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Experimental Cancer Therapy in Mice by Adenine Nucleotides

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Abstract—*Adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP), injected intraperitoneally into tumor-bearing (s.c. implanted footpad tumors) mice, exhibited significant anticancer activity. Daily treatments (for 10 days) inhibited the growth of the fast-growing, aggressive CT 26 colon adenocarcinoma in $CB6F_1$ mice. The growth-inhibitory activity of adenine nucleotides was also observed against a human pancreatic adenocarcinoma, CAPAN-1, xenografts in athymic nude mice. With low tumor burdens some 'cures' were obtained in both model systems. No inherent toxicity, as determined by changes in host weight, were observed during and after the period of treatment. Intraperitoneal injections of 1 ml of 50 mM AMP, ADP or ATP in saline, yielded elevated blood and plasma levels of ATP which lasted for several hours in both strains of mice. The growth-inhibitory activities of adenine nucleotides against tumor cells in vitro, have previously been demonstrated.*

INTRODUCTION

Low levels of adenine nucleotides were recently shown to inhibit the growth of several well-characterized human tumor cell lines in monolayer and soft-agar cultures [1, 2]. Exogenous ADP or ATP yielded accumulation and arrest of tumor cells in the S phase of their cycle followed by cell death [1]. Untransformed cells were not affected by this treatment [1]. The mechanism of the growth-inhibitory activities of adenine nucleotides was proposed to involve the disruption of the balance of cellular acid-soluble nucleotide pools, resulting in the inhibition of enzymatic activities which are essential to DNA replication and cellular growth [3, 4]. The effects of external ATP on the membrane permeability of transformed cells in culture have been studied extensively [5, 6]. Increases in plasma membrane permeability are produced by extracellular ATP in an alkaline medium, low in divalent cations, and are observed in transformed cells [5, 6]. Untransformed cells are generally not affected with the exception of cells possessing secretory functions [6, 7].

Ecto-enzymatic activities that catalyze the degradation of adenine nucleotides are responsible for the rapid clearance of adenine nucleotides from the vascular beds [8]. Nevertheless, the presence of submicromolar (0.1–0.5 μm) levels of ATP in human plasma has been established [9, 10] and extracellular ATP levels which are just one order of magnitude higher (1–5 μM), effectively inhibit the *in vitro* growth of human tumor cells [2].

This report indicates that adenine nucleotides may have a potential significance in cancer therapy. Treatment of a colonic murine tumor and a human pancreatic tumor xenograft grown in mice with adenine nucleotides resulted in inhibition of tumor growth with no apparent long term toxicity to the host. It is demonstrated that i.p. injections of mice with AMP, ADP or ATP result in increases in blood (total cellular) and plasma (extracellular) pools of ATP. It is important to note that the physiological (extracellular) effects of ATP on biological functions such as neurotransmission [11], cardiovascular functions [12], muscle contraction [13], platelet aggregation [14] or vascular tone [15] have been documented. The possibility thus exists that the anticancer properties of adenine nucleotides in live animals may be mediated through their effects on host functions in addition to their direct growth-inhibitory activities on tumor cells [1, 2].

MATERIALS AND METHODS

Materials

Adenine nucleotides (as sodium salts) were purchased from Sigma Chemical Co., St. Louis, MO. Adenine nucleotides obtained from other commercial sources were as effective. Commercial AMP,

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ADP and ATP which were further purified on DEAE cellulose columns [16] did not exhibit any increased or decreased activities as compared with the commercial (highest purity) preparations. The purity of adenine nucleotides was determined by high pressure liquid chromatography (HPLC) on ion exchange columns [4]. All solutions were more than 95% pure. Stock solutions of nucleotides in normal saline (500 mM) were adjusted to pH 5.5 with 10 N sodium hydroxide (only stock solutions of ADP and ATP needed to have their pH adjusted to 5.5). Sterile solutions of adenine nucleotides in saline (50 mM), or control saline solutions, were kept frozen until used for injections. Cells were cultured in MEM supplemented with antibiotics (100 units of penicillin per ml and 100 μg streptomycin per ml), L-glutamine (2 mM), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM) and 10% fetal bovine serum.

