Cell Death Induced by Uridine 5'-triphosphate (UTP) in Contrast to Adenosine 5'-triphosphate (ATP) in Human Epidermoid Carcinoma Cells (A-431)

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Key Words

UTP • ATP • Purines • Pyrimidines • Cell death • Apoptosis • P2 receptors

Abstract

Objective: Extracellular ATP has been reported as an important signaling molecule mediating quite divergent specific biological effects. Recent clinical trials suggest a potential role of ATP in cancer treatment. The aim of our study was to analyze the mechanisms of UTP in comparison to ATP-mediated cell death in an established cell line model (A-431). **Methods:** Cell culture and proliferation assays, separation of nucleotides by thin-layer technique, measurement of cytosolic free Ca^{2+} , flow cytometry analysis (annexin V), ultra structure, semi-quantitative RT-PCR, standard statistics. **Results:** ATP, when added as a single dose between 100 and 500 μM to A-431 cell cultures, showed increasing cytotoxicity, mainly as apoptosis, with a paradoxically decreasing effect at higher concentrations up to 1500 μM . Doses exceeding 1500 μM again led to increasing cytotoxicity. UTP at doses between 500 and 3000 μM resulted in increasing cell death following a normal sigmoid dose-response model. ATP and UTP were

degraded by membrane-bound ectoenzymes. ATP degradation products, e.g. adenosine, also induced cell death. Dipyridamole, an adenosine uptake inhibitor, was able to abolish ATP toxicity, which was also counteracted by the addition of uridine. In addition, we found functional and transcriptional evidence for P2 receptors on A-431 cells. **Conclusion:** Extracellular ATP seems to act via degradation to adenosine and consecutive induction of apoptosis. In contrast, we were not able to demonstrate analogous mechanisms for cell death mediated by extracellular UTP, but were able to provide some evidence of classical ligand-receptor action of uncleaved UTP on A-431 cells.

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Introduction

Extracellular ATP as well as other nucleotides have been reported as important signaling molecules that mediate various biological effects via cell surface receptors termed purinergic receptors or P2 receptors. A wide variety of physiological responses to ATP have

been described, including contraction of smooth muscles as well as rapid depolarization of neurons or stimulation of the inflammatory cells. Recent research also revealed UTP as an important ligand of P2Y receptors mediating a variety of physiological responses, e. g. epithelial cell mucociliary clearance or induction of hematopoietic stem cell migration. With the exception of erythrocytes, purinergic and pyrimidinergic receptors have been found in virtually every cell and tissue studied [1-3]. In line with previous preliminary data, a recent randomized clinical trial in 58 patients with advanced non-small-cell lung cancer (NSCLC) showed that intravenous ATP infusions had a favorable effect on fatigue, appetite, body weight, muscle strength, functional status and quality of life [4]. These efforts go back to early observations of ATP as a potential therapeutic agent with anticancer activities [5, 6].

P2 receptors are divided into two main classes, P2X and P2Y receptors [7]. P2X receptors are ATP-gated ion channels, which mediate rapid and selective permeability to cations [8]. Seven P2X subunits, P2X₁₋₇, define this ion channel family, of which probably three up to six subunits compose homomeric or heteromeric receptors [8], whereas recent data suggest that subunit P2X₆ does not form homomeric receptors [9]. All subunits were cloned from human tissues [8, 10]. The P2X₇ receptor is functionally unique among the P2X receptor family, as it can act as a non-selective membrane pore inducing apoptosis [11].

In contrast, P2Y receptors are purine and pyrimidine nucleotide receptors that are coupled to G proteins [2]. To date, the P2Y family is composed of eight human subtypes. P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors, all leading to stimulation of phospholipase C. The P2Y₁₁ receptor additionally mediates a stimulation of adenylate cyclase. In contrast, activation of the P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors causes an inhibition of adenylate cyclase activity. [12-16].

Magnitude and duration of purinergic and pyriminergic signaling are markedly influenced by the activity of nucleotide-metabolizing enzymes, such as NTPDase1 (CD39) and ecto-5'-nucleotidase (CD73) [17].

It has been shown in several reports that extracellular ATP has specific effects on cell proliferation as well as on cell death. As mentioned above, the P2X₇ receptor has been reported to mediate cell death by forming a non-selective pore allowing large molecules (<900 Dalton) to pass through. However, this may result in necrosis or apoptosis [2, 11, 18, 19].

Non-receptor-coupled mechanisms of ATP-mediated cell death have also been shown elsewhere [20]. In human leukemic cells (U-937), we have provided evidence that the continuous degradation of extracellular ATP to adenosine and its consecutive intracellular uptake is the mechanism leading to an intracellular pyrimidine starvation and activation of apoptosis [21].

Recent *in-vivo* data on non-melanoma skin cancer of Greig et al. showed that P2X₇ receptors are expressed in the necrotic center of nodular basal cell carcinomas and in apoptotic cells of superficial multifocal and infiltrative basal cell carcinomas. Furthermore, the P2X₇ receptor agonist benzoylbenzoyl-adenosine-5'-triphosphate and, alternatively, high concentrations of ATP caused a significant reduction in human epidermoid carcinoma (A-431) cell number *in-vitro*, whereas UTP caused a stimulation of proliferation. Therefore, these P2 receptors, e. g. P2X₇, have been discussed as potential targets for specific tumor treatment [19, 22].

The objective of our present study was (1) to analyze proliferation as well as cell death in an established cell line model (A-431) after addition of extracellular UTP, ATP and related nucleotides. (2) We aimed to identify underlying molecular mechanisms leading to cell death and to investigate the role of P2 receptor signaling.

Materials and Methods

Cell culture and proliferation assays

All chemicals were obtained from Sigma (Deisenhofen, Germany) except where otherwise stated. Human epidermoid carcinoma A-431 cells were obtained from the American Type Culture Center (ATCC number: CRL-1555; Rockville, USA) and were grown in RPMI 1640 containing 10% FCS (Boehringer, Mannheim, Germany), 2 mM glutamine and antibiotics (100 U/ml penicillin and 50 µg/ml streptomycin). Cultures were kept at 37°C in a humidified atmosphere with 5% CO₂. Cells were seeded at 6x10³/cm² and grown in 90 mm Petri dishes to confluence.

