# Identification of Potent and Selective Human Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase-3 (hNPP3) Inhibitors

Rabia Raza[a], Tashfeen Akhtar[b], Shahid Hameed[b], Joanna Lecka[c], Jamshed Iqbal[a,*] and Jean Sévigny[c,*]

[a]*Department of Pharmaceutical Sciences,COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan*

[b]*Department of Chemistry, Quaid-i-Azam University, Islamabad-45320, Pakistan*

[c]*Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec (pavillon CHUL), Québec, QC, Canada; and Département de Microbiologie-infectiologie et d'Immunologie, Faculté de Médecine, Université Laval, Québec, QC, Canada*

**Abstract:** NPP3 inhibitors are promising therapeutic agents due to their potential as anti-cancer, anti-metastatic and anti-neurodegenerative drugs. We have identified the first potent and selective inhibitors of human NPP3. We have also estimated the biochemical properties of the main NPP family members that can hydrolyse nucleotides using *p*-nitrophenyl-5'-thymidine monophosphate as substrate. $K_m$ values were found to be 20 ± 4 and 15 ± 6 μM for human NPP1 and NPP3, respectively. Maximum velocity ($V_{max}$) values calculated for NPP1 and NPP3 were 12 ± 2 and 5 ± 1 nmol *p*-nitrophenol released/min/mg of crude protein, respectively. A series of benzothiazole derivatives and a series of 1,3,4-oxadiazole-2-thiones was tested on hNPP1 and hNPP3. Benzothiazoles were the most potent non competitive inhibitors of hNPP3 described to date. 1,3,4-oxadiazole-2-thiones were also identified as compounds which inhibited specifically NPP3 over NPP1. The most potent compound was further characterized and found to exhibit a non competitive mechanism of inhibition.

**Keywords:** Benzothiazole Derivatives, Enzyme Inhibition, Enzyme Kinetics, Nucleotide pyrophosphatase/Phosphodiesterase, p-nitrophenyl-5'-thymidine monophosphate, 1,3,4-oxadiazole-2-thiones.

## INTRODUCTION

Nucleotide pyrophosphatases/phosphodiesterases (E-NPP; EC 3.1.4.1, EC 3.6.1.9) represent a family of ubiquitous and conserved eukaryotic enzymes. They exist as membrane glycoproteins with an active site facing the extracellular environment [1]. The family of NPPs consists of seven members, however, only NPP1-3 have the ability to hydrolyze nucleotides. NPP1, NPP2 and NPP3 have broad substrate specificity [2] and can hydrolyze nucleotides, dinucleotides, and nucleotide sugars, e.g. ATP, ADP, $NAD^+$, ADP-ribose, and diadenosine polyphosphates, resulting in the release of the respective nucleoside-5′-monophosphate derivatives plus the remaining part [3]. The enzymatic action of NPP1-3 directly results in the termination of nucleotide signaling, the salvage of nucleotides and/or the generation of new messengers like ADP or pyrophosphate [2, 4].

These enzymes are numbered according to their order of identification (cloning and characterization) [4]. NPP1 to -3 protein sequences feature a highly conserved endonuclease-like catalytic domain, and the two cysteine-rich somatomedin B-like domains that are exclusive to these three members. NPP1 and -3 are type II transmembrane glycoproteins featuring an intracellular N-terminus, a single membrane-spanning domain and an extracellular loop bearing the somatomedin B and catalytic domains [5]. The functionally active NPP2 is secreted upon maturation by proteolytic cleavage [4, 6]. NPP1-3 play a vital role in many important biological processes. Generation of $PP_i$ makes use of NPP1 in soft tissue calcification because $PP_i$ is an inhibitor of calcification [7]. NPP1 is also important in regulation of bone mineralization [8] and its loss causes hypermineralization abnormalities like osteoarthritis and ossification of the posterior longitudinal ligament of the spine [9]. As the high level of NPP1 is observed in membranes of aged rat brains and of NPP2 in the brain cortex of Alzheimer's disease patients, the inhibition of these enzymes has been suggested as a novel alternative for targeting neurodegenerative diseases [10]. NPP3 has significance as a tumor marker because its expression is associated with carcinogenesis and metastasis of cancer cells [11-13]. NPP3 also promotes the segregation and invasive properties of glial cells [14]. Thus, discovery of NPP1-3 inhibitors can be proposed as novel therapeutics for cancer, metastatic, or neurodegenerative diseases [10]. Although NPP2 is the most known NPP member, however, to our knowledge there are no functions reported so far related to the hydrolysis of nucleotides by this enzyme. Indeed this enzyme appears to have a weak activity towards nucleotides in comparison to NPP1 and NPP3, and in contrast to the latter two enzymes, it prefers lysophospholipids or choline phosphodiesters as substrates [4].