Cell lines

The two tumor lines used in this study were CT26, a mouse undifferentiated, aggressive colon carcinoma [17] and CAPAN-1, a well-differentiated human pancreatic adenocarcinoma [1, 2, 18]. Cells were shown to be free of *Mycoplasma* contamination. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Animals

Females and males $CB6F_1$ (a standard F_1 hybrid of BALB/c and C57BL/6) were obtained from Jackson Laboratories, Bar Harbor, Maine. Athymic outbred NCr *nu/nu* mice (males and females) were purchased from Mr. Robert Sedlacek, Department of Radiation Medicine, Massachusetts General Hospital, Boston, MA 02114. All the mice were kept at our facility for at least 2 weeks before inoculation of the tumors and the start of treatment and were 7–9 weeks old ($CB6F_1$) or 6–7 weeks old (athymic *nu/nu*) at the start of the procedure. Animals weighed 20–25 g (females) or 23–28 g (males), were housed 5–12 per cage, and had *ad libitum* access to food and water.

Experimental protocol

Tumor cells were removed from subconfluent cultures by a mild trypsin–EDTA treatment, the cell pellet was washed once with MEM without serum and the cells were suspended at the desired cell numbers in PBS (phosphate-buffered saline, 8 g NaCl, 6.2 g KCl, 1.15 g $Na_2HPO_4.7H_2O$, and 0.2 g KH_2PO_4 per liter). Cell suspensions were shown to be at least 90% viable by trypan blue exclusion. Mice were injected subcutaneously in the right hind footpad with tumor cells suspended in 50 μl of PBS. The regular protocol (unless otherwise stated) consisted of daily i.p. injections of 1 ml of 0.85% NaCl solution or 1 ml of 50 mM of AMP, ADP or ATP in 0.85% NaCl (from stock solutions adjusted to pH 5.5) starting 1 day after tumor inoculation and given for 10 consecutive days. Injections were administered with 30 gauge needles and mice were lightly anesthetized with ether. Mice were weighed before the start of treatment and once a week during and after treatment. After tumors became palpable, tumor sizes were determined every 3 days. Animals were sacrificed, tumors were excised and weighed before they reached 10% of the animals' weight [19].

Analysis of blood and plasma ATP levels

Blood (0.25 ml) was collected into syringes (25 gauge needles) containing citrate (0.05 ml of 93 mM sodium citrate, 7 mM citric acid, 140 mM dextrose, pH 6.5) from the inferior vena cava after i.p. injections of 1 ml of saline or 1 ml of 50 mM adenine nucleotides in saline. Mice were anesthetized with ether during the procedure. Plasma was prepared by immediate centrifugation of the blood in a Beckman microfuge (30 s at 8000 ***g***). Blood (20 μl) or plasma (100 μl) aliquots were added to 1 ml of cold 7% trichloroacetic acid. Acid-soluble nucleotides were extracted for 30 min on ice and after removal of the precipitate by centrifugation, trichloroacetic acid was removed from the aqueous phase by vigorous extraction of the aqueous solution with 2 ml of 0.5 M tri-*n*-octylamine in Freon-113 [4]. Determination of ATP levels in the extracts was performed by bioluminometry (luciferin–luciferase) [20] using a Turner Designs Bioluminescence Photometer.

RESULTS

Inhibition of tumor growth with adenine nucleotides

Toxicity. Treatment of $CB6F_1$ with daily i.p. injections of 1 ml of 50 mM of adenine nucleotides in saline (for 10 consecutive days) did not produce any weight losses during or after the treatment period (Fig. 1). Occasionally, an animal treated with ATP or ADP would die a few hours after injection. However, healthy, non-stressed animals tolerate these doses and completely recover a few hours after treatment. Injections of AMP did not cause any deaths. The relatively faster loss of body-weight in control animals at the end of experiments where relatively large tumors were involved (Fig. 1) is attributed to the larger tumors of the control groups as compared to the tumor sizes among the adenine nucleotide-treated groups.

