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ORIGINAL ARTICLE

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Tumour cell uptake of the metastasis inhibitor ruthenium complex NAMI-A and its in vitro effects on KB cells

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Abstract *Purpose:* The uptake of NAMI-A (imidazolium *trans*-imidazoledimethylsulphoxidetetrachlororuthenate) by KB cells in vitro was compared with the effects of this compound on the cell cycle phase distribution of the cells. *Methods:* NAMI-A uptake was determined by flameless atomic absorption spectroscopy, and the cell cycle phase distribution was determined by flow cytometry. *Results:* NAMI-A uptake was proportional to its concentration in the incubation medium. The use of a number of incubation conditions showed that NAMI-A uptake from MEM was independent of the presence of serum and dependent on the presence of amino acids in the incubation medium, and that NAMI-A uptake was markedly higher when the cells were incubated in PBS. The uptake increase observed in PBS did not occur when the cells were kept at 0–4°C, suggesting the presence of active transportation of NAMI-A into cells. In addition, the presence of divalent cations such as Ca^{2+} and Mg^{2+}, appeared to facilitate NAMI-A uptake. The anionic substance transport inhibitor probenecid significantly reduced the active transportation of NAMI-A into cells. The effects of NAMI-A on cell cycle distribution were strictly dependent on its uptake by tumour cells and not on its extracellular concentration. *Conclusions:* These findings suggest the interaction of NAMI-A with biological components resulting in possible consequences for the distribution of the compound itself. Furthermore, NAMI-A enters tumour cells both by passive diffusion and by active transportation.

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Introduction

Complexes containing atoms of different metals are used in anticancer therapy for their inhibitory action on cell proliferation. Complexes based on platinum and chromium can exhibit these effects after cell uptake and DNA interaction [18, 23], whereas complexes containing metals such as copper, cobalt and thallium exert their action by a multiple mechanism of suppression of the synthetic steps that inhibit nucleic acid metabolism, specifically DNA and purine de novo synthesis [16, 17]. Furthermore, interaction of a metal complex such as cisplatin with the nucleic acids present on the surface of tumour cells has been suggested by Juckett and Rosenberg based on their results with the mouse tumour Sarcoma 180 [19].

Ruthenium complexes with sulphoxide ligands with the metal in the +3 oxidation state are probably the most recent group of metal-based compounds which deserve consistent attention in preclinical studies mainly because of their antimetastatic properties [24]. Ruthenium complexes show their antineoplastic action via several mechanisms: they can either bind to DNA [20] or inhibit DNA recognition and cleavage by some restriction enzymes [13]. Para-substituted derivatives of oxoruthenium can oxidize oligonucleotides and cause failure at the guanine level of the binding of the bases compromising the sugar function [12]. The ruthenium atom inserts into the DNA double helix and the ligands of the metal atom form crosslinks with topoisomerase II [15]. However, complexes of ruthenium can also act without penetrating the cellular membrane by binding to extracellular sites and inducing conformational modifications that can have an antineoplastic effect [10, 14].

Imidazolium *trans*-imidazoledimethylsulphoxidetetrachlororuthenate (NAMI-A) is an antineoplastic ruthenium compound which shows a specific antimetastatic activity [25]. NAMI-A reduces the growth of lung

metastases without significantly affecting primary tumour growth [26, 27, 28] and without showing cytotoxicity against tumour cells in vitro, besides the transient arrest of cell cycle in the premitotic phase [3], and inhibition of spontaneous matrigel crossing [34]. Among other effects, NAMI-A is active in metastatic cells [5], modulates the activity of ERK1/2 pathways at a high level (Pintus et al., in preparation), and produces a ruthenium content in the lungs related to type I collagen binding (Callerio Foundation, data to be published 2002). These properties may account for the increase in life expectancy in tumour-bearing hosts in vivo [3].

Experimental results suggest that the antimetastatic activity of NAMI-A might not require internalization of the compound by tumour cells [2] and that the compound may act because of its interaction at the extracellular level [4, 5]. Assuming that the in vitro effects on the cell cycle are related to the in vivo effects [34], the aim of this study was to examine the influence of NAMI-A uptake by tumour cells on cell cycle distribution.

The amount of NAMI-A uptake by tumour cells was examined under different experimental conditions in order to highlight the processes of NAMI-A internalization by tumour cells.