*Address correspondence to these authors at the COMSATS Institute of Information Technology, Abbottabad, Postal Code 22060, Pakistan;
Tel: +92-992-383591-96; Fax: +92-992-383441; E-mail: drjamshed@ciit.net.pk
and 2705 boulevard Laurier, local T1-49, Québec, QC, Canada G1V 4G2;
Tel: +1 418-654-2772; Fax: +1 418-654-2765;
E-mail: Jean.Sevigny@crchul.ulaval.ca

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The field of NPP inhibition is at its embryonic stage and limited work has been reported in this context. There is a therapeutic potential for NPP inhibitors in the treatment of chondrocalcinosis [15] and cancer [16, 17]. The identification of new NPP inhibitors should help define selective molecules to discriminate between the different ectonuleotidases. Potent and selective NPP inhibitors should help determine the roles of these enzymes in the control of P1 and P2 receptor signalling. They should also be helpful to explore the structure-activity relationship of NPPs.

Recently a series of quinazolin-4-piperidin-4-ethyl sulfamide has been reported as NPP1 inhibitors [18]. However, it has high affinity binding to hERG potassium channels, which results in drug-induced QT prolongation [18].

Among the standard inhibitors of ectonucleotidases like suramin and sodium azide (inhibitors of some NTPDases), [19] levamisole (alkaline phosphatase inhibitor) [20] and gadolinium chloride that is considered as the most potent inhibitor for both soluble and membrane-bound NTPDases, [21] only suramin is reported to has some inhibitory effect on NPPs [22]. Recently, Diadenosine 5′,5″-(boranate) polyphosphonate analogues are reported as selective NPP inhibitors [23]. Biscoumarin derivatives have been reported to be non competitive inhibitors of snake venom and human NPP1 enzymes, with $K_i$ and $IC_{50}$ values in the range of 50 to 1000 and 164 to >1000 μM, respectively [24]. 1,3,4-Oxadiazole-2(3H)-thiones and its analogues have also been reported as non competitive inhibitors of NPP1 with $IC_{50}$ values in the range of 66 to > 1000 μM [25, 26]. However the $K_i$ and $IC_{50}$ values of those inhibitors are quite high so there is still a need for selective and potent inhibitors of NPPs.

In the present study, we have compared the biochemical properties of NPP1 and NPP3 using *p*-nitrophenyl thymidine 5′- monophosphate (*p*-Nph-5′-TMP) as a synthetic substrate of these enzymes. Minor variations were observed for the hydrolysis of *p*-Nph-5′-TMP among NPP1 and NPP3. We have tested Benzothiazole and 1,3,4-oxadiazole-2-thione derivatives (Fig. **1**) as inhibitors of hNPP1 and hNPP3. We also identified new potent and selective inhibitors of hNPP3. To the best of our knowledge, it is a first report on potential and selective inhibitors of human NPP3.

## MATERIAL AND METHODOLOGY

### Materials

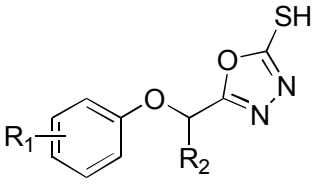
Adenosine 5′-triphosphate (ATP), adenosine 5′-monophosphate (AMP), *p*-Nitrophenyl-5′-thymidine monophosphate, calcium chloride and Tris (Trizma base) were obtained from Sigma (St. Louis, USA). DMEM/F-12, Lipofectamine, fetal bovine serum (FBS) and phenylmethanesulfonyl fluoride (PMSF) were purchased from Invitrogen (Burlington, ON, Canada).