Treatment of CT26 footpad tumors with i.p. injections of adenine nucleotides

The growth inhibitory properties of adenine nucleotides against CT26 murine colon carcinoma,

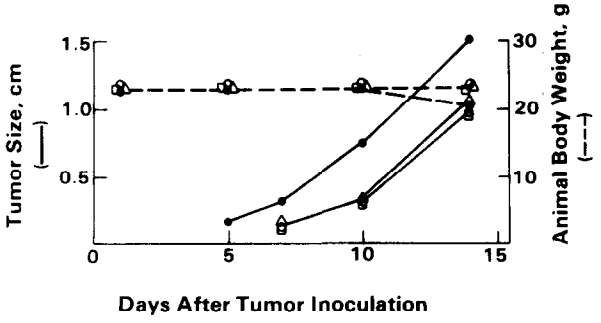

Fig. 1. Inhibition of growth of CT26 tumors in CB6F$_1$ mice and changes in host body weights during and after treatment with adenine nucleotides. Tumor-bearing mice were treated with saline (●), AMP (○), ADP (△) or ATP (□) following procedures described in the text. The figure presents data from experiment number 4 of Table 1. Values are the mean of 11 animals (per group). The standard deviations did not exceed 30% of the mean for any of the data points presented.

which was inoculated s.c. in the hind footpad of CB6F$_1$ mice, are illustrated in Table 1. The efficacy of adenine nucleotides was demonstrated against high, intermediate and low tumor burdens. No significant differences between males and females were observed either in the rate of tumor growth or in the tumor's response to adenine nucleotides treatment. The growth inhibitory activities of ATP–$MgCl_2$ were also evaluated. ATP–$MgCl_2$ was reported to be effective in improving recovery after hepatic [21] and renal [22] failures. A feature of ATP–$MgCl_2$ that may be related to anticancer therapy is the improved reticuloendothelial function after administration of ATP–$MgCl_2$ [23]. Recently, ATP–$MgCl_2$ was reported to reduce the nephrotoxicity induced by *cis*-diaminedichloroplatinum, a widely used cancer chemotherapeutic agent [22].

Table 1. Effects of adenine nucleotides treatment on the growth of CT 26 tumors in CB6F$_1$ mice

Experiment (No. of animals per group, sex)	Treatment*	No. of tumor cells inoculated	Time of analysis (days after inoculation)	Tumor diameter† (cm)	Tumor weight‡ (g)	Inhibition of tumor growth§ (% of saline-treated)
1 (10,F)	Saline	0.5×10^6	15	1.8 ± 0.2	1.93 ± 0.23	
	ATP–$MgCl_2$			1.4 ± 0.2	1.59 ± 0.24**	18
	ATP			1.3 ± 0.2	1.34 ± 0.33‡‡	31
2 (10,M)	Saline	0.5×10^6	15	1.9 ± 0.3	1.85 ± 0.37	
	ATP–$MgCl_2$			1.4 ± 0.3	1.38 ± 0.40¶	26
	ATP			1.4 ± 0.2	1.30 ± 0.29††	30
3 (12,M)	None	0.5×10^6	13	1.6 ± 0.3	1.16 ± 0.20	−1
	Saline			1.5 ± 0.3	1.14 ± 0.41	
	AMP			1.2 ± 0.2	0.65 ± 0.21‡‡	43
	ADP			1.3 ± 0.2	0.73 ± 0.23**	36
	ATP			1.2 ± 0.2	0.70 ± 0.18††	39
4 (11,F)	None	0.5×10^6	14	1.6 ± 0.3	1.46 ± 0.25	−7
	Saline			1.5 ± 0.3	1.36 ± 0.27	
	AMP			1.0 ± 0.3	0.88 ± 0.31‡‡	36
	ADP			1.1 ± 0.2	0.86 ± 0.27‡‡	37
	ATP			1.0 ± 0.2	0.84 ± 0.23§§	39
5 (12,F)	None	0.5×10^5	16	1.1 ± 0.2	0.76 ± 0.16	−7
	Saline			1.0 ± 0.2	0.71 ± 0.19	
	AMP			0.6 ± 0.2	0.41 ± 0.18‡‡	43
	ATP			0.5 ± 0.1	0.38 ± 0.10§§	47
6 (10,F)	Saline	0.5×10^5	18	1.3 ± 0.2	0.94 ± 0.20	
	AMP			0.8 ± 0.2	0.56 ± 0.21‡‡	41
	ATP			0.8 ± 0.1	0.51 ± 0.18§§	46
	AP_4A‖‖			0.9 ± 0.3	0.74 ± 0.21‖	22
7 (10,F)	Saline	0.1×10^5	22	1.5 ± 0.3	1.19 ± 0.26	
	AMP			0.9 ± 0.5	0.51 ± 0.28§§¶¶	57
	ATP			0.9 ± 0.5	0.68 ± 0.36††¶¶	43

*Treatment was performed as described in the text. In experiments 1 and 2 animals were treated with 1 ml of 25 mM of ATP–$MgCl_2$ or 1 ml of 25 mM of ATP. In experiments 3–7, animals were treated with 1 ml of 50 mM of AMP, ADP or ATP.