For proliferation assays, cells were seeded at 2.5x10⁴ cells per well in 24-well flat bottom plates (1.9 cm²/well) containing 2 ml medium per well. Cell counts after the addition of nucleotides or other agents were obtained by harvesting the A-431 cultures after trypsinization at designated times, usually after 24, 48, and 72 hours, using a Fuchs-Rosenthal chamber, and excluding trypan blue-stained cells. The cell counts of the controls in the proliferation assays were 2.87±0.21 (24h), 5.85±0.40 (48h), and 8.22±0.52 (72h) [x10⁵ cells per mL], respectively.

For inhibiting serum-derived enzymes, RPMI 1640 with 10% FCS was heated for 1 h at 56°C [23] and then cooled down to a temperature of 37°C for cell culture. For assessment of morphology after treatment with different agents, cells were photographed with a Leitz camera mounted on a Leitz

microscope Diavert 975800. Additionally, their ultrastructure was studied using standard methods for embedding in Epon, ultrathin sectioning and staining of sections with uranyl acetate and lead citrate [24]. All experiments were repeated at least fourfold, each performed in duplicate.

Assay of extracellular nucleotide metabolism (thin-layer technique)

In order to measure extracellular nucleotide concentration and metabolism, tritium-labeled nucleotides ($[^3\text{H}]\text{-ATP}$, $[^3\text{H}]\text{-UTP}$, 1 mCi/ml each) were incubated in 96-well plates containing 50 μL of medium with or without cells (seeded at 6×10^3 cells per well). Thin-layer chromatography was performed by a procedure as described elsewhere (Wiendl *et al.* 1998 [20]). In brief, at times indicated, 3 μL of the supernatants were taken and directly spotted onto a PEI-cellulose thin-layer sheet, thus stopping the enzymatic reactions as previously shown [25]. Marker nucleotides were spotted at each starting point, and the nucleotides were separated with water and 0.75 M LiCl at room temperature. After drying, the separated nucleotide spots were visualized under ultraviolet light and cut out. Radioactivity was measured in a liquid scintillation counter in a mixture consisting of toluene, 0.5% PPO (2,5-diphenyloxazole) and 0.03% dimethyl-POPOP [1,4-bis(4-methyl-5-phenyloxazol-2-yl)]-benzene.

Measurement of cytosolic free Ca^{2+} in intact cells

A sample containing 10^7 cells was centrifuged and incubated in 1 ml of RPMI 1640 medium supplemented with 50 mM HEPES and 2.5 mM probenecid (pH 7.4). Then, 4 μL of a Fura-2/AM solution (1.0019 mg/ml DMSO; Sigma) was added and incubated for 15 min. at 37°C in the dark. Cells were diluted 1:5 with medium supplemented as described above and incubated for another 15 min. After one washing step, cells were resuspended in 6 ml of assay medium (140 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM NaH_2PO_4 , and 5.5 mM glucose, pH 7.4; overall $[\text{Ca}^{2+}]$: 1.0 mM by calcium-free medium. "Low- Ca^{2+} " medium was diluted to a final Ca^{2+} concentration of 50 nM). For fluorescence measurement, 1 ml of cell suspension was diluted with 1 mL of assay medium. The fluorescence intensity was followed under permanent stirring with a Kontron analytical fluorimeter (SFM 25) equipped with a chart recorder. Excitation wavelength was set at 340 nm and emission wavelength at 510 nm. All measurements were carried out at room temperature. Maximum fluorescence was determined by adding Triton X-100 at a final concentration of 0.1%, minimum fluorescence by adding Tris-EGTA to a final concentration of 12 mM, pH 7.4. Intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) were calculated using the formula below (where F is fluorescence and k_d (225 nM) is the dissociation constant for Fura-2/ Ca^{2+} interaction at 37°C [26]):

$$[\text{Ca}^{2+}]_i = k_d \cdot \frac{F - F_{\min}}{F_{\max} - F}$$

Flow cytometry analysis 1: propidium iodide staining

At least 10^6 cells were harvested by trypsinization or gentle collection with a cell scraper, washed once in phosphate-

buffered saline (PBS) and resuspended in 1 ml of DNA-staining solution (0.1% sodium citrate, 0.1% (v/v) Triton X-100, 50 $\mu\text{g}/\text{ml}$ propidium iodide). After incubation for 30 min on ice, analysis was performed with FACScanTM (BD, NJ, USA).

Flow cytometry analysis 2: annexin V-FITC-propidium iodide staining

At least 10^6 cells were harvested by trypsinization, since we were able to show that gentle collection with a cell scraper produced a considerable amount of cell fragments as well as aggregates of two or more cells (Fig. 1a). The next steps followed the annexin V-FITC staining protocol (BD Pharmingen, NJ, USA). The measurements were made with FACScanTM (BD, NJ, USA).

Semi-quantitative reverse transcription-coupled PCR (RT-PCR)

Total RNA was isolated from collected cells using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). The quantity of RNA was determined photometrically at 260 nm.

5 μg of total RNA were reverse-transcribed using the SuperscriptTM II RNase H⁻ RT (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. In brief, 5 μg total RNA were mixed with 250 ng random primers, 1 μL dNTPs (10 mM), and DEPC-treated water to a total volume of 12 μL . After heating the mixture at 65°C for 5 min, 4 μL first-strand buffer (5x), 2 μL DTT (0.1 M), 1 μL RNaseOUTTM recombinant ribonuclease inhibitor (40 U/ μL), and 200 U of RT were added. The reaction mix was incubated at 42°C for 50 min. The concentration of the obtained cDNA was measured photometrically and diluted to 5 ng/ μL .