### Cell Culture and Transfection of NPP1 and NPP3

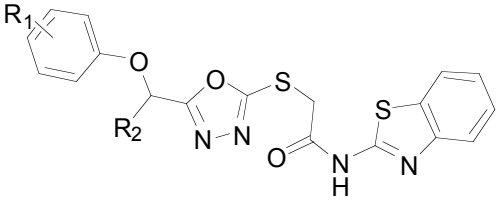
Human NPPs were produced by transiently transfecting COS-7 cells in 10-cm plates using Lipofectamine, as previously described [27] with plasmids expressing either human NPP1 [28] or NPP3 [29]. Briefly, 80–90% confluent cells were incubated for 5 h at 37°C in DMEM/F-12 in the absence of FBS with 6 μg of plasmid DNA and 24 μL of Lipofectamine reagent. Then, an equal volume of DMEM/F-12 containing 20% FBS was added to stop the reaction, and 48–72 h later cells were collected.

### Preparation of Membrane Fractions

NPP1 and NPP3-transfected cells were washed three times with Tris–saline buffer at 4°C, harvested by scraping in 95 mM NaCl, 0.1 mM PMSF, and 45 mM Tris buffer, pH 7.5, and washed twice by centrifugation at 300×g for 5 min at 4°C. Cells were resuspended in the harvesting buffer containing 10 μg/mL aprotinin and sonicated. Nuclear and




(a)




(b)

| | | | |
|---|---|---|---|
| **1a** | $R_1$= 4Br $R_2$=$C_2H_5$ | **1b** | $R_1$= 4Br $R_2$=$C_2H_5$ |
| **2a** | $R_1$= 4$CH_3$ $R_2$=$C_2H_5$ | **2b** | $R_1$= 4$CH_3$ $R_2$=$C_2H_5$ |
| **3a** | $R_1$= 3,4-diCl $R_2$=$CH_3$ | **3b** | $R_1$= 3,4di-Cl $R_2$=$CH_3$ |
| **4a** | $R_1$= 4Br $R_2$=$CH_3$ | **4b** | $R_1$= 4Br $R_2$=$CH_3$ |
| **5a** | $R_1$= 4Cl $R_2$=$C_2H_5$ | **5b** | $R_1$= 4Cl $R_2$=$C_2H_5$ |
| **6a** | $R_1$= 3,4-diCl $R_2$=$C_2H_5$ | **6b** | $R_1$= 3,4-diCl $R_2$=$C_2H_5$ |
| **7a** | $R_1$= 4Cl $R_2$=$CH_3$ | **7b** | $R_1$= 4Cl $R_2$=$CH_3$ |
| **8a** | $R_1$= 4$CH_3$ $R_2$= $CH_3$ | **8b** | $R_1$= 4$CH_3$ $R_2$= $CH_3$ |

**Fig. (1).** 1,3,4-oxadiazole-2-thiones (**a**) and benzothiazoles (**b**) investigated in this study.

cellular debris were discarded after another centrifugation step and the supernatant (crude protein extract) was aliquoted. Glycerol was added to the resulting supernatant at a final concentration of 7.5%. Samples were kept at -80°C until used. Protein concentration was estimated by the Bradford microplate assay, using bovine serum albumin as the standard of reference [30].

### Assay of Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase

Reactions for enzyme kinetics and inhibition studies were performed in a 96 well plate in a total volume of 100 µl. Assay buffer containing 1 mM $CaCl_2$, 140 mM NaCl, 50 mM Tris base, (pH 8.7) was used. For inhibition studies, various concentrations of the indicated inhibitors were preincubated with 5 µl of the membrane preparation of either hNPP1 or hNPP3 for 10 minutes at 37°C. After preincubation, the reaction was initiated with 10 µl of *p*-Nph-5′-TMP (to achieve a final concentration of 0.5 mM/well) and again it was incubated for 30 minutes at 37°C. After completion of incubation, the amount of product that was *p*-nitrophenol was measured at 405 nm using Bio-Tek Elx800 TM, Instruments, Inc. USA.

The activity with non-transfected cells was performed as a control and it was negligible in comparison with the activity obtained for transfected cells. It never exceeded 5% of the activity with transfected cells with *p*-Nph-5′-TMP as the substrate.

