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Multidrug resistance modifies polyamines uptake in P388 murine lymphoma cells: experimental and modeling approach

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Abstract

Polyamines (putrescine, spermidine and spermine) are ubiquitous compounds, essential for cell growth. This paper compares the polyamine transport between sensitive P388 murine lymphoma cells and two multidrug resistant P388 sublines with the assistance of an experimental model. This new model allows the characterisation of the whole polyamines uptake and efflux. Three parameters are identified by the model: two rate constants (K_+ for the uptake and K_- for the efflux) which are considered as physical constants specific to the transport of one polyamine in one cell type, and $Ci(o)$ which represents the initial intracellular concentration. This model well describes our experimental results of polyamine transport across the P388 cell plasma membrane. Multidrug resistant P388 cells exhibit spermine uptake significantly higher than that of sensitive cells when on the opposite, putrescine enters more rapidly into the sensitive P388 cells. In conclusion, comparison of polyamine transport between sensitive and multidrug resistant P388 phenotypes shows large and significant differences.

Keywords: Spermidine; Spermine; Putrescine; P388 cells; Multidrug resistance

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1. Introduction

Polyamines (putrescine, spermidine and spermine) are ubiquitous intracellular components, essential for cell growth and differentiation. Their intracellular levels are well regulated by a biosynthesis from ornithine and an uptake from circulation. The fact that DFMO (difluoromethylornithine), which inhibits the ornithine decarboxylase, induced in vitro a rapid decrease of the polyamine intracellular level followed by cell growth arrest, led to a particular interest for polyamine in cell proliferation phenomena including tumour cells. Indeed, most of cancer cells are growing faster than their normal neighbours and various types of cancer cells were described to require a higher polyamine level than normal cells [1,2].

Nevertheless, in vivo studies showed that DFMO treated-cells are able to take up polyamines from the extracellular medium. This result involved the study of polyamine transport in different cell types [3–8] as well as its inhibition by different molecules [9,10].

Actually, many new molecules like polyamine analogues and polyamine metabolism inhibitors, are synthesised in order to block tumoral development [10–14]. Moreover, some of these new molecules were shown to be particularly effective against multidrug resistant (MDR) cells [12]. Acquired or intrinsic resistance has been, for several years, a major problem in cancer chemotherapy. This resistance might occur for various antitumoral agents after a contact with only one of them, this has been called multidrug resistance (MDR). Multidrug resistance is characterised by the expression (or surexpression) of a membrane glycoprotein, the P-gp170 which acts as a 'pump' expulsing the chemotherapeutic agent out of the cell [15–17]. Nevertheless, multidrug resistance can also be accompanied by cell membrane and/or protein modifications.

The purpose of this study was to evaluate in vitro the potential influence of multidrug resistance on the natural polyamine transport. Indeed, putrescine and spermidine transport were previously compared between sensitive and multidrug resistant K562 cells [18] but no study concerned spermine.

Most of the polyamine transport studies were described by the Michaëlis–Menten model [18–20].

$$\frac{\mathrm{d}Ci(t)}{\mathrm{d}t} = \frac{V_{\max} \cdot Ce(t)}{K_m + Ce(t)}$$

where $Ci(t)$ and $Ce(t)$ are respectively the intracellular and extracellular polyamine concentrations.

The kinetic parameters $V_{\max}$ (maximal rate of uptake or reaction) and K_m (concentration for which $V = V_{\max}/2$; $1/K_m$ represents the affinity of the carrier), of this model do not inform on the compounds efflux. Moreover, results must present a saturation to allow the determination of $V_{\max}$ and K_m separately. Indeed, following the Euler approximation, the Michaëlis–Menten model can be written:

$$Ci(t_1) \cong Ci(o) + \frac{V_{\max} \cdot t_1}{K_m + Ce(o)} \cdot Ce(o) \qquad \text{(with } t_1 \text{ close to 0)}$$

which becomes, with low extracellular concentrations $Ce(o) \ll K_m$,

$$Ci(t_1) \cong Ci(o) + \frac{V_{\max}}{K_m} \cdot t_1 \cdot Ce(o)$$

In this latter case, a linear regression allows one to identify only the ratio $V_{\max}/K_m$ and not both values separately. For those different reasons, it is useful to develop a theoretical model in which a global constant for the uptake (K_+) as well as for the efflux (K_-) will be identified for a given polyamine (putrescine, spermidine or spermine) and a given cell line (sensitive or resistant) on the basis of three experiments, each one giving $Ci(t_1)$ as a function of $Ce(o)$.