Materials and methods

Tumour line for in vitro testing

An established KB cell line (ECACC no. 86103004) derived from a human oral carcinoma was cultured according to standard procedures [8]. Vials of the original cell line were maintained in liquid nitrogen. Cells were obtained once a week by routine subculturing, and used for the experiments reported here. The cell line was maintained in Eagle's minimum essential medium (MEM) [11], supplemented with 10% newborn calf serum (GIBCO BRL) with 10 ml/l penicillin and streptomycin solution (Sigma Chemical Company, St. Louis, Mo.) (100 U/ml penicillin G and 100 µg/ml streptomycin) and buffered with 3 m*M* Tris(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid, 3 m*M* *N*,*N*-bis(2-hydroxyethyl)-2-aminoethane-sulphonic acid, 3 m*M* *N*-2-hydroxyethyl piperazine-*N*′-2-ethane-sulphonic acid, and 3 m*M* Tricine (Sigma). The medium used for assays was antibiotic-free. The cell population doubling time was about 24 h. Cells from confluent monolayers were removed with 2–3 ml 0.05% trypsin solution (Sigma). For the determination of ruthenium, cells were sown at a density of 24×10^4 cells per 3-ml per well in a six-well plate (Corning Costar) 5 days before the experiments. The amino acids used in these experiments (glutamine and histidine) and probenecid were purchased from Sigma. For the propidium iodide test, cells were sown at a density of 4×10^4 cells in a 24-well plate (Corning Costar) 5 days before the experiments.

Propidium iodide test

Viable cells (0.5×10^6) of a monocellular suspension, as determined by the trypan blue exclusion test, were fixed in 70% ethanol at 4°C for at least 1 h. Before analysis, ethanol was removed by decanting after centrifugation to pellet the cells and these were washed twice with phosphate-buffered saline (PBS). Cells were resuspended in PBS containing 1 mg/ml RNase at 37°C for 30 min and further stained for at least 30 min at room temperature in the dark with 40 µg/ml propidium iodide (Sigma) (modified from the method of Crissman and Steinkamp [9]). Red fluorescence (610 nm) was analysed using a peak fluorescence gate to discriminate aggregates. Analyses were carried out at Fondazione Callerio with an EPICS XL flow cytometer. The cell cycle distribution was determined by analysis of data with Multicycle software (Phoenix Flow Systems, San Diego, Calif.).

Preparation of samples for atomic absorption spectroscopy

Samples were processed using the procedure of Tamura and Arai [32] with modification. After treatment, cells were washed four times and dried in Nalgene cryogenic vials (a first drying step was performed overnight at 80°C and a second step at 105°C until the samples reached a constant weight). The dried cells were decomposed by the addition of 150 µl tetramethylammoniumhydroxide (TMAH; Aldrich Chimica, Italy) and 150 µl FAAS buffer (prepared by dissolving 9.0 g NaCl in 1.0 l Millipore milliQ water containing 16 ml 37% hydrochloric acid) directly into the vials. Volumes were adjusted to 1 ml for FAAS.

Quantification of ruthenium

Concentrations of ruthenium were measured in triplicate by means of graphite furnace atomic absorption spectrometry (GFAAS). The GFAAS instrument used was a Zeeman graphite tube atomizer, model SpectrAA-300, supplied with a specific ruthenium emission lamp (hollow cathode lamp P/N 56-101447-00; Varian, Mulgrave, Victoria, Australia). To adjust for possible deterioration of the furnace during a daily work session, a reslope standard was measured every six samples. Changes in the readings of this reslope standard were included in the calculation of the NAMI-A concentrations in the samples. The values of two subsequent reslope readings were not allowed to account for a deviation higher than 20%. The lowest and highest limits of quantitation (LLQ, GLQ) were set at concentration levels which corresponded, respectively, to the lowest and highest standard concentrations. The limit of detection (LOD) was estimated according to the EURACHEM guide. For the fitness for purpose of the analytical method, the LLQ, GLQ and LOD were respectively 12.5, 200 and about 8 ng Ru/ml of sample. Quantification of ruthenium was carried out in 10-µl samples at 349.9 nm with an atomizing temperature of 2500°C using argon as carrier gas at a flow rate of 3.0 l/min (for further details concerning the furnace parameter settings, see reference 6). Before each daily analysis session, a five-point calibration curve was obtained using ruthenium custom-grade standard 998 mg/ml (Inorganic Venturesb, Lakewood, N.J.). The raw data printed out on paper were processed using Microsoft Excel.