PCR reactions were performed using 10 ng cDNA as a template in 50 μL PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, 2 mM MgCl_2 , 250 μM each dNTP, 7.5 pM each specific primer, 2 U *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany) in a programmable thermocycler (GeneAmp[®] PCR System 9700, Applied Biosystems, CA, USA) using the following parameters: initial denaturation for 5 min at 94°C followed by usually 23 cycles with 45 s at 94°C , 60 s at primer-specific annealing temperature, and 90 s at 72°C , and a final incubation for 10 min at 72°C . The specific annealing temperatures were 60°C for GAPDH, 62°C for CD39, 64°C for CD73, 64°C for P2X_1 , 60°C for P2X_2 , 62°C for P2X_3 , 54°C for P2X_4 , 57°C for P2X_5 , 64°C for P2X_6 , 54°C for P2X_7 , 64°C for P2Y_1 , 67°C for P2Y_2 , 56.5°C for P2Y_4 , 56.5°C for P2Y_6 , 57°C for P2Y_{11} , and 57°C for P2Y_{12} . The following primers were used (5' $\rightarrow$ 3'; size, NIH GenBank[®] accession number): GAPDH sense CAC CAC CAT GGA GAA GGC TGG, GAPDH antisense GAA GTC AGA GGA GAC CAC CTG (553 bp, BC001601); CD39 sense GAC CCA GAA CAA AGC ATT GCC, CD39 antisense TGT AGT CCT TGC CAT AGA GGC G (629 bp, NM_001776) [27]; CD73 sense CAC CAA GGT TCA GCA GAT CCG, CD73 antisense GTT CAT CAA TGG GCG ACC GG (1007 bp, XM_015643) [27]; P2X_1 sense CTG TGA AGA CGT GTG AGA TCT TTG G, P2X_1 antisense TTG AAG AGG TGA CGG TAG TTG GTC C (465 bp, XM_008506); P2X_2 sense GCG CCA TGG CCG CCG CCC AGC CCA AGT A, P2X_2 antisense GGA AAG GAG CTC AGA GTT GAG CCA AAC C (1428 bp, AF260426)

Fig. 1. Methodology. (a) Flow cytometric analysis (DNA staining with propidium iodide) of differences between cells harvested with a cell scraper (upper panel) or by trypsinization (lower panel). The arrow indicates significant fragmentation of scraped cells. At the other end, aggregates of two or more cells are marked by an arrow head. (b-d) Evaluation of PCR conditions and data analysis. Representative PCR products are shown in the upper panels, while the lower panels summarize the data of four experiments (median $\pm$ quartiles). (b) Linear amplification for the house keeping gene GAPDH was determined between 20 and 25 cycles. (c) Linear correlation between different amounts of template cDNA with the concentration of the resulting PCR products for GAPDH using 23 cycles of amplification (d) Correlation between increasing concentrations of the GAPDH PCR product and detected DNA signals using agarose gel electrophoresis and quantification of the signals with an appropriate acquisition and analysis software.

[10]; P2X₃ sense ATC AAC CGA GTA GTT CAG C, P2X₃ antisense GAT GCA CTG GTC CCA GG (695 bp, XM_006347); P2X₄ sense GAG ATT CCA GAT GCG ACC, P2X₄ antisense GAC TTG AGG TAA GTA GTG G (296 bp, XM_006593); P2X₅ sense TCG ACT ACA AGA CCG AGA AG, P2X₅ antisense CTT GAC GTC CAT CAC ATT G (595 bp, XM_008509) [28]; P2X₆ sense CTG CAG AGG CTG CTG CAG TT, P2X₆ antisense GCC ACT CTC CAC TGG GCA C (426 bp, AF065385); P2X₇ sense AAC ATC ACT TGT ACC TTC C, P2X₇ antisense TGT GAA GTC CAT CGC AGG (671 bp, XM_012121); P2Y₁ sense CTA CAT CTT GGT ATT CAT CAT CGG, P2Y₁ antisense GAG ACT TGC TAG ACC TCT TGT CAC C (775 bp, XM_003033) [27]; P2Y₂ sense CTC TAC TTT GTC ACC ACC AGC GCG, P2Y₂ antisense TTC TGC TCC TAC AGC CGA ATG TCC (638 bp, XM_006367) [27]; P2Y₄ sense CCA CCT GGC ATT GTC AGA CAC C, P2Y₄ antisense GAG TGA CCA GGC AGG GCA CGC (425 bp, XM_010396) [29]; P2Y₆ sense CGC TTC CTC TTC TAT GCC AAC C, P2Y₆ antisense CCA TCC TGG CGG CAC AGG CGG C (365 bp, BC009391) [29]; P2Y₁₁ sense CAG CGT CAT CTT CAT CAC C, P2Y₁₁ antisense GCT ATA CGC TCT GTA GGC (274 bp, BC009877); P2Y₁₂ sense CCA GAA TCA ACA GTT ATC AGG TAA CC, P2Y₁₂ antisense GTC AGT TAA TAT TTT TAC TTAGCG CTT TGC (1145 bp, NM_022788) [30]. Oligonucleotides were obtained from Invitrogen, Karlsruhe, Germany.

Each RT-PCR experiment was performed at least three times. The identity of all PCR products was verified by DNA sequencing (ABI377 Automatic Sequencer, Perkin Elmer, Weiterstadt, Germany). Controls were treated as above without

adding template and/or reverse transcriptase and showed no PCR products. Five microliters of each PCR product were analyzed on 1.5% agarose gels, stained with ethidium bromide and photographed using a computer-assisted gel documentation system (BioDocAnalyse 1.0; Biometra, Göttingen, Germany). Intensities of PCR signals were determined with the beta 4.0.2 version of the data acquisition and analysis software from Scion Corporation (Scion Corporation, Frederick, MD, USA).

In order to reliably detect changes in gene expression levels using semi-quantitative RT-PCR techniques, diverse control experiments were performed. First, to ensure a linear correlation of the original mRNA concentration with the amount of generated PCR product, we determined the range of linear amplification for the highly expressed house keeping gene GAPDH between 6 and 40 PCR cycles under the used conditions (Fig. 1b). Assuming that gene expression levels for P2 receptors are similar or lower compared to GAPDH, subsequent PCR products were generated using 23 cycles of amplification.

In a second control experiment, different amounts of template cDNA were correlated with the concentration of the resulting GAPDH PCR products, using 23 amplification cycles. Between 0.1 ng and 10 ng, the amount of cDNA correlated in a linear way with the concentration of the generated PCR products (Fig. 1c). Thus, detected differences in the concentrations of PCR products corresponded in a linear way with differences in the original mRNA concentrations. In all subsequent PCR amplifications, 10 ng of cDNA were used.

