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5′-Nucleotidases: specific assays for five different enzymes in cell extracts☆

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Abstract

Several mammalian 5′-nucleotidases (5-NTs), attached to membranes or present in the cytosol or in mitochondria, remove the phosphate from ribo- and deoxyribonucleotides with different specificities for the sugar and base moieties. Some enzymes probably participate in signaling functions by producing adenosine from AMP. A more general function may be to prevent overproduction of deoxyribonucleotides. 5-NTs may affect the pharmacological activity of nucleoside analogs and also be involved in their mitochondrial toxicity. Here we describe for five cloned 5-NT specific assays that largely rely on new inhibitors for some of the enzymes. The assays can be used to quantitate each enzyme in crude cell extracts. To ascertain their validity we applied each assay to extracts from genetically modified cells that overproduce separately each of the five enzymes. The methodology should be useful in further studies of the physiological function of 5-NTs and their influence on the clinical use of nucleoside analogs.

Keywords: Nucleoside analogs; Enzyme overproduction; Substrate cycles; Signaling

5′-Nucleotidases (5-NTs) are a group of enzymes that with different specificities catalyze the dephosphorylation of commonly occurring ribo- and deoxyribonucleoside 5′-phosphates. These catabolic enzymes received little attention until recently. However, during the last few years cDNAs of several mammalian 5-NTs were cloned and their catalytic properties were described in some detail.

The physiological role of most 5-NTs is as yet poorly understood. In some cases the product of their action may be a signal substance [1,2]. In other instances, the fact that their substrates are retained inside cells whereas the products diffuse into the extracellular space suggests that the enzymes might participate in substrate cycles [3] and thus have a regulatory function, depleting cells from an excess of nucleotides [4,5].

Contributing to the recent interest in 5-NTs is the realization that the enzymes are also of medical interest. In cases of severely combined immunodeficiency caused by the genetic loss of either adenosine deaminase [6] or purine nucleoside phosphorylase [7] the specific involvement of the immune system may depend on the low level of a 5-NT in immune cells [8] leading to an accumulation of toxic levels of dATP or dGTP. Nucleoside analogs used to treat malignancies and viral diseases become therapeutically effective only after phosphorylation to the triphosphate level by a series of kinase reactions. 5-NTs counteract this activation at the monophosphate level. We proposed that the intramitochondrial 5-NT (dNT-2) [9] may be of interest in connection with the mitochondrial toxicity shown by several nucleoside analogs, e.g., azidothymidine [10]. A low susceptibility of the monophosphate of the analog for dephosphorylation by dNT-2 may contribute to the toxicity. Still another clinical connection between

☆ *Abbreviations:* 5-NT, 5′-nucleotidase; dNT-1, cytosolic 5′(3′)-deoxyribonucleotidase; dNT-2, mitochondrial 5′(3′)-deoxyribonucleotidase; cN I, cytosolic 5′-nucleotidase I (AMP-specific); cN II, cytosolic 5′-nucleotidase II (IMP/GMP specific); eNT, 5′-ectonucleotidase (membrane-bound); BPE-T(1-[2-deoxy-3,5,-*O*-(2-bromo-1-phosphono) ethylidene-β-D-*threo*-pentofuranosyl]thymine); PMcH-U ((±)-1-*trans*-(2-phosphonomethoxycyclohexyl)uracil).

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nucleoside analogs and 5-NT comes from reports that link the development of resistance to analogs to increase 5-NT activity [11].

In most of these studies the specific nature of the 5-NT was not well defined. Several of the enzymes show overlapping substrate specificities and it is difficult to distinguish their activities in tissues or cell extracts. For our own work we wished to develop a general methodology to measure specifically the various known 5-NTs in cell extracts to determine inter alia if overproduction of one or the other enzyme in some cases may be responsible for the development of resistance against nucleoside analogs. This communication describes the results of our efforts.