†Tumor diameter was calculated from $\frac{L+W}{2}$. Mean ± S.D.

‡The weight of footpad tumors immediately after excision. Mean ± S.D.

§Inhibition was based on tumor weights.

‖–¶¶Statistically significant differences from saline-treated group by Student's t test. ‖$P < 0.01$; ¶$P < 0.05$; **$P < 0.025$; ††$P < 0.01$; ‡‡$P < 0.005$; §§$P < 0.001$. ‖‖AP_4A, a nucleotide that is degraded to ATP and AMP by ecto-enzymatic phosphodiesterases [32] was injected at a concentrated of 10 mM. ¶¶Included one complete cure.

As shown in Table 1 the efficacy of ATP–$MgCl_2$ against CT26 footpad tumors is not greater than that of the sodium salt of ATP. ATP–$MgCl_2$, however, is much more toxic to the host as compared to ATP (Na^+ salt) (data not shown).

The data reported in Table 1 and Table 2 suggest that the efficacy of adenine nucleotides in inhibiting the growth of CT26 tumors is increased when the inoculated tumor burden is intermediate or low. When 10^4 or 5×10^3 tumor cells were implanted, treatment with adenine nucleotides resulted in a few 'cures'. 'Cured' mice were kept alive for 4 months without appearance of a tumor. Extensive unpublished data demonstrate that concentrations of AMP and ATP which are lower than 50 mM, are as effective against CT26 footpad tumors in $CB6F_1$ mice when administered by daily intraperitoneal injections in volumes of 1.4–1.8 ml of saline. The start of the treatment schedule can be delayed until the tumors are palpable (6–10 days after tumor inoculation, depending on the number of tumor cells implanted), without any significant effects on the magnitude of tumor growth inhibition. The efficacy of the inhibition of tumor growth is also dependent to a small extent on the pH of the AMP and ATP solutions which are administered i.p., with pHs 5.5–6.2 being the most effective (data not shown).

Treatment of human pancreatic adenocarcinoma xenografts with adenine nucleotides

The effects of AMP, ADP and ATP on human tumor xenografts in nude mice were evaluated utilizing the pancreatic ductal adenocarcinoma CAPAN-1 [18]. Treatment schedules consisted of single daily i.p. injections for 10 consecutive days, starting 1 day after tumor inoculation. Tumor burden was relatively low ($0.5–1 \times 10^5$ cells implanted) and tumor growth was slow (tumors became palpable 4–6 weeks after inoculation). The effects of AMP, ADP and ATP were evaluated by the total arrest of tumor growth for a period of 6–10 weeks (lack of palpable footpad tumors), and in all cases, the tumor-free mice did not develop any tumors for a period of 4 months. The data presented in Table 2 demonstrate the effects of adenine nucleotides on the arrest of CAPAN-1 xenografts in athymic nude mice. Since CAPAN-1 tumors grow at a slow rate under the inoculation conditions used in this study, the lack of palpable tumors 4 months after tumor implantation, may not imply an actual 'cure', but rather a tumor growth-arrest or a delay of tumor growth.

Increases in blood and plasma ATP levels after i.p. injections of adenine nucleotides

In order to demonstrate that intraperitoneally introduced adenine nucleotides can elevate blood and plasma ATP levels after entry into the systemic circulation, blood samples were withdrawn from the inferior vena cava at several time points after the i.p. injections. The data reported in Table 3 show that administration of AMP, ADP and ATP yield elevated blood and blood plasma ATP levels and that total cellular as well as extracellular ATP

Table 2. Arrest of growth of CT26 tumors in $CB6F_1$ mice and CAPAN-1 xenografts in athymic nu/nu *mice by adenine nucleotides treatment*