Statistical analysis

Data were analysed using ANOVA followed by the post-multiple comparison Newman-Keuls test. Comparison between regression lines was carried out by the parallelism test, the Pharmacological Calculation System [31]. Significance was accepted for P values < 0.05.

Results

Time-dependence of NAMI-A uptake by KB cells

The in vitro exposure of KB cells to 100 µ*M* NAMI-A (minimal effective concentration on cell cycle [3]) caused the intracellular accumulation of ruthenium depending both on the length of the cell challenge and on the incubation medium used, i.e. PBS or MEM. The amount of ruthenium in the tumour cells increased with time,

from 0.032 ± 0.007 μg per million cells after 15 min incubation to 0.528 ± 0.021 μg per million cells after 240 min incubation in PBS. When incubation was carried out in MEM, the amount of ruthenium was significantly lower and was only of 0.041 ± 0.001 μg per million cells ($P < 0.001$; Fig. 1) after 240 min incubation.

In order to determine whether the reduced tumour cell uptake of NAMI-A in MEM was due to the presence of serum in the medium, KB cells were also challenged with NAMI-A in a solution of serum-free MEM. Under these conditions, the amount of ruthenium measured in the cells after incubations for up to 120 min did not increase as compared to that measured in MEM with serum. A slight but significant ($P < 0.05$ vs MEM) increase in ruthenium uptake was measured after 240 min incubation. Also the uptake of ruthenium by KB cells incubated with 100 μ*M* NAMI-A in calcium/magnesium-free PBS (PBS-CMF) was less than that by cells incubated in PBS, and the reduction was greater, and statistically significant ($P < 0.01$ vs PBS), from 30 min onwards.

In equivalent experiments carried out at 0–4°C, the amount of ruthenium taken up by the tumour cells remained constant independently of the incubation time and approximated the amounts measured when the experiments were carried out in MEM at 37°C (Fig. 2).

On the basis of these results, all the following experiments were carried out for 60 min using the two opposite conditions of tumour cell incubation, i.e. PBS and MEM with serum.

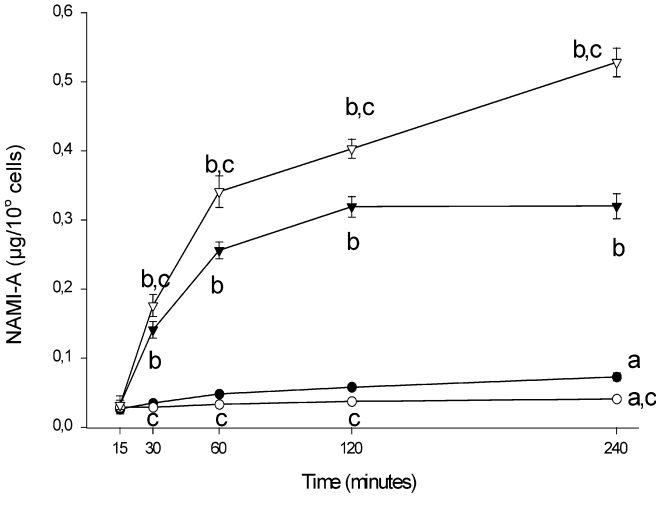

Fig. 1. Influence of cell medium on time course of NAMI-A uptake by KB cells. Cells, sown in six-well plastic plates 5 days before, were exposed to 100 μ*M* NAMI-A for 15, 30, 60, 120 and 240 min in different media (*closed circles* serum-free MEM, *open circles* MEM with serum, *closed triangles* PBS-CMF, *open triangles* PBS). After an adequate time, cells were detached with trypsin 0.05% (w/v) and processed as described in Materials and methods for the evaluation of intracellular ruthenium. Values are means ± SEM from two separate experiments, each performed in triplicate. The means indicated with same letter are significantly different: *a* $P < 0.05$, *b* $P < 0.01$, *c* $P < 0.001$ (Student's unpaired *t*-test)