Experimental procedures

Cell lines

eNT overproducers were derivatives of Jurkat cells, all other cells were derivatives of 293 human embryonic kidney cells. The dNT-2 overproducing cell line was prepared for this work. The cDNA for dNT-2 (AF210652) was subcloned into an inducible vector pIND (Invitrogen) as a *Hin*dIII–*Bam*HI fragment obtained by PCR from the full-length cDNA with the forward primer 5′-CATCAAGCTTC TGGGCCATGATCCG-3′ and with 5′-CTGAGGATCCAGCCCCA CAGAGGA-3′ as reverse primer. The recombinant plasmid was transfected with calcium phosphate into cells of clone 293-2.100 [12]. A clone of ponasterone-inducible cells (dNT-2/clone 3A) was selected with 0.8 mg/ml of G418. The overproducers for dNT-1 (dNT-14) [13], dNT-2 (dNT-2/clone 3A), cN II (Hkig-2) [12], were prepared in this laboratory, the cN I-overproducer (cN I clone 5) [14] was a gift from Dr. J. Spychala, Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, USA. In the first three lines enzyme overproduction was induced with 4 μM ponasterone, the cN I line was a constitutive overproducer. The constitutive eNT-overproducer (BNT5.1) [15] was a gift from Dr. L. Thompson, Oklahoma Medical Research Foundation, Oklahoma City, USA. Controls for the induced dNT-1, dNT-2 clone3A, and Hkig-2 cells were the non-induced cells. Controls for the overproducing cN I clone 5 and BNT5.1 were the starting cells transfected with an empty vector.

Inhibitors of dNT-2 (BPE-T) or of both dNT-2 and dNT-1 (PMcH-U) were provided by Dr. J. Balzarini, Department of Virology, Katholieke Universiteit Leuven, Leuven, Belgium.

Preparation of cell extracts

All manipulations were done at close to 0 °C. Before reaching confluency, monolayer cells on typically one 10 cm dish were washed with two changes of ice-cold phosphate saline, carefully drained, and then scraped off the dish with 0.3 ml of a hypotonic buffer containing Triton-X100 (0.2% for cN I cells, 0.5% for dNT-2 cells, and 1% for remaining cells). For dNT-1 and cN II cells this buffer contained in addition 20 mM Tris–HCl, pH 7.8, 2 mM EDTA, 1 mM DTT, and 15% glycerol. dNT-2 Cells were extracted without glycerol and with addition of NaCl (final concentration of 0.2 M) before centrifugation. cN I cells were extracted as described in [14]. The cell homogenates were centrifuged at 6000*g* for 10 min and the supernatant solutions were used for enzyme assays.

Enzyme assays

An earlier publication described conditions for assays of eNT, cN II, and dNT-1 in crude cell extracts [16]. At that time cN I and dNT-2 were not known. Much of the then described considerations, in particular the choice of the radioactive substrates, were retained in the present work. Some modifications were introduced, and in particular the the new inhibitors proved to be important and made possible a better distinction between the enzymes. All incubations were in 0.02 ml at 37 °C for 30 min with two different amounts of cell extract to ascertain proportionality. Several experiments with multiple overproducing lines gave consistent results. The substrate in the assays was a radioactive nucleotide (500–1000 cpm/nmol), the product a radioactive nucleoside. Substrate and product were separated by passing the solution after incubation through a 2 ml column of AG1-X2 (Biorad) [16]. One milliunit of enzyme activity is defined as the amount of enzyme that produced 1 nmol nucleotide per minute. Specific activity is here defined as milliunits per milligram of protein, not as units per milligram protein as in earlier work.

dNT-1/dNT-2 [9,13]. The assay measured the dephosphorylation of 5 mM (^{3}H)dUMP in 0.25 mM acetate buffer, pH 5.5, 20 mM $MgCl_2$, 5 mM DTT, 30 mM KCl, and 0.2 mg/ml bovine serum albumin. The reaction was terminated by addition of 1 ml of ice-cold 50 mM acetic acid and the solution was passed through the AG1-X2 column that was further washed with 2 ml of 50 mM acetic acid. The runthrough and wash fractions were collected directly in a vial and the radioactivity was determined by liquid scintillation counting.

cN II [12,16]. The assay measured the dephosphorylation of 5 mM (^{14}C)IMP in 50 mM imidazol buffer, pH 6.5, 10 mM $MgCl_2$, 5 mM DTT, 0.5 M NaCl, and 0.2 mg/ml bovine serum albumin. Separation of labeled inosine from IMP was made as described above under dNT-1/dNT-2.

cN I [14,17]. The assay measures the dephosphorylation of 1.0 mM (^{3}H)AMP in 50 mM MOPS buffer, pH 6.9, 100 mM KCl, 3 mM ADP, 6 mM $MgCl_2$, 0.1 mM DTT, 0.05 mM β-methyleneADP, and 0.2 mg/ml bovine serum albumin. The reaction was terminated by addition of 1 ml 20 mM MOPS buffer, pH 6.9, 50 mM NaCl and the solution was applied to the AG1-X2 column. The column was washed with 9 ml of the MOPS–NaCl solution and runthrough and washings were combined. Three ml of the combined runthrough and washings was used for liquid scintillation counting.

eNT [15,16]. The assay measured the dephosphorylation of 0.5 mM (^{3}H)CMP in 50 mM Tris–HCl buffer, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and 0.2 mg/ml bovine serum albumin. The reaction was terminated by addition of 1 ml of water. The amount of cytidine formed was determined by chromatography on AG1-X2 as described for dNT-1/dNT-2, using water instead of acetic acid as eluant.