Experiment (host, No. of animals per group, sex)		Treatment*	No. of tumor cells inoculated	Time of analysis (weeks after inoculation)	Arrested tumors/implanted tumors
1	($CB6F_1$,10,F)	Saline	0.5×10^4	5	0/10
		AMP			2/10†
		ATP			4/10§
2	(Athymic, *nu/nu* 5,F)	Saline	1×10^5	7	1/5
		ATP			4/5
3	(Athymic, *nu/nu* 5,F)	Saline	1×10^5	6	1/5
		ATP			3/5
4	(Athymic, *nu/nu* 11,F)	Saline	0.5×10^5	10	3/11
		AMP			7/11‡
		ATP			8/11§
5	(Athymic, *nu,nu* 10,F)	Saline	0.5×10^5	9	2/10
		AMP			6/10‡
		ADP			6/10‡
		ATP			8/10‖

*Treatment was performed as described in the text. Intraperitoneal injections of 1 ml of saline or 1 ml of 50 mM of adenine nucleotides were administered.

†–‖Differences between these groups and the saline treated groups of the same experiment are significant by the χ^2 test. †$P < 0.5$; ‡$P < 0.25$; §$P < 0.1$; ‖$P < 0.05$.

Table 3. ATP levels in mouse blood (total cellular) and plasma after i.p. injections of adenine nucleotides

Host	Treatment*	Blood ATP levels† (hours after treatment) (mM)	Plasma ATP levels† (hours after treatment) (μM)
$CB6F_1$	Saline	0.65 ± 0.16 (1)	0.87 ± 0.25 (1)
		0.69 ± 0.15 (5)	0.94 ± 0.21 (5)
$CB6F_1$	AMP	1.23 ± 0.21 (1)	1.75 ± 0.36 (1)
		1.36 ± 0.24 (5)	1.69 ± 0.19 (5)
$CB6F_1$	ADP	1.28 ± 0.30 (1)	2.66 ± 0.46 (1)
		1.65 ± 0.41 (5)	2.75 ± 0.87 (5)
$CB6F_1$	ATP	2.53 ± 0.61 (1)	3.99 ± 0.81 (1)
		3.47 ± 0.57 (5)	5.14 ± 0.71 (5)
Athymic *nu/nu*	Saline	0.83 ± 0.25 (1)	1.10 ± 0.36 (1)
		0.91 ± 0.37 (4)	0.99 ± 0.43 (4)
Athymic *nu/nu*	AMP	1.87 ± 0.46 (1)	2.17 ± 0.36 (1)
		2.35 ± 0.67 (4)	1.97 ± 0.41 (4)
Athymic *nu/nu*	ATP	2.71 ± 0.54 (1)	2.93 ± 0.69 (1)
		4.26 ± 0.79 (4)	3.01 ± 0.76 (4)

*Each mouse (7–9 week old females) received i.p. injections of 1 ml of saline or 1 ml of 50 mM AMP, ADP or ATP in saline and the procedures performed as described in the text.

†Each group consisted of three mice and data are expressed as mean ± S.D. Blood withdrawal was performed within 15 min of the 1 h time points and within 30 min of the 4 and 5 h time points. The corresponding blood and plasma ATP levels were determined on the same blood samples in all cases.

concentrations in the circulation remain higher than its levels in normal controls for a period of several hours. The biochemical mechanism responsible for elevated blood and plasma ATP levels after i.p. injections of AMP and ADP has not yet been established. An elevation of portal venous plasma ATP concentrations in dogs, after intraperitoneal injections of ATP (50 ml of 120 mM of ATP in 18–20 kg animals) has been reported [24]. Increases in ATP concentrations of portal venous plasma, from 0.2 to 1.7 μM, were found 10 min after the i.p. injection of ATP [24]. The data outlined in Table 3 indicate that the magnitude of the increases in plasma ATP concentrations is linked to the increases in blood (total cellular) ATP concentrations. Thus, in the case of AMP and ADP treatments, elevated plasma ATP levels could be the result of increases in ATP levels in specific cells, achieved due to the increased supply of precursors, followed by the release of some cellular ATP. Unpublished results show that the increase in total blood ATP levels after i.p. injections of AMP or ATP last for more than 5 h and less than 18 h at which point total blood ATP pools drop back to normal levels. All the expanded ATP pools are accounted for in red blood cells and are stable after extensive washing of the isolated red blood cells in Hanks' balanced salt solution. Slow release of ATP from red blood cells containing expanded ATP pools is responsible for the increases in the plasma ATP levels (data not shown).