Finally, we correlated known concentrations of the GAPDH PCR product with our ability to detect these DNA signals using agarose gel electrophoresis and quantification of the data with the acquisition and analysis software from Scion corporation (Scion Corporation, Frederick, MD, USA). Between 0 and approximately 100 ng, we observed a linear relation between DNA concentration and quantification of the photographed signal, while above 100 ng, our detection system became saturated (Fig. 1d).

Statistical analysis

To compare different variables, two-sided Mann-Whitney U-test was used where appropriate. $P < 0.05$ was considered to be significant. Non-linear regression analysis following a sigmoidal dose-response model was used to determine EC_{50} concentrations:

$$y = [\text{bottom}] + \frac{[\text{top}] - [\text{bottom}]}{1 + 10^{(\log EC_{50} - x) \cdot [\text{slope}]}}$$

For calculation and presentation, we used the GraphPad Prism™ software version 4.03.

Results

Cytotoxic effects: ATP, UTP

ATP, when added as a single dose to exponentially growing A-431 cell cultures, showed dose-dependent effects after 24, 48, and 72 hours (h). Doses between 100 and 500 μM ATP resulted in cell growth inhibition following a sigmoidal dose-response model with an EC_{50} of 123 to 152 μM (μM , 95% confidence interval, CI; 24 h: 152, 122 to 181; 48 h, 152, 131 to 173; 72 h, 123, 85 to 161). Below 250 μM ATP cell growth was retarded but still exponential (data not shown). Atypically, at concentrations exceeding 500 μM ATP, the cytotoxic effect of ATP diminished to a relative maximum at 1500 μM . Doses exceeding 1500 μM again led to increasing cytotoxicity (Fig. 2a; Tab. 1). ATP γ S (0.5, 1.5, 3.0 mM), α , β meATP (0.5, 1.5, 3.0 mM), Ap3A (0.5 mM), Ap4A (0.5 mM), and GTP (0.5, 1.5, 3.0 mM) showed similar cytotoxic effects (Tab. 1). Adenine dinucleotides (Ap3A, Ap4A) are physiological purinergic components which exhibit divergent biological effects on a variety of target cells and organs. The dinucleotides are metabolized by soluble enzymes in the blood plasma as well as by membrane-bound ectoenzymes. The enzymatic cleavage of the dinucleotides plays a dual role for their biological function: (a) termination of the signal; and (b) generation of purinergically active products such as ATP, ADP and finally adenosine. In contrast to ATP, the dinucleotides are long-lived purine nucleotides in the blood. The potential role of the dinucleotides as signaling molecules has been demonstrated in several systems. However, it needs

Fig. 2. Proliferation of A-431 cells in culture after addition of a single Dose ATP (a) or UTP (b) harvested after 24, 48, and 72 hours (h). The range of $\log EC_{50}$ (24-72 h) is shown as gray-filled area. The area of the relative maximum of the 'paradox' ATP effect is hatched gray. Additionally, the results of non-linear regression analysis following a sigmoidal dose-response model are shown (median $\pm$ quartiles of 4 experiments).

further investigation to identify the underlying mechanisms of dinucleotides in apoptosis [31].

In contrast, UTP doses between 500 and 3000 μM resulted in increasing cell growth inhibition perfectly following a sigmoid dose-response model with an EC_{50} of 1.70 to 1.86 mM (mM, 95% CI; 24 h: 1.86, 1.62 to 2.09; 48 h, 1.70, 1.59 to 1.81; 72 h, 1.75, 1.64 to 1.86) (Fig. 2b; Tab.1). Below 1.0 mM UTP, cell growth was gradually reduced but still exponential (data not shown).

Cytotoxic effects: nucleotide degradation products

Extracellular ATP is degraded by membrane-bound ectoenzymes and/or by serum-derived enzymes [17]. All the experiments unless otherwise indicated) were performed in RPMI medium supplemented with 10% of

Table 1. Detailed proliferation data (given as percent of the mean of controls, 4 experiments each). *significantly different from the control ($p < 0.05$), †significantly different from Ara-C ($p < 0.05$).