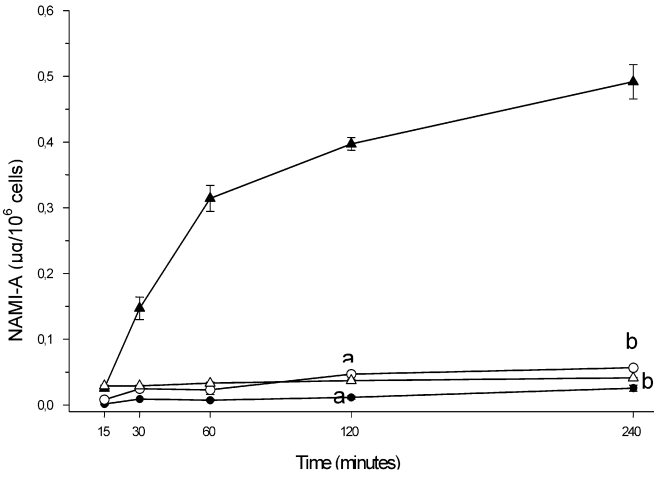

Fig. 2. Influence of temperature on the time course of NAMI-A uptake by KB cells. Cells, sown in six-well plastic plates 5 days before, were exposed to 100 μ*M* NAMI-A for 15, 30, 60, 120 and 240 min in MEM (*open circles*, *open triangles*) or PBS (*open circles*, *closed triangles*), at 4°C (*open circles*, *closed circles*) or 37°C (*open triangles*, *closed triangles*). After appropriate times, cells were detached with trypsin 0.05% (w/v) and processed as described in Materials and methods for the valuation of intracellular ruthenium. Values are means ± SEM from two separate experiments, each performed in triplicate. Means indicated with same letter are significantly different: *a* $P < 0.05$, *b* $P < 0.01$ (Student's unpaired *t*-test)

Concentration dependence of NAMI-A uptake

The dependence of NAMI-A uptake on the concentrations in the incubation medium was determined by exposure of KB cells to NAMI-A at concentrations in the range 10 μ*M* to 1 m*M* for 60 min both in PBS and in MEM. In cells incubated with PBS, NAMI-A uptake increased from 0.058 ± 0.001 μg per million cells at 10 μ*M* NAMI-A to 6.665 ± 0.503 μg at 1 m*M* NAMI-A. In cells incubated with MEM, NAMI-A uptake increased from 0.023 ± 0.004 μg per million cells at 10 μ*M* NAMI-A to 2.689 ± 0.179 μg at 1 m*M* NAMI-A (Fig. 3a).

The fraction of NAMI-A that went into the cells was constant in cells incubated in MEM (Fig. 3b), while the fraction that went into cells incubated in PBS reduced as the NAMI-A concentration in the incubation medium increased. The number of cells that recovered after challenge with NAMI-A in PBS was less than the number that recovered in untreated control cultures and among cells exposed to NAMI-A in MEM with increasing NAMI-A concentrations in the medium (Fig. 3c). Cells exposed to NAMI-A in MEM showed a significant reduction in recovery at the end of incubation only at a NAMI-A concentraiton of 1 m*M* ($P < 0.001$).

Effects of probenecid on NAMI-A uptake

The effects of the anionic substance transport inhibitor probenecid on the uptake of NAMI-A by KB cells