### Determination of Kinetic Parameters

The kinetic parameters Michaelis-Menten constants ($K_m$) and the maximum velocity ($V_{max}$) of human NPP1 and NPP3 were determined in a final assay volume of 100 µl. After addition of 5 µl of either hNPP1 or hNPP3 in enzyme assay buffer (1 mM $CaCl_2$, 140 mM NaCl, 50 mM Tris base, pH 8.7) it was preincubated for 10 minutes at 37°C. Then 10 µl of substrate (*p*-Nph-5′-TMP, final concentrations ranging from 2 to 500 µM) dissolved in enzyme assay buffer was added and again incubated for 30 minutes at the same temperature. The amount of enzymatic product (*p*-nitrophenol) was measured and data were fitted to the Michaelis-Menten equation, using the software package Graphpad prism version 5.0. The experiments were repeated three times in triplicates.

### Determination of hNPPs Inhibition

The compounds used in this study were benzothiazoles and their derivatives were synthesized according to the published protocol [31]. For inhibition studies, 10 mM stock solutions of all inhibitors were prepared in DMSO. These stock solutions of inhibitors were further diluted to obtain 1 mM solutions in enzyme assay buffer. The 1 mM solutions were further diluted in the same buffer as required for the enzyme inhibition curves. Primarily all compounds were screened at a final concentration of 1 mM. The compounds that inhibited 50% or more hydrolysis of *p*-Nph-5′-TMP, were further evaluated for their inhibitory potency by testing 6-8 different concentrations of each inhibitor spanning three orders of magnitude. Inhibitors were preincubated with enzyme for 10 minutes at 37°C, prior to addition of substrate. After addition of 10 µl of *p*-Nph-5′-TMP, it was again incubated for 30 minutes at 37°C. The Cheng–Prusoff equation was used to calculate the $K_i$ values from the $IC_{50}$ values, determined by the nonlinear curve fitting program PRISM 5.0 (GraphPad, San Diego, CA). The mechanism of inhibition of hNPP3 was determined using the enzyme inhibition assay protocol; different concentrations of *p*-Nph-5′-TMP as a substrate were used in the absence and presence of two different concentrations of inhibitor.

## RESULTS

### Determination of Michaelis–Menten Constant ($K_m$) and Maximum Velocity ($V_{max}$)

Michaelis–Menten constants ($K_m$) and maximal velocity ($V_{max}$) for NPP1 and NPP3 were determined and the results are presented in Table **1**. The hydrolysis rate increased with increasing *p*-Nph-5′-TMP concentration, with saturation at 500 µM and above. Control experiments were also performed in the absence of substrate and in the absence of enzyme to check the spontaneous hydrolysis of *p*-Nph-5′-TMP during the assay, which was less than 2%.

Different concentrations of substrate *p*-Nph-5′-TMP were used to determine $K_m$ of NPPs. Estimated $K_m$ values of 20 ± 4 and 15 ± 6 µM were obtained for NPP1 and NPP3, respectively. However, initial reaction velocities were calculated from the amount of the product (*p*-nitrophenol) formed. The $V_{max}$ values were found to be 12 ± 2 and 5 ± 1 nmol *p*-nitrophenol/min/mg of protein, for NPP1 and NPP3, respectively. For the determinations of NPP kinetics, less than 10% of *p*-Nph-5′-TMP was hydrolyzed over the course of the reaction to ensure the optimal assay conditions of initial velocity. Kinetic parameters ($K_m$ and $V_{max}$) were calculated using GraphPad Prism software.

**Table 1. Kinetic Parameters of Human NPPs**

| Enzyme | $K_m$ (µM) | $V_{max}$ (nmol/min/mg Protein) |
|---|---|---|
| hNPP1 | 20 ± 4 | 12 ± 2 |
| hNPP3 | 15 ± 6 | 5 ± 1 |

$K_m$ and $V_{max}$ values were determined with *p*-Nph-5′-TMP as a substrate concentration ranging from 2 to 500 µM by regression analysis using GraphPad Prism software. Results are expressed as the mean ± SEM of three separate experiments, each performed in triplicate.