		24 h	48 h	72 h
	<i>conc.</i>	<i>median [quartiles]</i>	<i>median [quartiles]</i>	<i>median [quartiles]</i>
Control		101 [†] [94.1, 106]	97.8 [†] [96.6, 104]	99.9 [†] [98.1, 102]
Ara-C	10 μ M	20.2* [15.9, 22.0]	2.43* [2.03, 2.84]	0.86* [0.57, 1.15]
	0.5 mM	29.2* [†] [28.3, 31.1]	7.71* [†] [6.49, 9.33]	2.59* [†] [2.01, 3.16]
ATP	1.5 mM	45.7* [†] [43.9, 46.6]	15.8* [†] [14.2, 18.3]	19.5* [†] [17.8, 21.2]
	3.0 mM	19.2* [16.5, 22.9]	2.84* [2.43, 3.66]	2.18* [0.94, 3.43]
	0.5 mM	32.9* [†] [27.4, 38.4]	8.60* [†] [7.74, 10.3]	2.18* [†] [1.24, 3.12]
ADP	1.5 mM	42.9* [†] [35.6, 44.8]	14.2* [†] [10.8, 15.9]	7.16* [†] [6.54, 7.78]
	3.0 mM	35.7* [†] [31.1, 39.3]	8.42* [†] [6.88, 10.5]	5.60* [†] [4.36, 5.92]
	0.5 mM	62.1* [†] [55.7, 68.5]	52.9* [†] [46.8, 60.5]	25.5* [†] [24.3, 26.8]
AMP	1.5 mM	54.8* [†] [41.1, 69.5]	31.8* [†] [27.8, 38.6]	15.6* [†] [13.7, 17.4]
	3.0 mM	52.1* [†] [38.4, 63.1]	25.0* [†] [21.5, 27.5]	13.4* [†] [12.1, 14.6]
	0.5 mM	70.4* [†] [63.0, 75.8]	36.1* [†] [33.6, 38.7]	41.4* [†] [38.0, 44.5]
Adenosine	1.5 mM	58.5* [†] [48.4, 69.5]	25.9* [†] [20.9, 31.0]	17.4* [†] [15.9, 18.4]
	3.0 mM	52.1* [†] [40.2, 63.1]	12.5* [†] [9.46, 15.1]	0.94* [0.62, 1.56]
	0.5 mM	101 [†] [91.3, 108]	104 [†] [96.8, 109]	89.7* [†] [83.9, 94.4]
Inosine	1.5 mM	98.7 [†] [92.3, 105]	105 [†] [94.9, 114]	90.6* [†] [88.3, 94.9]
	3.0 mM	92.3 [†] [86.8, 96.8]	104 [†] [95.4, 111]	91.2* [†] [87.5, 96.8]
	0.5 mM	62.1* [†] [53.9, 66.7]	57.7* [†] [53.5, 64.2]	59.3* [†] [55.0, 63.7]
GTP	1.5 mM	60.3* [†] [53.0, 71.3]	69.1* [†] [63.4, 79.1]	66.0* [†] [63.4, 79.1]
	3.0 mM	36.6* [†] [29.3, 43.9]	3.72* [1.86, 6.05]	2.32* [1.16, 3.76]
	0.5 mM	18.3* [12.8, 23.8]	3.68* [2.30, 5.06]	0.83* [0.55, 1.11]
ATP γ S	1.5 mM	32.9* [25.6, 40.2]	6.44* [†] [5.06, 7.83]	3.05* [†] [2.50, 3.33]
	3.0 mM	11.0* [9.10, 14.6]	2.76* [1.84, 4.14]	0.55* [0.28, 0.83]
	0.5 mM	79.5* [†] [73.1, 86.8]	70.2* [†] [68.0, 73.2]	59.6* [†] [56.9, 62.4]
α , β meATP	1.5 mM	19.2* [18.3, 28.3]	10.3* [†] [8.05, 12.5]	2.78* [†] [1.94, 3.61]
	3.0 mM	22.9* [16.5, 29.3]	6.10* [†] [4.72, 8.05]	1.11* [0.83, 13.9]
Ap3A	0.5 mM	12.8* [9.15, 16.5]	1.38* [0.92, 2.30]	0.55* [0.28, 0.83]
Ap4A	0.5 mM	8.20* [5.50, 11.0]	1.38* [0.92, 1.84]	0.55* [0.28, 0.83]
	0.5 mM	94.8 [†] [90.0, 99.5]	99.1 [†] [95.8, 102]	86.8* [†] [84.9, 88.6]
UTP	1.5 mM	59.1* [†] [55.2, 62.9]	69.6* [†] [67.7, 71.4]	63.4* [†] [61.4, 66.3]
	2.5 mM	7.15* [†] [5.75, 8.55]	5.10* [†] [4.50, 5.75]	2.20* [†] [2.05, 2.40]
	0.5 mM	96.4 [†] [90.4, 104]	86.8 [†] [84.0, 96.4]	86.0* [†] [81.4, 90.1]
UDP	1.5 mM	91.8 [†] [88.6, 100]	88.6 [†] [85.9, 95.0]	82.7* [†] [76.1, 87.3]
	2.5 mM	92.7 [†] [87.7, 98.6]	83.1* [†] [77.7, 88.6]	75.4* [†] [70.3, 80.0]
	0.5 mM	94.1 [†] [87.7, 103]	87.7 [†] [81.3, 94.1]	87.5* [†] [83.9, 92.6]
UMP	1.5 mM	92.7 [†] [82.2, 100]	87.5 [†] [82.2, 95.0]	86.6 [†] [82.7, 94.3]
	2.5 mM	91.8 [†] [88.6, 99.5]	84.0* [†] [79.5, 88.6]	79.9* [†] [75.3, 85.0]
	0.5 mM	99.6 [†] [95.0, 106]	101 [†] [92.5, 106]	99.6 [†] [95.0, 106]
Uridine	1.5 mM	98.9 [†] [93.9, 106]	92.7 [†] [88.1, 97.2]	83.1* [†] [77.7, 87.7]
	2.5 mM	86.3 [†] [81.2, 93.9]	84.0* [†] [74.8, 89.5]	70.3* [†] [63.9, 80.4]

native, not heat-inactivated FCS to allow extracellular nucleotide metabolism, as *in-vivo*. All the adenine nucleotides as well as adenosine showed cytotoxicity (0.5, 1.5, 3.0 mM), which was less for adenosine than for the nucleotides (Tab.1). Inosine, the product of degradation of adenosine by adenosine deaminase, showed only slight cytotoxicity (significant only at 72 h, Tab. 1). Inhibition of serum-derived enzymes in order to inhibit extracellular

nucleotide metabolism by heating the medium prior to the addition of cells and nucleotides did not abolish the cytotoxic effects of adenine nucleotides (data not shown).

Analogous experiments with UDP, UMP and, alternatively, uridine revealed much less cytotoxicity than UTP (Tab. 1). Heat-inactivation of medium had almost no influence on the cytotoxic effects of uridine nucleotides (data not shown).

Fig. 3. Morphology of cell death in A-431 cells in culture after addition of a single Dose ATP or UTP (24 hours of incubation). (a) Controls (light microscopy, LM, original magnification, OM, x250). (b) Controls (LM, OM x400). (c) One healthy control cell (Electron microscopy, EM, OM x5,000). (d-i) Addition of ATP. There is a reduction in cell number, loss of normal intercellular contacts, budding and formation of apoptotic bodies (d, LM, OM x250; e, LM, OM x400; f, LM, OM x250). The ultrastructural analysis of representative cells shows apoptotic (g, h) as well as necrotic cells (i) confirming the LM findings. (g) Chromatin condensation and formation of cytoplasmic vacuoles (EM, OM x4,500). (h) Disintegration of the cell into apoptotic bodies, which contain parts of the cellular material (EM, OM x4,200). (i) Necrotic disintegrated cell (EM, OM x4,600). (j-m) Addition of UTP. There are analogous morphological findings as after addition of ATP. (j, LM, OM x250; k, LM, OM x400; l, EM, OM x3,600, m, EM, OM x4,600). Representative pictures of data of four experiments.