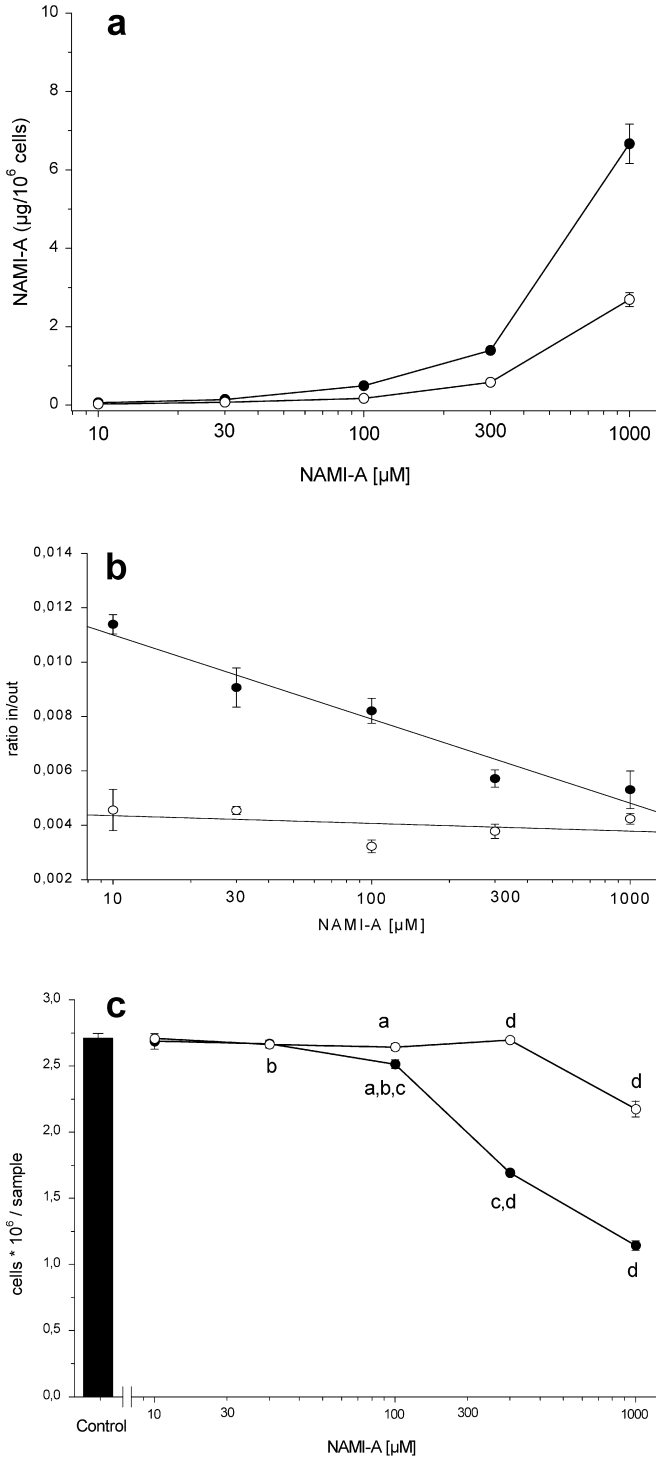

Fig. 3a–c. Dependence of NAMI-A uptake by KB cells on its concentration in the culture medium. Cells, sown in six-well plastic plates 5 days before, were exposed for 60 min to different NAMI-A concentrations (10, 30, 100 and 300 µ*M*, and 1 m*M*) in MEM (*open circles*) or PBS (*closed circles*) at 37°C. Cells were detached with trypsin 0.05% (w/v) and processed as described in Materials and methods. Values are means ± SEM from two separate experiments, each performed in triplicate. **a** Evaluation of intracellular ruthenium. **b** Ratio intracellular/extracellular ruthenium concentration (data plotted are from **a**); $P < 0.01$, test for parallelism. **c** Evaluation of cellular vitality; Student's unpaired *t*-test. Means indicated with same letter are significantly different: *a*, *b* $P < 0.05$; *c*, *d* $P < 0.001$

incubated in PBS or MEM are shown in Table 1. KB cells were pre-exposed to 100 µ*M* probenecid or vehicle for 60 min, and the culture medium was then replaced with PBS or MEM containing 100 µ*M* NAMI-A with or without 100 µ*M* probenecid. The presence of probenecid significantly reduced NAMI-A uptake by cells incubated in PBS by 20% as compared to the relevant controls ($P < 0.01$). Probenecid had no effect on NAMI-A uptake by cells incubated in MEM.

Effects of glutamine and histidine on NAMI-A uptake

To investigate the possible influence of some amino acids on NAMI-A uptake, KB cells were incubated for 60 min in PBS containing 100 µ*M* NAMI-A and histidine (42 mg/l) or glutamine (292 mg/L), i.e. the same concentrations of these amino acids present in MEM. The presence of histidine and glutamine in the PBS significantly reduced the uptake of NAMI-A by KB cells, by 45% ($P < 0.01$) and 51% ($P < 0.01$), respectively (Table 2). These reductions are comparable to that observed when NAMI-A uptake from MEM was investigated (reduction by 67% vs the relevant controls).