The use of citrate–dextrose as an anticoagulant, as compared to heparin, yields higher levels of plasma ATP pools (data for heparin is not shown). Citrate (but not heparin) was shown recently to inhibit the activity of a blood plasma phosphodiesterase which is active in catalyzing the degradation of ATP, by chelating divalent cations required for the enzymatic activity [25]. Inhibition of this phosphodiesterase activity during the short period of plasma preparation is presumably responsible for the higher plasma ATP levels obtained by utilizing this anticoagulant.

DISCUSSION

The efficacy of adenine nucleotides in experimental tumor therapy in murine models is demonstrated in this report. The growth of murine colon carcinoma CT26 (classified as undifferentiated [17]) in $CB6F_1$ mice as well as the growth of a differentiated human pancreatic adenocarcinoma of ductal origin, CAPAN-1 [18], xenografts in athymic nude mice, were significantly inhibited. The mechanism of the anticancer activity of adenine nucleotides may be related to the growth-inhibitory activity of ATP, which was previously demonstrated against a variety of tumor cells *in vitro*, in monolayers [1] and soft-agar cultures [2]. The biochemical mechanisms of the inhibition of DNA synthesis and cellular proliferation by extracellular ATP, as well as the specificity of the effects towards transformed cells, have previously been indicated [1–6, and references cited therein]. It is shown in this study that AMP and ADP, as well as ATP, administered intraperitoneally in a relatively large volume, yield increases in blood (total cellular) and plasma (extracellular) levels of ATP. The elevated ATP levels were maintained for several hours, and are presumably respon-

sible for the antitumor activities of adenine nucleotides.

The procedures utilized for the administration of adenine nucleotides for the purpose of achieving effective sustained ATP plasma levels over a period of time are of primary importance in potential tumor therapy. Although ATP can be introduced in humans by intravenous infusions [26, 27], in this study a large volume intraperitoneal administration was utilized because of the obvious technical reasons. The administration of anticancer drugs in large volumes into the peritoneal cavity affords reliably uniform distribution of the drugs [28] as well as slow passage of the drugs from the peritoneal cavity into the systemic circulation, a property which is beneficial if the rate of plasma clearance is high [29]. Other modes of delivery of adenine nucleotides may, however, prove to be more effective.

The preclinical studies on murine tumor models provide favorable implications with regard to potential beneficial therapeutic indexes in the treatment of human cancer with adenine nucleotides. The reason for this is that human tissues possess significantly lower phosphomonoesterase activities, which catalyze the non-specific degradation of ATP, than comparable animal tissues [30, 31]. In addition, human blood *in vitro* has lower ecto-ATPase activities than rodents blood [8]. Thus, the stability of extracellular (plasma) ATP pools in the circulation is expected to be greater in humans than it is in rodents. Prospective human therapy would therefore require the administration of lower levels of adenine nucleotides for achieving effective elevated blood and plasma ATP concentrations, which in turn would reduce side effects. Recently, intravenous infusions of low levels of ATP (5 μmol/kg/20 min) for treatment of chronic obstructive pulmonary disease in humans yielded significant (50–70%) increases in the arterial and mixed venous blood ATP levels [26]. The effects of injections or infusions of ATP on the systemic and pulmonary circulation have been studied [27, 33, 34].

Because of the high catalytic activities of blood plasma phosphodiesterases [25] and ecto-enzymes such as ecto-ATPases, ecto-ADPases, ecto-5′-nucleotidases and non-specific phosphomonoesterases and phosphodiesterases, which are present on blood and vascular cells, adenine nucleotides are very effectively removed from the vascular beds. Nevertheless, this report demonstrates that blood plasma levels of ATP of 1–5 μM are attainable in mice by i.p. injections of adenine nucleotides and that these exogenously administered adenine nucleotides inhibit tumor growth in the murine model. Because of the significant metabolic lability of extracellular (plasma compartment) ATP pools, the anticancer activities of adenine nucleotides are expected to be extremely dependent on their doses and schedules of administration as well as on the mode of delivery of these agents.

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