### Influence of Synthesized Inhibitors on Activity of hNPPs

We have investigated the inhibitory effects of two series of compounds, 1,3,4-oxadiazole-2-thiones (**1a** – **8a**) and their benzothiazole derivatives (**1b** – **8b**) on human recombinant enzymes NPP1 and NPP3. Using defined enzymes we can clearly show that hNPP1 and 3 reveal a differential susceptibility to benzothiazole inhibitors. Compound **2b** was found to be the most potent inhibitor of hNPP1 and hNPP3 with $IC_{50}$ values 1.7 ± 0.3 and 1.9 ± 0.4 µM (Table **2**), respectively. On the other hand 1,3,4-oxadiazole-2-thiones (**1a** – **8a**) showed selective inhibition towards hNPP3 as all of the investigated compounds of the **a** series were weak inhibitors of hNPP1 but they showed strong inhibition on hNPP3 (except **7a** and **8a** that exhibited a nonselective

**Table 2. Effect of 1,3,4-oxadiazole-2-thiones and their Benzothiazoles Derivatives on hNPP3 and hNPP1 Activity**

| Compound Code | Formula | hNPP3 $IC_{50}$[a] (μM) ± SEM[b] | hNPP1 $IC_{50}$[a] (μM) ± SEM[b] or (% Inhibition)[c] |
|---|---|---|---|
| 1a | $C_{11}H_{11}BrN_2O_2S$ | 13 ± 4 | (15 ± 5)[c] |
| 2a | $C_{12}H_{14}N_2O_2S$ | 33 ± 2 | (8 ± 3)[c] |
| 3a | $C_{10}H_8Cl_2N_2O_2S$ | 27 ± 5 | (10 ± 3)[c] |
| 4a | $C_{10}H_9BrN_2O_2S$ | 46 ± 6 | (10 ± 2)[c] |
| 5a | $C_{11}H_{11}ClN_2O_2S$ | 35 ± 5 | (17 ± 4)[c] |
| 6a | $C_{11}H_{10}Cl_2N_2O_2S$ | 20 ± 6 | (5 ± 1)[c] |
| 7a | $C_{10}H_9ClN_2O_2S$ | 544 ± 36 | (9 ± 2)[c] |
| 8a | $C_{11}H_{12}N_2O_2S$ | 520 ± 47 | (10 ± 3)[c] |
| 1b | $C_{20}H_{17}BrN_4O_3S_2$ | 3.1 ± 6.1 | 2.6 ± 5.3 |
| 2b | $C_{21}H_{20}N_4O_3S_2$ | 1.7 ± 0.3 | 1.9 ± 0.4 |
| 3b | $C_{19}H_{14}Cl_2N_4O_3S_2$ | 3.5 ± 0.6 | 3.9 ± 0.3 |
| 4b | $C_{19}H_{15}BrN_4O_3S_2$ | 3.6 ± 0.4 | 5.5 ± 0.6 |
| 5b | $C_{20}H_{17}ClN_4O_3S_2$ | 3.4 ± 0.8 | 2.2 ± 0.4 |
| 6b | $C_{20}H_{17}Cl_2N_4O_3S_2$ | 2.1 ± 0.7 | 2.4 ± 0.5 |
| 7b | $C_{19}H_{15}ClN_4O_3S_2$ | 3.6 ± 0.6 | 5.5 ± 0.6 |
| 8b | $C_{20}H_{18}N_4O_3S_2$ | 2.9 ± 0.5 | 2 ± 0.8 |

a The $IC_{50}$ presented here is the concentration that inhibited 50% of the hydrolysis of 0.5 mM *p*-Nph-5′-TMP in 30 min.
b SEM = Standard mean error of 3 experiments.
c The % inhibition of the hydrolysis of 0.5 mM *p*-Nph-5′-TMP caused by 1 mM of the indicated compounds (**a** compounds) is given in parentheses.

inhibition pattern). Selected concentration-response curves of potent inhibitors **2a** and **2b** are shown in Fig. (**2**).