Table 1. NAMI-A uptake in the presence of 100 µ*M* probenecid. Cells, sown in six-well plastic plates 5 days before, were exposed to 100 µ*M* probenecid or incubation medium (MEM or PBS). After 60 min preincubation the medium was removed and the cells were exposed for 60 min to 100 µ*M* NAMI-A with or without probenecid. Cells were detached with trypsin 0.05% (w/v) and processed as described in Materials and methods. Values are means ± SEM from two separate experiments, each performed in triplicate

Treatment	Intracellular NAMI-A (µg/10^6 cells)	
	PBS	MEM
NAMI-A 100 µ*M*	0.654 ± 0.043	0.220 ± 0.011
NAMI-A plus probenecid 100 µ*M*	0.522 ± 0.018*	0.207 ± 0.017

*$P < 0.01$ vs NAMI-A only in PBS; ANOVA plus Newman-Keuls test

Table 2. NAMI-A uptake in the presence of amino acids. Cells, sown in six-well plastic plates for 5 days before, were exposed for 60 min to 100 µ*M* NAMI-A in PBS, with or without histidine 42 mg/l or glutamine 292 mg/l, and in MEM. Cells were detached with trypsin 0.05% (w/v) and processed as described in Materials and methods. Values are means ± SEM from two separate experiments, each performed in triplicate

Treatment	Intracellular NAMI-A (µg/10^6 cells)	
	PBS	MEM
NAMI-A 100 µ*M*	0.761 ± 0.021	0.252 ± 0.007
NAMI-A plus histidine	0.421 ± 0.005*	
NAMI-A plus glutamine	0.373 ± 0.057*	

*$P < 0.01$ vs NAMI-A only in PBS; ANOVA plus Newman-Keuls test

Effects of NAMI-A uptake on the cell cycle

Analysis of the cell cycle distribution 24 h after exposure of KB cells to NAMI-A showed a statistically significant cell arrest in the G_2-M premitotic phase (Table 3). In PBS, this effect was seen at 100 μ*M* NAMI-A in the incubation medium: the percentage of cells decreased by 24% ($P < 0.01$) in the G_0/G_1 phase and increased by 127% ($P < 0.01$) in the G_2-M phase. In MEM, similar effects were seen only at 1 m*M* NAMI-A in the incubation medium: the percentage of cells decreased by 10% ($P < 0.01$) in the G_0/G_1 phase and increased by 87% ($P < 0.01$) in the G_2-M phase.

When cells were exposed for 120 min to 100 μ*M* NAMI-A in either PBS or MEM at 37°C and 0–4°C there were statistically significant changes vs untreated controls only when KB cells were kept at 37°C and exposed to NAMI-A in PBS. The percentage of cells decreased by 59% in the G_0/G_1 phase ($P < 0.01$) and increased by 304% ($P < 0.01$) in the G_2-M phase. When the cells were maintained at 0–4°C, the percentage in the different phases of the cell cycle did not differ between any of the treatment conditions (Table 4).

Discussion

The amount of NAMI-A that enters tumour cells affects the cell cycle distribution. It was strictly dependent on the nature of the vehicle used for cell challenge, i.e. MEM or PBS. NAMI-A uptake by tumour cells in MEM was significantly lower than in PBS and was not affected by temperature or by serum proteins. We can exclude the involvement of PGP in this process, since it is known that this protein is not expressed in KB cells [33]. Moreover, in experiments carried out with the PGP inhibitors verapamil and nifedipine (data not shown), we found no difference in NAMI-A uptake.

It is possible that NAMI-A uptake by tumour cells is affected by some components of the incubation medium, as already shown under other circumstances by Nieboer et al. [22]. Confirmation of this is provided by the noteworthy increase in NAMI-A uptake when equivalent experiments were carried out in PBS. However, in this case, uptake of NAMI-A appeared to be temperature- and calcium/magnesium-dependent. Since MEM contains histidine, and it is known that ruthenium compounds may bind to this molecule [1, 21], we investigated the effects of the addition of histidine or glutamine at the concentrations at which they are found in MEM. In this case, NAMI-A uptake was markedly reduced, and this result may explain the lower uptake of NAMI-A by cells challenged with the compound in MEM. However, interactions with other components cannot be excluded, and it would be interesting to ascertain the reversibility of these interactions that might modify the concentration of the free compound. Furthermore, it cannot be excluded that the components of MEM compete with NAMI-A for transport through the cell membrane and therefore reduce the latter's cell uptake. This hypothesis might explain the change in the rate of NAMI-A uptake from MEM which becomes linear and independent of its concentration in the medium. Entry of NAMI-A into tumour cells should be favoured by its interaction with the intracellular target(s) responsible for the pharmacological effects.