Results

General procedures

Our experiments included five 5-NTs whose cDNAs have been cloned. A sixth enzyme was not included as it is reported to be found exclusively in erythrocytes [18]. The relevant enzymes are the eNT [1,15] located on the plasma membrane, three cytosolic 5-NTs (cN I [14,17,19], cN II [20,21], and dNT-1 [13]), and the mitochondrial dNT-2 [9]. Of the three cytosolic enzymes, cN I is most abundant in skeletal and heart muscles but is also found in brain. cN I dephosphorylates AMP and is believed to participate in muscle contraction [2]. The two other cytoplasmic enzymes, cN II and dNT-1, occur ubiquitously, with cN II specifically hydrolyzing guanine- and hypoxanthine-containing nucleotides [12,21] and dNT-1 showing a preference for

deoxyribonucleotides (132). The mitochondrial dNT-2 shows a high specificity for deoxyribonucleotides of uracil and thymine [9].

Our work depended on the availability of the inhibitors BPE-T and PMcH-U [22] that made it possible to distinguish between the activity of some enzymes. A detailed description of their action on dNT-1 and dNT-2 will be given elsewhere (Chiara Rampazzo, Radek Liboska, Magda Endova, Jan Balzarini, Peter Reichard, and Vera Bianchi, to be published). A second prerequisite was the availability of cultured cell lines overproducing each of the 5 enzymes.

Assay conditions considered to be specific for each 5-NT as described under Experimental procedures were applied to extracts from each overproducing line and tested for their specificity. In all cases tests with the inhibitors were also made. If an assay is completely specific it should show an increase of 5-NT activity compared to the control cells only in the cell line that overproduces the 5-NT for which the assay was designed.

Overproduction of dNT-1 or dNT- 2

Table 1 shows the dephosphorylation of dUMP in extracts from either dNT-1 or dNT-2 overproducing cells. The dNT-1 overproducing cells showed a 20-fold increase of activity, the dNT-2 overproducer a 15-fold increase (first row). A distinction between dNT-1 and dNT-2 is made from the effects of inhibitors (next three lines). To minimize the consumption of inhibitors these experiments were made at a suboptimal concentration of dUMP. PMcH-U strongly decreased the enzyme activity in the extracts from the control and both overproducing lines. BPE-T inhibited the dNT-2 overproducer strongly and had only a very small effect on dNT-1. The results show that the major part of the activity found in the control cells was dNT-1. The assays specific for cN I and eNT showed no increase in activity in extracts from the overproducer. The cN II assay showed a small increase in the dNT-1 overproducer, but not in the dNT-2 overproducer. These results are in agreement with the known base specificities of the two enzymes [9,13].

Overproduction of cN I

In the cN I assay with AMP as substrate, the control cells were essentially inactive but the cN I overproducing cells gave a very high activity (Table 2) that depended on the presence of ADP. If this nucleotide was not present during incubation the activity decreased to between 5% and 10%. PMcH-U was a strong and BPE-T a weaker inhibitor. With dUMP as substrate, considerable

Table 1
Application of four different assays for 5-NT activity to extracts from cells overproducing dNT-1 or dNT-2

Assay condition	Substrate	Inhibitor	Specific enzyme activity		
			Control	dNT-1 Overproducer	dNT-2 Overproducer
dNT-1/dNT-2	dUMP (5 mM)	–	13	270	180
dNT-1/dNT-2	dUMP (0.2 mM)	–	3	60	67
dNT-1/dNT-2	dUMP (0.2 mM)	PMcH-U (1 mM)	1	15	5
dNT-1/dNT-2	dUMP (0.2 mM)	BPE-T (1 mM)	2	51	6
cN I	AMP (1 mM)	–	1	1	1
cN II	IMP (5 mM)	–	4	12	6
eNT	CMP (0.5 mM)	–	0.7	1	2

The assays were constructed for the determination of specific 5-NTs as described in the Experimental procedures. When indicated, inhibitors of dNT-1 and/or dNT-2 were present during incubation. All assays were done in duplicates with two different amounts of protein (0.5–10 μg). Specific enzyme activity is nmol of nucleoside formed per minute and per milligram of protein.