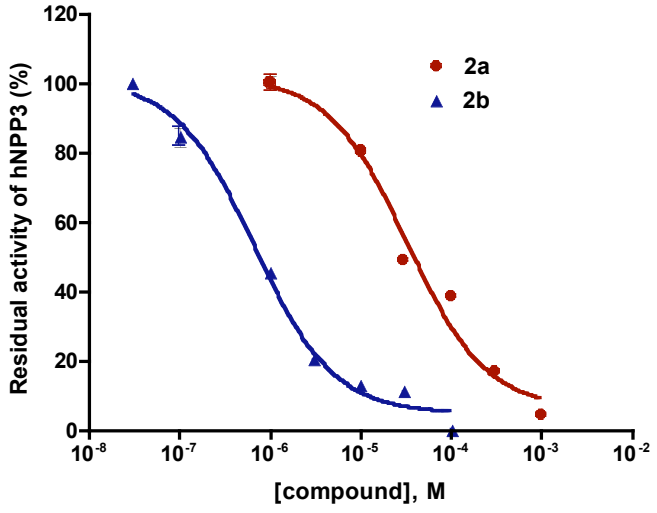



**Fig. (2).** Concentration-response curves for hNPP3 inhibitors **2a** and **2b**.

### Mechanism of Inhibition

Compound **2b**, the most potent inhibitor at hNPP3 was further investigated for the mechanism of enzyme inhibition. The kinetics of NPP3 was determined in the absence and in the presence of various concentrations of inhibitor **2b** (0, 2 and 4 μM) (data not shown). The $K_m$ value was not affected by different concentrations of inhibitor; however, the $V_{max}$ was decreased with increasing inhibitor concentration, indicating a non competitive mechanism of inhibition.

## DISCUSSION

### Determination of Kinetic Parameters $K_m$ and $V_{max}$

The kinetic parameters Michaelis–Menten constants ($K_m$) and maximal velocity ($V_{max}$) for hNPP1 and hNPP3, well defined members of NPP family were determined. The $K_m$ value of hNPP1 is slightly higher than its orthologue hNPP3. The $K_m$ values, we have obtained are in very good agreement with literature values. However, $V_{max}$ values differ from the already reported values of NPPs [2, 32, 33]. The difference is due to the different origins of enzymes and also due to the different purification levels. Furthermore, a very few studies have used defined recombinant enzymes [23, 28], whereas in most cases undefined enzymes were used to determine their catalytic properties [20, 22, 34]. The enzymes used in this study were from human while the reported enzymes were obtained from other species [2, 20, 22, 34].

### Influence of Synthesized Inhibitors on Activity of hNPPs

Benzothiazole derivatives such as, pyrimido benzothiazole, [35] quinoline derivatives, [36] imidazo benzothiazoles, [37] and polymerized benzothiazoles [38] showed potent antitumor activities. In the previous studies benzothiazoles were reported as potent antitumor and antiviral agents [31]. In this study benzothiazole bearing amido-mercapto substituted 1,3,4-oxadiazoles (**1b** – **8b**) were found to be potent inhibitors of both tested NPPs. The most potent com-

pound was 2b and its $IC_{50}$ value was 1.7 μM for hNPP1 which was several hundred folds higher than previously reported values that were 164 μM [24] and 368 μM [25] for hNPP1. All compounds of **b** series were also potent inhibitors of hNPP3 and it is a first report of hNPP3 inhibitors. The lower $IC_{50}$ value of **2b** may be attributed to the greater lipophilicity and increased electron density on the benzene ring because of the electron donating effect of methyl group. Another factor that may be responsible was that, in compounds of **a** series there was SH, which in **b** series was substituted by an alkyl group. Furthermore, the amide group in the benzothiazole group may be another contributing factor towards the enhanced activity of **b** series compounds, along with the benzothiazole nucleus. This may be augmented by a marked reduction in the $IC_{50}$ values of **7a** and **8a**.

## CONCLUSION

In conclusion, we found a novel class of potent inhibitors of hNPP1 and hNPP3. Benzothiazoles (**1b** – **8b)** were the most potent non competitive inhibitors of hNPP3 described to date. Additionally, another class of compounds (1,3,4-oxadiazole-2-thiones) was identified that inhibited specifically NPP3 over NPP1. These new potent inhibitors of NPP3 and NPP1 can be further tested as tools to explore the therapeutic potential of such molecules in functions regulated by these NPPs. More specifically NPPs inhibitors have potential as drugs to prevent the development of cancer, metastases and neurodegeneration.

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## ABBREVIATIONS

| | | |
|---|---|---|
| DMEM/F-12 | = | Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 |
| FBS | = | Fetal bovine serum |
| PMSF | = | Phenylmethanesulfonyl fluorid |
| *p*-Nph-5′-TMP | = | *p*-nitrophenyl thymidine 5′- monophosphate |
| $PP_i$ | = | Inorganic pyrophosphate |

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