min. The purified compounds were eluted with 5% MeOH, concentrated at reduced pressure, and stored as 10 mM aqueous stock solutions at −70 °C.

The analytical HPLC system for the monitoring of product formation progress and its purity as well as the preparative HPLC system for product purification consisted of a L7100 pump, a L7400 variable wavelength UV-detector, and a D 7500 Integrator (Merck-Hitachi, Darmstadt, Germany). Mass spectra of the reaction products by ESI-MS were measured in isopropanol–H_2O–HCOOH (50:49.9:0.1, v/v/v). Helios β spectrometer (Spectronic Unicam, Cambridge, UK) was used to record UV-spectra of the compounds in aqueous phosphate buffer, pH 7.

4.2.1. N^6-(Δ²-Isopentenyl)adenosine-5'-O-diphosphate (iPRDP)

iPRDP was synthesized from 6-chloropurine-9β-D-riboside-5'-*O*-diphosphate, triethylammonium salt, and 2-isopentenylamine by nucleophilic substitution with 2-isopentenylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. The formation of the product was monitored by analytical HPLC. The reaction was quenched by adding formic acid and subsequently cooling to −70 °C, then the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase and 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. Fractions containing product were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). The triethylammonium salt of iPRDP was isolated with >98% purity (yield: 32%). iPRDP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 268 (4.228) nm; ESI-MS (pos.) *m/z*: 494.1 ($[M+H]^+$); ESI-MS (neg.) *m/z*: 496.1 ($[M-H]^-$); Mr calculated for the free acid ($C_{15}H_{23}N_5O_{10}P_2$): 495.32.

4.2.2. N^6-(Δ^2-Isopentenyl)adenosine-5'-O-triphosphate (iPRTP)

iPRTP was synthesized from 6-chloropurine-9β-D-riboside-5'-*O*-triphosphate, triethylammonium salt, and isopentenylamine by nucleophilic substitution with 2-isopentenylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. Again, product formation was monitored by analytical HPLC. The reaction was quenched by adding formic acid and subsequent cooling to −70 °C, the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase, using 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. Again, fractions containing product were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). The triethylammonium salt of iPRTP was isolated with a purity of >98% (yield: 42%). iPRTP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 268 (4.228) nm; ESI-MS (pos.) *m*/*z*: 576.1 ([M+H]$^+$); ESI-MS (neg.) *m*/*z*: 574.2 ([M−H]$^-$); Mr calculated for the free acid ($C_{15}H_{24}N_5O_{13}P_3$): 575.30.

4.2.3. N^6-(2-Hydroxybenzyl)aminopurine riboside-5'-O-monophosphate (oTRMP)

oTRMP was prepared from 6-chloropurine-9β-D-riboside-5'-*O*-monophosphate, disodium salt dihydrate, by nucleophilic substitution with 2-hydroxybenzylamine in the presence of *N*,*N*-diisopropyl-*N*-ethylamine in methanol. The reaction was carried out at 90 °C for 12 h in a nitrogen atmosphere. The solvent was removed by evaporation under vacuum and raw *o*TRMP was purified by *RP* C18 flash chromatography (mobile phase 15% methanol in water) followed by crystallization from propan-2-ol. The purity of the final product was 95% (HPLC) and the yield 80%. oTRMP sodium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 270 (4.312) nm; ESI-MS (pos.) *m*/*z*: 454.0 ([M+H]$^+$); ESI-MS (neg.) *m*/*z*: 452.0 ([M−H]$^-$); Mr calculated for the free acid ($C_{17}H_{20}N_5O_8P$): 453.35.

4.2.4. N^6-(2-Hydroxybenzyl)aminopurine riboside-5'-O-diphosphate (oTRDP)

oTRDP was synthesized from 6-chloropurine-9β-D-riboside-5'-*O*-diphosphate, triethylammonium salt, by nucleophilic substitution with 2-hydroxybenzylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. The formation of product was monitored by analytical HPLC. The reaction was, again, quenched by addition of formic acid and subsequent cooling to −70 °C, then the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase using 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. The fractions containing product were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). The triethylammonium salt of oTRDP was isolated with a purity of >99% (yield: 38%). oTRDP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 270 (4.312) nm; ESI-MS (pos.) *m*/*z*: 534.1 ([M+H]$^+$); ESI-MS (neg.) *m*/*z*: 532.0 ([M−H]$^-$); Mr calculated for the free acid ($C_{17}H_{21}N_5O_{11}P_2$): 533.32.