Table 2
Application of the four assays for 5-NT activity to cells overproducing CN I

Assay condition	Substrate	Inhibitor	Specific enzyme activity	
			Control	cN I Overproducer
cN I	AMP (1 mM)	–	2	141
cN I	AMP (0.2 mM)	–	0.1	22
cN I	AMP (0.2 mM)	PMcH-U (1 mM)	0.1	0.2
cN I	AMP (0.2 mM)	BPE-T (1 mM)	0.1	8
dNT-1/dNT-2	dUMP (5 mM)	–	22	46
dNT-1/dNT-2	dUMP (0.2 mM)	–	3	4
dNT-1/dNT-2	dUMP (0.2 mM)	PMcH-U (1 mM)	0.5	2
dNT-1/dNT-2	dUMP (0.2 mM)	BPE-T (1 mM)	3	3
cN II	IMP (5 mM)	–	5	5
eNT	CMP (0.5 mM)	–	1	19

Assays were made and results are presented as described for Table 1.

dephosphorylation was found in extracts from both controls and overproducing cells. Also in the overproducer the activity was strongly inhibited by PMcH-U (but not BPE-T) showing that dUMP served as a substrate for cN I. IMP showed no increased dephosphorylation by the extracts from the overproducer suggesting that the small amount of activity found in both control and overproducing cells was due to cN II. In contrast, CMP dephosphorylation was increased 20-fold in the overproducer demonstrating that CMP served as substrate for cN I. In conclusion, cN I cross-reacted in the assays designed for dNT-1 and eNT, but not in the cN II assay. In the discussion we will outline ways to distinguish between the three enzymes.

Overproduction of cN II

IMP dephosphorylation was increased 15-fold in extracts from the overproducing cells (Table 3) and this increase also occurred in the presence of the two inhibitors. dUMP showed good and equal activity in control and overproducing cells and in both cases the activity was strongly inhibited by PMcH-U but not by BPE-T indicating that the responsible enzyme was dNT-1. Both AMP and CMP were only poorly dephosphorylated by control and overproducing cells. Thus cN II does not dephosphorylate any of the labeled nucleotides used in the other assays.

Overproduction of eNT

eNT shows no pronounced base specificity in its choice of substrate. We used CMP for the assay of the enzyme, as CMP is a poor substrate for most other nucleotidases. Experiments were made both with the membrane and with the high speed supernatant fractions of the cell extracts. Overproduction was 7.5-fold (Table 4). eNT activity was strongly inhibited by ATP, in accordance with the known effect of this nucleotide, but not by PMcH-U or BPE-T. The high speed supernatant of both control and overproducing cells also showed activity with dUMP. This activity was, however, inhibited by PMcH-U (but not by BPE-T) demonstrating that it was caused by dNT-1. In the membrane

Table 3
Application of the four assays for 5-NT activity to cells overproducing cN II

Assay condition	Substrate	Inhibitor	Specific activity	
			Control	CN II overproducer
cN II	IMP (5 mM)	–	4	65
cN II	IMP (0.2 mM)	–	1	22
cN II	IMP (0.2 mM)	PMcH-U (1 mM)	1	22
cN II	IMP (0.2 mM)	BPE-T (1 mM)	1	21
dNT-1/dNT-2	dUMP (5 mM)	–	17	21
dNT-1/dNT-2	dUMP (0.2 mM)	–	3	2
dNT-1/dNT-2	dUMP (0.2 mM)	PMcH-U (1 mM)	1	1
dNT-1/dNT-2	dUMP (0.2 mM)	BPE-T (1 mM)	2	2
cN I	AMP (1 mM)	–	0.3	5
eNT	CMP (0.5 mM)	–	2	2

Assays were made and results are presented as described for Table 1.

Table 4
Application of the four assays for 5-NT activity to cells overproducing eNT

Assay condition	Substrate	Inhibitor	Specific enzyme activity			
			Membranes		Cytosol	
			Control	eNT Overproducer	Control	eNT Overproducer
eNT	CMP (0.5 mM)	–	2	15	0.8	1
eNT	CMP (0.5 mM)	ATP (5 mM)	0.5	2	0.3	0.4
eNT	CMP (0.5 mM)	PMcH-U (1 mM)	2	13	0.5	1
eNT	CMP (0.5 mM)	BPE-T (1 mM)	2	13	0.6	1
dNT-1/dNT-2	dUMP (5 mM)	–	7	9	23	21
dNT-1/dNT-2	dUMP (0.2 mM)	–	1	1	3	3
dNT-1/dNT-2	dUMP (0.2 mM)	PMcH-U (1 mM)	0.6	0.8	0.7	0.6
dNT-1/dNT-2	dUMP (0.2 mM)	BPE-T (1 mM)	0.7	1	3	3
cN I	AMP (1 mM)	–	1	2	1	0.7
cN II	IMP (5 mM)	–	1	2	5	5
cN II	IMP (5 mM)	ATP (5 mM)	0.7	0.8	8	7

Assays were made and the results are presented as described for Table 1.