Table 3. Dose- and incubation medium-dependence of the effects of NAMI-A on the distribution of KB cells among the cell cycle phases. KB cells were first sown in a 24-well plastic plate and incubated for 120 h, and then exposed for 60 min to 10, 30, 100 and 300 μ*M* and 1 m*M* NAMI-A in PBS or MEM. The drug was then removed and the cells were incubated in fresh medium for a further 24 h. The cells were then processed as described in Materials and methods for the propidium iodide test. Values are mean ± SEM percentages of cells in each phase of the cell cycle as indicated from two separate experiments, each performed in triplicate

NAMI-A concentration (m*M*)	Cell cycle phase		
	G_0/G_1	S	G_2-M
Incubation in PBS			
0	69.5 ± 1.0	20.4 ± 0.7	10.1 ± 0.5
0.01	68.8 ± 1.4	20.2 ± 1.2	11.0 ± 0.7
0.03	68.8 ± 1.1	20.1 ± 0.9	11.1 ± 1.5
0.1	52.5 ± 1.0*	24.5 ± 1.6	23.0 ± 3.9*
0.3	32.7 ± 2.5*,**	22.8 ± 0.7	44.5 ± 4.5*,**
1	21.1 ± 0.4*,**	35.7 ± 3.7*	43.2 ± 5.8
Incubation in MEM			
0	70.2 ± 1.7	21.2 ± 0.8	8.6 ± 0.9
0.01	74.8 ± 1.3	18.7 ± 1.1	6.6 ± 0.5
0.03	74.0 ± 2.0	19.4 ± 1.7	6.6 ± 0.4
0.1	72.4 ± 5.6	20.2 ± 4.4	7.5 ± 1.3
0.3	73.3 ± 1.6	19.0 ± 1.4	7.6 ± 0.5
1	63.0 ± 1.4*	20.9 ± 1.0	16.1 ± 0.6*

*$P < 0.01$ vs zero NAMI-A concentration, **$P < 0.01$ vs next lower NAMI-A concentration; ANOVA plus Newman-Keuls *t*-test

Table 4. Effect of NAMI-A on the distribution of cells among the cell cycle phases. Cells, sown in 24-well plastic plates, were exposed to 100 μ*M* NAMI-A for 120 min in MEM or PBS at 4°C and 37°C. The drug was then removed and the cells were incubated in fresh medium for 24 h. The cells were then processed as described in Materials and methods for the propidium iodide test. Values are mean ± SEM percentages of cells in each phase of the cell cycle as indicated from two separate experiments, each performed in triplicate

Treatment group	Cell cycle phase		
	G_0/G_1	S	G_2-M
Incubation at 0–4°C			
Control-PBS	67.4 ± 0.6	26.5 ± 2.0	8.4 ± 0.3
NAMI-A-PBS	67.9 ± 1.0	22.3 ± 0.8	9.7 ± 0.2*
Control-MEM	67.1 ± 0.5	24.9 ± 0.3	8.0 ± 0.3
NAMI-A-MEM	67.3 ± 0.4	24.4 ± 0.6	8.2 ± 0.2
Incubation at 37°C			
Control-PBS	70.8 ± 1.6	18.9 ± 0.7	10.2 ± 0.9
NAMI-A-PBS	29.0 ± 1.5*	28.8 ± 2.2*	41.2 ± 3.7*
Control-MEM	71.6 ± 0.4	19.9 ± 0.4	8.4 ± 0.1
NAMI-A-MEM	69.3 ± 0.7	19.9 ± 0.4	10.8 ± 0.4

*$P < 0.01$ vs respective control; ANOVA plus Newman-Keuls test

On the basis of the results of the present investigation, we can hypothesize that there is an active process of intracellular transport of NAMI-A into the cells, associated with a process of passive diffusion. That NAMI-A is transported into the target cells by these two processes is also favoured by the complex metabolism of this compound in aqueous solution which leads to a number of charged species which might encounter difficulties in passively crossing the cell membrane [29, 30]. The consequences related to these events are important for the understanding of the in vivo activity of NAMI-A, particularly considering that a pharmacokinetic study of NAMI-A has confirmed its transportation through the biological barriers [7], suggesting a good mobility of the compound in vivo.

In conclusion, the findings of this investigation show that NAMI-A enters and accumulates in tumour cells, although this uptake may be only one of the factors that account for the antimetastatic activity of this compound in vivo.

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