4.2.5. N^6-(2-Hydroxybenzyl)aminopurine riboside-5'-O-triphosphate, (oTRTP)

oTRTP was synthesized from 6-chloropurine-9β-D-riboside-5'-*O*-triphosphate, triethylammonium salt, by nucleophilic substitution with 2-hydroxybenzylamine in the presence of sodium hydroxide (pH 11) in water at 40 °C. Analytical HPLC was used to monitor the progress of the reaction. The reaction was quenched by the addition of formic acid and subsequent cooling to −70 °C, then the product was purified by preparative MPLC with DEAE Sepharose as the stationary phase, using 300 mM triethylammonium bicarbonate (pH 8) as the mobile phase. The relevant fractions were collected, concentrated under reduced pressure, and desalted by preparative HPLC (trapping on reversed phase silica gel, washing with water then eluting with 5% MeOH). oTRTP was isolated as the triethylammonium salt with a purity of >97% (yield: 42%). oTRTP triethylammonium salt: white solid; UV–VIS (phosphate buffer pH 7.0) λ_{max} (log ε) 270 (4.312) nm; ESI-MS (pos.) *m*/*z*: 614.0 ([M+H]$^+$); ESI-MS (neg.) *m*/*z*: 612.0 ([M−H; Mr calculated for the free acid ($C_{17}H_{22}N_5O_{14}P_3$): 613.35.

4.2.6. 2-Methylthio-6-(2-hydroxybenzyl)aminopurine riboside (2MeSoTR)

2MeSoTR was prepared from 2-methylthio-6-chloropurine-9β-D-riboside and 2-hydroxybenzylamine. The reaction was carried out, at 90 °C for 20 h, in methanol in the presence of triethylamine in nitrogen gas. The reaction mixture was then evaporated in a vacuum evaporator and residue that was insoluble in 25% methanol was re-crystallized from 70% methanol. Purity of the final product was determined to be 97% (HPLC) and the calculated yield 50.5%.

2MeSoTR: white solid; ESI-MS (pos.) *m*/*z*: 420.3 ([M+H]$^+$); Mr calculated for the free acid ($C_{18}H_{21}N_5O_5S$): 419.46.

4.3. Capillary electrophoresis

CEM cells were harvested by centrifugation (500*g*, 4 °C, 5 min), washed twice in an excess of ice cold phosphate buffered saline (pH 7.2) and then flash frozen in liquid nitrogen. Cell extracts were analysed using a capillary electrophoresis system supplied by Agilent Technologies (Waldbronn, Germany) equipped with an uncoated fused silica column (80.5 cm total length, 72 cm effective length, 75 μm I.D.). Parameters for sample processing and separation were adapted from those used by Friedecký et al. (2007). Briefly, the background electrolyte consisted of 40 mM citrate, 0.8 mM cetrimonium bromide (CTAB) adjusted to pH 4.3 with γ-butyric acid (GABA). Each new capillary was washed with 1 M NaOH (30 min) followed by water (30 min) and then running buffer (30 min). At the beginning of every day, the capillary was washed with solutions in the following order: 1 M NaOH (10 min), water (10 min) and the running buffer (20 min). Between each run capillary was washed with the running buffer for 2 min. Samples were injected under low pressure (50 mbar, 5 s). ATP, ADP, AMP and oTRMP were identified by spiking with standard solutions. Quantification was done using corrected peak areas at detection wavelength 254 nm. The intracellular concentration of oTR-MP was calculated using the following formula: (concentration of the analyte in the cell extract × volume of the cell extract/number of extracted cells) × (1/average cell volume). Taking CEM cells to be spherical, with a diameter of 11.2 μm (the median value as measured by cell counter Z2, Beckman), the average cell volume was calculated to be 735.2 μm^3.

4.4. Cell cultures

The following cell lines – RPMI 8226 (multiple myeloma), CEM (T-lymphoblastic leukaemia), K562 (chronic myelogenous leukaemia), HL-60 (promyelocytic leukaemia), MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma), G361 (malignant melanoma), HOS (human osteosarcoma) and BJ (human foreskin fibroblasts) – were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in standard DMEM or RPMI medium (Sigma, MO, USA) supplemented with heat-inactivated fetal bovine serum (10%) 2 mM L-glutamine and penicillin–streptomycin (1%) under standard cell culture conditions (37 °C, 5% CO_2 in a humid environment) and sub-cultured two or three times per week using the standard trypsinization procedure.