Cytotoxic effects: identification of apoptosis

Changes in cell morphology like cell shrinkage, loss of normal intercellular contacts, budding of the cell surface, and apoptotic body formation are considered as hallmarks of apoptosis [32]. These changes were observed by light microscopy after the addition of ATP and UTP at cytotoxic concentrations (Fig. 3). The observations were confirmed by ultrastructural analysis. Apoptotic signs as condensation of nuclear chromatin, occasional cytoplasmic vacuoles, disintegration of the cell into

Fig. 4. Flow cytometric analysis (staining with propidium iodine and annexin V-FITC) of cell death induced by ATP or UTP (incubation 24 hours). Ara-C served as positive control. The upper panel shows representative charts of different conditions. The lower left sector of each chart indicates viable cells, the lower right sector apoptotic, and the upper right sector necrotic and late apoptotic cells. The lower panel shows the detailed results of apoptotic (black columns) and necrotic/late apoptotic (white columns) cell counts (median $\pm$ quartiles of three experiments).

apoptotic bodies, which could contain any part of the cellular material, were detectable (Fig. 3) [24, 32].

Fig. 5. Kinetics of degradation of ATP and UTP by A-431 cells as measured by thin-layer technique. The figure shows representative examples of at least three equivalent experiments.

To confirm these morphological findings, flow cytometric analysis with propidium iodide (PI) and annexin V staining was performed to detect apoptosis by targeting for the loss of phospholipid asymmetry of the plasma membrane [33]. Cytosine arabinoside (Ara-C), a known apoptosis-inducing antimetabolite, was used as positive control [34]. After 24 hours of incubation there was a clear increase in annexin V-positive and PI-negative cell

fractions, as an early sign of apoptosis. With increasing dosage of ATP (UTP), the annexin V- and PI-positive fraction also increased, suggesting late apoptosis and additional occurrence of necrosis (Fig. 4). The earliest detectable shift from annexin V-negative and PI-negative cell fractions to an annexin V-positive cell fraction occurred after 6 hours of incubation (ATP 0.5; UTP 2.0 mM).

Fig. 6. Proliferation of A-431 cells in culture after addition of a single Dose ATP (a) or UTP (b) with and without addition of the uptake inhibitors dipyridamole (adenosine) and verapamil (uridine) or addition of adenosine and uridine, respectively (median $\pm$ quartiles of 4 experiments, asterisks indicate significance, $p < 0.05$).

Mechanisms: role of extracellular nucleotide metabolism

ATP is metabolized to ADP, AMP and finally to adenosine, whereas UTP is degraded to UDP, UMP and finally uridine by membrane-bound and soluble nucleotidases [17]. Thus, extracellular ATP (UTP), 1.0 μ M, was mainly metabolized to AMP (UMP) in cell-free RPMI medium containing FCS (10%), but not degraded in RPMI medium alone. By adding heat-inactivated FCS, ATP was not degraded, and UTP was only partially degraded to UDP, but not to UMP or uridine (Fig. 5, upper panel).

Fig. 7. Responses of cytosolic free Ca^{2+} after application of a single dose of ATP or UTP with or without receptor antagonists (PPADS, suramin). The upper panel shows the results of four experiments (median median $\pm$ quartiles, asterisks indicate significance, $p < 0.05$). The lower panel shows representative response curves (for comparability of the different conditions, the graphs are computer-assisted adapted to the given scale).

When cells were incubated with labeled ATP (UTP), 100 μ M, in medium without serum, there was no accumulation of AMP (UMP) in the medium. This indicates that cell-bound ecto-nucleotidases were responsible for the further degradation to adenosine

Fig. 8. RT-PCR analysis of P2 receptors, CD39 and CD73. The figure shows the mRNA expression profiles of three experiments (median $\pm$ quartiles). The results are given as relative expression against GAPDH (=1).

(uridine), which obviously is taken up by the cells, since the sum of radioactive products in the cell supernatants was greatly diminished (Fig. 5, lower panel).

As ATP showed a biphasic cytotoxic effect on growing cells with a relative maximum of cell growth at a concentration of 1.5 mM, the ATP metabolism was followed at different initial ATP concentrations (0.5, 1.5, and 3.0 mM). At 1.5 mM ATP, an accumulation of adenosine in the medium was measured. It seemed to be taken up rather slowly as compared to the samples with ATP at 0.5 mM and 3.0 mM, respectively (data not shown).

In addition, an expression profile of the nucleotidase NTPDase1 (CD39; $\text{NTP} \rightarrow \text{NMP} + 2 \text{P}_i$, $\text{NDP} \rightarrow \text{NMP} + \text{P}_i$) and the ecto-5'-nucleotidase (CD73; $\text{NMP} \rightarrow \text{nucleoside} + \text{P}_i$) was performed by semi-quantitative RT-PCR. Both enzymes are expressed (relative to GAPDH; CD39, 74%, CD73, 85.5%; Fig. 8).

Mechanisms: effects of cellular uptake inhibitors for adenosine and uridine

The addition of dipyridamole, an inhibitor of the adenosine transmembrane carrier [35, 36] prior to treatment with ATP (0.5 mM, cell number given as % of control after 72 h: 2.59%), and adenosine (0.5mM, 41.4%), respectively, significantly prevented cytotoxicity, i. e. antagonized these effects (+10 μM dipyridamole: ATP 80.0%, adenosine 82.3%, all for $p < 0.05$; Fig. 6). Dipyridamole alone had a mild growth inhibitory effect itself (5 μM 80.9%, 10 μM : 79.4%).

Since adenosine cytotoxicity can be mediated by intracellular nucleotide imbalance and consecutive pyrimidine starvation, which can be overcome by adding extracellular uridine to the medium as a pyrimidine source [23, 37, 38], we added uridine as a single dose (100 μM) prior to adding ATP and adenosine, respectively.

This significantly antagonized the cytotoxicity of ATP and adenosine, respectively (ATP 67.3%, adenosine 87.0%, all for $p < 0.05$; Fig. 6). By adding 1000 μM of uridine prior to 0.5 mM ATP, cytotoxicity was completely prevented (99.1%, $p < 0.05$; Fig. 6).