Table 5
Interferences between assays for 5-NTs and strategies for overcoming interferences

5-NT tested	Labeled nucleotide	Interfering 5-NT	Strategy for discrimination
dNT-1/dNT-2	dUMP	cN I	ADP-dependence of cN I
cN I	AMP	cN II	PMcH-U inhibition of cN I
cN II	IMP	dNT-1/dNT-2	PMcH-U inhibition of dNT-1 ATP stimulation of cN II
eNT	CMP	cN I	PMcH-U inhibition of cN I

fraction none of the other assays showed a clear increase in activity in the eNT overproducer. Both dUMP and IMP were dephosphorylated in the cytosolic fraction due to the presence of dNT-1 and cN II. Thus eNT does not dephosphorylate any of the other labeled nucleotides under the conditions of the assays in which they are used.

Discussion

From the reported specific activities of the pure recombinant 5-NTs and the specific activity of the same enzyme measured by our assays we calculate that each enzyme, when present, constitutes between 0.001% and 0.01% of the total soluble protein in crude extracts from cultured cells. It is therefore not trivial to measure the levels of these enzymes in cell extracts. Cell extracts also catalyze competing reactions and the substrate specificities of the known 5-NTs overlap to some extent.

The presence and amount of an enzyme in a crude cell preparation can be demonstrated either from its catalytic activity or by immunological means. We have prepared both peptide and affinity-purified polyclonal antibodies against dNT-1 and dNT-2 and have available polyclonal antibodies against cN II. Western blots suffered, however, from a lack of sensitivity. We therefore decided to construct enzyme assays to quantitate the activity of the various 5-NTs. Our final methodology depends on the specific properties of each class of enzymes. The two inhibitors obtained from Dr. Balzarini not only permitted a distinction between the activities of dNT-1 and dNT-2 but also made possible a clearer distinction between them and other NTs. Molecular genetics finally provided the required validation via the application of all assays to a series of cell extracts that each contained an excess of one of the enzymes.

In the following we discuss the validity of the proposed methodology for each separate enzyme and begin with dNT-1 and dNT-2. Their combined activities were determined from the dephosphorylation of dUMP and a distinction between them could be made from the action of inhibitors (Table 1). From the inhibition by BPE-T one can estimate the amount of dNT-2. There is no cross-reaction with dUMP dephosphorylation in the cN II or eNT assays, but there was some in the cN I assay (Table 2). However, only cN I shows activity with AMP as substrate and is stimulated by ADP.

The assay for cN I activity posed some problems. The strong activation of cN I by ADP [14,19] provides a hallmark for the enzyme. In earlier described assays, ATP was used during incubation to inhibit eNT activity. However, we found that inclusion of ATP removed the radioactive AMP substrate, probably via the myokinase reaction and therefore we excluded ATP. eNT activity was instead inhibited by the β-methyleneADP present during incubation. Dephosphorylation of AMP was not catalyzed by dNT-1, dNT-2, or eNT. There was, however, a minor cross-reaction with cN II (Table 3).

The only complication in the assay for cN II comes from the limited dephosphorylation of IMP by dNT-1 (Table 1). cN I or eNT did not cross-react. A distinction between cN II and dNT-1 can be made from the stimulation of cN II by ATP and from the action of PmCH-U.

Dephosphorylation of CMP occurs with eNT and cN I, but not with dNT-1 or cN II. We have already discussed how the distinction between eNT and cN I can be made. An additional distinguishing feature is the localization of eNT to the cell membrane.

Table 5 summarizes the possible cross-reactions between the different assays and also indicates how problems arising from them can be overcome. We have used the described methodology extensively during more than one year for 5-NT assays of cultured cells and to a minor extent in preparations from mammalian organs. dNT-1 and cN II activities dominate in extracts from cultured cells. We were not able to find cN I activity, except in the transfected overproducing line, but we have not tested muscle or heart cells. Measurements of eNT activity are best done in isolated membranes. The methodology should be of use in further work aimed at the elucidation of the physiological function of 5-NTs and in experiments connected with the clinical use of nucleoside analogs.

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