4.5. Calcein AM cytotoxicity assay

Approximately 10,000 cells in 80 μl of medium were seeded into 96-well microtitre plates. After 12 h incubation, compounds to be tested were added in 20 μl portions. Control cultures were treated with DMSO alone. The final concentration of DMSO in the medium did not exceed 0.5%. Serial, triplicate 3-fold dilutions (six in total, highest concentration in incubations 166 μM) of each compound were tested. After 72 h incubation, Calcein AM solution (Molecular Probes) was added to a final concentration of 1 μg/ml, and the cells were incubated for a further hour. The fluorescence of free calcein was then quantified using a Fluoroscan Ascent fluorometer (Microsystems), and the percentage of surviving cells in each well was calculated by dividing the fluorescence obtained from each cell with exposed cells by the mean fluorescence obtained from control wells × 100%. Finally, IC_{50} values (the concentrations causing a 50% decrease in cellular esterase activity) were calculated for each compound from the generated dose–response curves (Kryštof et al., 2002). The IC_{50} values presented here are averages obtained from at least three independent experiments, where individual replicate values fell within 25% of the average.

4.6. NCI_{60} cytotoxicity assay

Tests of toxicity on NCI_{60}, a set of 59 human cancer cell lines derived from nine tissue types, were performed at the Developmental therapeutics program (DTP) of the National Cancer Institute (Bethesda, USA). The cytotoxicity of oTR was evaluated by measuring total cell protein using the sulforhodamine B method according to the standard DTP protocol (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html) at both time 0 and after 48 h. GI_{50} values (concentration of a drug inducing 50% reduction of growth) values were estimated from the dose response curves.

4.7. Analysis of the correlation between NCI_{60} activity and gene expression patterns

The activity pattern (GI_{50} values for individual NCI_{60} cell lines) of oTR was correlated with those of 214 antineoplastics in the "Approved Oncology Drugs" and "Standard Agents" DTP datasets and with expression patterns (gene expression measurements for individual NCI_{60} cell lines) of adenosine kinase (the probes 204119_s_at and 204120_s_at on U133A Affymetrix arrays, Shankavaram et al., 2007). If a compound had been tested over several ranges of concentration, the pattern from the greatest number of repetitions was used. Pearson correlation coefficients were calculated on a log–log scale. All calculations and manipulations of data were carried out using statistical software R. The list of "Standard Agents" and "Approved Oncology Drugs" can be accessed at http://dtp.nci.nih.gov/docs/cancer/searches/standard_agent_table.html and http://dtp.cancer.gov/branches/dscb/oncology_drugset_explanation.html, respectively. Relevant activity patterns were extracted from DTP Cancer Screening Data May 2009 Release (http://dtp.nci.nih.gov/docs/cancer/cancer_data.html). Microarray data normalized using the RMA algorithm were downloaded from the CellMiner database (Shankavaram et al., 2009).

4.8. Hollow fibre assays

The effect of two doses (100 and 150 mg/kg/day) of oTR on implanted tumours was determined in hollow fibre assays. The doses selected were derived from acute toxicity experiments that had been previously carried out (results not shown). The assays were performed and evaluated at DTP, according to a standard protocol (http://dtp.cancer.gov/branches/btb/hfa.html). The following cell lines were used: MDA-MB-231, NCI-H23, SW-620, COLO 205, LOX IMVI, OVCAR-3, NCI-H522, U251, UACC-62, MDA-MB-435, OVCAR-5 and SF-295. Each mouse was implanted with three cell lines, as both intraperitoneal (ip) implants and subcutaneous (sc) implants (six implants in total). The compound was administered intraperitoneally for 4 subsequent days. The activity against the xenografted cells was assessed by MTT assay. A 50% or greater reduction in xenograft growth was considered to be a positive result, and each positive result was given a score of two. The sum of the scores was then calculated for all implants, giving a total score, and separate scores for both intraperitoneal (ip) and subcutaneous (sc) implants for each cell line. Hence, the maximum possible score was 96 (12 cell lines × two implantation sites × two dose levels × two). A compound is categorised as active if the total score is at least 20, or the sc score is equal to or greater than eight. The DTP scoring system has been designed so that standard anticancer drugs are classified as active.

Acknowledgements

Tests on NCI60, the acute toxicity study and hollow fibre assays were performed at the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, USA).

We would like to thank Olga Hustáková and Dita Parobková for superb technical assistance. We are grateful to Sees-editing Ltd. (UK) for editing this manuscript to an excellent standard. This work was supported financially by the Czech Grant Agency (grants 206/06/1284, 301/08/1649) and the Czech Ministry of Education (grant MSM6198959216).

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