Analogously, verapamil as a cellular uptake inhibitor for uridine [39] was added simultaneously with UTP (2.0 mM, 29.0%). However, this showed a significant gain of cytotoxicity (+40 μM verapamil: UTP 7.77%, $p < 0.05$) as well as the addition of adenosine (+1000 μM adenosine: UTP 12.5%, $p < 0.05$; Fig. 6). Verapamil alone showed only mild growth-inhibitory effects itself (20 μM 89.9%, 40 μM : 86.1%).

However, the addition of verapamil abolished the relieving effect of uridine significantly in cultures treated with ATP (Fig. 6). This confirms the role of verapamil as cellular uptake inhibitor of uridine.

Mechanisms: involvement of nucleotide receptors (P2X and P2Y)

P2X receptors are ATP-gated ion channels, which mediate rapid and selective permeability to cations (Na^+ , K^+ and Ca^{2+}) [2, 40], whereas P2Y receptors are coupled to G proteins. Most P2Y receptors then act via G activating phospholipase-C leading to the formation of IP₃, and mobilization of intracellular Ca^{2+} . Coupling to adenylate cyclase by some P2Y receptors, however, has also been described [2].

Therefore, in a first series of experiments, cytosolic calcium concentrations ($[\text{Ca}^{2+}]_i$) in response to ATP and UTP were measured by the fura-2 method. ATP and UTP showed significant bursts of $[\text{Ca}^{2+}]_i$ followed by a slower decrease remaining on an elevated level (plateau). Using low- Ca^{2+} medium (50 nM), this plateau soon returned to the zero line (Fig. 7). PPADS (Tocris, Bristol, UK), a specific antagonist at P2X receptors [41, 42], was added

to the cells prior to applying ATP or UTP. This resulted in a significant decrease in the initial $[Ca^{2+}]_i$ burst as well as a modification of shape of the following $[Ca^{2+}]_i$ decrease (Fig. 7). Similar results were obtained for the P2Y receptor antagonist suramin [2]. Thus, functional evidence of the presence of ATP and UTP receptors in A-431 cells was provided.

Secondly, an expression profile of P2X (subtypes 1-7) and P2Y (subtypes 1, 2, 4, 6, 11, 12) receptors on the mRNA-level was performed by semi-quantitative RT-PCR. There was a detectable expression of P2X₁ (relative to GAPDH: 98.9%), P2X₃ (22.5%), P2X₄ (93.9%), P2X₅ (96.9%), P2X₆ (25.5%), P2X₇ (88.9%), P2Y₁ (94.4%), P2Y₂ (96.9%), P2Y₄ (98.5%), P2Y₆ (98.8%), and P2Y₁₁ (10.0%) (Fig. 8), but not for P2X₂ and P2Y₁₂ (=P2Y_{ADP}; positive controls with genomic DNA, data not shown).

Thirdly, the specific antagonists PPADS (250 μ M) and suramin (40 μ M) were given to the cells in culture prior to adding ATP (0.5 mM), and UTP (2.0 mM), respectively. However, no significant reduction in cytotoxicity was observed. Receptor-specific antagonists at P2X₁ (PPNDS, 20 μ M [43]; Tocris, Bristol, UK) and P2X₇ (KN-62, 25 μ M [44]; Tocris, Bristol, UK) also showed no protection from cell death.

Discussion

The objective of our study was to investigate the molecular and biochemical mechanisms underlying ATP-mediated and UTP-mediated cytotoxicity in the established human epidermoid carcinoma cell line A-431. We provide evidence that cytotoxicity mediated by extracellular ATP was mainly mediated by its metabolite adenosine, whereas UTP acts differently, probably as an uncleaved ligand via P2 receptors.

Data on U-937 cells demonstrate that in heat-inactivated medium, where rapid degradation of ATP via serum nucleotidases was slowed down, the cytotoxic ability of ATP was completely abolished [21]. In our cell model, however, the metabolism of ATP yielding adenosine by membrane-bound ecto-nucleotidases seems predominant. In fact, ATP as well as its degradation products mediated cytotoxicity. However, ATP was more potent than ADP, AMP, and adenosine. This might be due to additional receptor-based signaling. In addition, a constant 'flow' of adenosine by gradient degradation might have a stronger effect, than a single dose of adenosine.

Since the addition of dipyrindamole as an inhibitor of the nucleoside transmembrane carrier strongly prevented

cell growth retardation, the uptake of adenosine into the cells seemed to be essential for triggering cell death. These findings are consistent with previous studies on 3T6 transformed mouse fibroblasts and chick embryonic sympathetic neurons [23, 45] where this mechanism for adenosine-mediated cell death has been described.

The addition of uridine abolished adenosine cytotoxicity in A-431 cells indicating that the cells died as a consequence of pyrimidine starvation. This mechanism has been discussed to be responsible for the lymphocyte death in patients with ADA deficiency [46]. Very similar mechanisms have also been reported for human leukemic cells (U-937) [21].

To further characterize cytotoxicity in A-431 cells induced by ATP, we analyzed cells by flow cytometry using an annexin V-propidium iodine assay. The results confirmed our morphological observations that mainly apoptosis was responsible for the cytotoxic effects of ATP.

Testing a broad range of ATP concentrations for the inhibition of cell growth and monitoring the extracellular metabolism of nucleotide degradation, we observed that a putative "forward inhibition" of extracellular ATP metabolism at higher ATP concentrations led to a reduced generation of adenosine. This inhibition most likely accounts for the unusual biphasic dose-response kinetic in A-431 cells. In YAC-1 cells, a reduced ^{51}Cr release used as a marker for ATP-triggered lysis was observed at rising ATP concentrations [47]. Since Ca^{2+} supplementation was able to overcome this effect, the availability of extracellular Ca^{2+} seemed to be a limiting factor for the cytotoxic ATP effect. In U-937 cells, where cytotoxicity of ATP is mediated by adenosine generation and its intracellular uptake, supplementation of the cell culture medium with Ca^{2+} did not enhance cytotoxicity at higher ATP concentrations, but abolished cytotoxicity at all tested ATP concentrations, most likely due to chelation of ATP in the culture supernatant [21]. Another explanation for the observed biphasic dose response of A-431 cells might be the blockade of a nucleoside transmembrane carrier by adenine at higher concentrations. This degradation product of adenosine has been shown to block a low affinity subtype of the nucleoside transmembrane carrier in smooth muscle [34]. Another example of an ATP effect with a biphasic dose-response phenomenon has been reported for its transient contractile effect on rat airways. ATP acts directly on airway myocytes. Opening of P2X receptors triggers external Na^+ entry that depolarizes the plasma membrane and activates L-type voltage-operated Ca^{2+} channels.

In addition to these mechanisms, ATP also acts on P2Y receptors and induces Ca^{2+} release from intracellular Ca^{2+} stores. Thus, the transient effect of ATP is not due to ATP degradation but can be attributed, at least partially, to purinergic receptor desensitization [48]. Such an explanation of observed biphasic cytotoxicity of ATP on A-431 cells may be ruled out, since, as mentioned above, adenosine itself most likely is the metabolite responsible for ATP toxicity. This means that ATP and adenosine might act concertedly on A-431 cells. This biphasic dose response to cytotoxic ATP treatment is a highly cell-specific phenomenon, which most likely is related to a cell-specific distribution and activity of ectoenzymes for the degradation of ATP. In contrast to A-431 cells, U-937 cells, for example, cannot generate enough adenosine from ATP in a heat-inactivated medium [21].

Moreover, this specific dose-response curve might also reflect the involvement of different receptor types at distinct concentrations of ATP exhibiting different effects on apoptosis signaling. We have investigated the potential role of the P2 receptor family in ATP-mediated cytotoxicity. Functionally, we were able to show that there are ATP-coupled receptors mediating significant intracellular Ca^{2+} responses, which could be antagonized in part by receptor-specific antagonists (PPADS, suramin). In contrast, the cytotoxic effects of ATP and UTP in cultured A-431 cells could not be abolished by PPADS [41, 42], suramin [2], PPADS (P2X₁-specific [43]) or KN-62 (P2X₇-specific [44]), but were inducible by the agonists ATP γ S, α,β meATP, Ap3A, and Ap4A. To date, among the P2 receptors, only the role of the P2X₇ receptor as mediating purinergic cytotoxicity by inducing apoptosis and/or necrosis has been well established [11, 18, 49-51]. In this study, we determined the mRNA expression levels of most cloned P2X and P2Y receptor subtypes. The indirect, semi-quantitative RT-PCR method may be used with sufficient accuracy as shown by direct quantification of specific mRNA by dot-blot hybridization [52]. We found significant expression levels of P2X₁, P2X₃, P2X₄, P2X₅, P2X₆, P2X₇, P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁. This is in agreement with recent data from Greig et al., showing the expression of P2X₅, P2X₇, P2Y₁, and P2Y₂ by immunostaining [22].

The investigation of UTP-mediated toxicity showed several differences with respect to ATP. Extracellular UTP was also efficiently degraded by membrane-bound ecto-nucleotidases in our cell model. In contrast to ATP, however, UTP-degradation products like UDP, UMP as

well as uridine were almost ineffective in exhibiting cell toxicity, suggesting a major role of uncleaved UTP. In line with these results, addition of verapamil as a putative uridine-uptake inhibitor [39] did not show any relief on UTP-mediated cytotoxicity. In addition, we found a sigmoid dose-response for different UTP concentrations, which underscores a classical ligand-receptor action of UTP. In contrast, recently published data reported on an increase in the A-431 cell number at a concentration of 1 mM UTP. Other concentrations of UTP have not been investigated) [22].

Furthermore, we provided evidence of the expression of UTP-sensitive P2 receptors on A-431 cells. The cytotoxic effects of UTP are mainly due to apoptosis induction, similarly to the effects observed with ATP.

In conclusion, several potential mechanisms of purinergic cell death in A-431 cells are assumed. Extracellular ATP seems to act via degradation to adenosine, which, after its cellular uptake, leads to pyrimidine starvation and consecutive induction of apoptosis. However, our data do not exclude some involvement of the P2 receptors. ATP analogs, which are not degraded by nucleotidases, may act via other pathways, probably solely via P2 receptors. E. g., the non-hydrolysable ATP analogue ATP γ S was extremely potent in causing growth retardation and cell death what is likely that some additional direct ATP effect is involved in ATP-induced cell death, probably via P2X₅ [19]. In contrast, we found no evidence of analogous mechanisms for cell death mediated by extracellular UTP, but some evidence of ligand-receptor action on A-431 cells.

Considering the potential application of ATP as an antitumoral agent, millimolar concentrations might be achieved *in-vivo* by intrathecal injection of the nucleotide [5, 6, 53-55]. Such concentrations may be harmful to non-transformed cells while, at the same time, certain tumor cells, e. g. tumor cells complying with A-431, would escape cytotoxicity due to the described biphasic effect. Our results prompt the notion that the degradation to adenosine and its intracellular uptake should be considered as an important mechanism triggering 'ATP effects'. On the other hand, recent randomized clinical trials have reported that ATP infusions contribute to maintenance of body weight, muscle strength, and quality of life but do not affect tumor response nor increase overall survival in patients suffering from non-small cell lung cancer (NSCLC, stage IIIB or IV) [4, 56].

Abbreviations

α,β meATP (α,β -methylene ATP); ADP (adenosine 5'-diphosphate); AMP (adenosine 5'-monophosphate); Ara-C (cytarabine); Ap3A (diadenosine 5'-triphosphates); Ap4A (diadenosine 5'-tetraphosphates); ATP (adenosine 5'-triphosphate); ATP γ S (adenosine 5'-[γ -thio]triphosphate); CD39 (NTPDase1); CD73 (ecto-5'-nucleotidase); FCS (fetal calf serum); GAPDH (glyceraldehyde 3-phosphate dehydrogenase); GTP (guanosine 5'-triphosphate); PPADS (pyridoxal-

phosphate-6-azophenyl-2',4'-disulfonate); RT-PCR (reverse transcription-coupled PCR); UDP (uridine 5'-diphosphate); UMP (uridine 5'-monophosphate); UTP (uridine 5'-triphosphate).

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