Moreover, experimental variations (within each set of three experiments) will be taken into account thanks to the identification of a third parameter, namely the initial intracellular concentration $[Ci(o)]$ of each experiment.

In this paper, this model is applied in order to differentiate sensitive and multidrug resistant P388 cell lines from the total putrescine, spermidine and spermine transport viewpoint, by the determination of the above mentioned rate constants.

2. Materials and methods

2.1. Materials

Cell culture medium and additives were purchased from Gibco-Life Technologies (Paisley, Scotland). The culture flasks were coming from Falcon (Becton-Dickinson, Lincoln Park, NJ, USA). Putrescine, spermidine and spermine were obtained from Fluka (Buchs, Switzerland) and diaminohexane from Aldrich (Steinheim, Germany). Perchloric acid, NaOH and liquid paraffin were purchased from Merck (Darmstadt, Germany), $CHCl_3$ was obtained from Lab-Scan (Dublin, Ireland) and dibutylphtalate and β-mercaptoethanol from Sigma (St Louis, MO, USA).

2.2. Cell culture

The sensitive and multidrug resistant P388 cell lines were graciously provided by Dr Gh. Atassi (Institut de Recherches Servier, Suresnes, France). Cells were cultured at 37°C in humidified 5% CO_2 atmosphere. Cells were passaged twice per week, in 75-cm^2 flasks. For uptake experiments, cells were placed in 25-cm^2 flasks in concentrations of 4×10^6 cells/ml, 1.5 ml final volume. The medium consisted of RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer (pH 7.4) and 20 μM β-mercaptoethanol. Multidrug resistant cell lines were cultured in presence of doxorubicin at concentrations of 1 and 10 μM, respectively for P388/adr-1 and P388/adr-10. To avoid interference, doxorubicin was not added in the culture medium the last week before the experiment. The presence of P-glycoprotein was previously detected in multidrug resistant P388 by the Institut de Recherches Servier.

2.3. Polyamine uptake in P388 cells

Cells placed in 25-cm^2 flasks were incubated 1 h at 37°C before adding putrescine, spermidine or spermine. The seven concentrations studied were in the range from 50 to 1500 μM for putrescine and 100 to 1500 μM for spermidine and spermine. One flask was used per concentration. Experiments were repeated on 3 different days. For each experiment, two blanks (cells without polyamine added) were prepared in parallel in order to determine the endogenous polyamine content. Contact time between polyamines and cells were 5 min (putrescine in all three P388 cell lines, spermine in multidrug resistant cells) and 10 min (spermidine in all three P388 cell lines, spermine in sensitive cells).

After that time, 1 ml of cell suspension was centrifuged in an Eppendorf tube on a 0.5-ml layer of dibutylphtalate (85%) and liquid paraffin (15%), for 2 min at $4000 \times g$. The supernatant was discarded, 1 ml perchloric acid 8% and 500 μl $CHCl_3$ were added to the cells. The mixture was vortexed before being centrifuged 5 min at $500 \times g$.

2.4. Polyamine determination

The intracellular polyamine analysis was performed using an HPLC procedure employing pre-column derivatisation with benzoyl chloride as described by Schenkel et al. [21]. Diaminohexane was used as an internal standard.

2.5. Statistical analysis

The parametric Student test [22] was used for statistical evaluation of the data after determination of the parametric error as described in Section 3.3.

3. Original model

3.1. Model description

The intracellular accumulation of a compound (putrescine, spermidine or spermine) can be described as follows:

$$\frac{dn_i(t)}{dt} = \underbrace{k_+ \cdot N \cdot S \cdot Ce(t)}_{\text{(in)}} - \underbrace{k_- \cdot N \cdot S \cdot \alpha \cdot Ci(t)}_{\text{(out)}} \quad (1)$$

where n_i (t) is the total intracellular quantity of the compound at instant t, N is the number of cells and S the membrane surface of one cell. $Ce(t)$ is the total extracellular molar concentration of the compound when $Ci(t)$ is the total intracellular molar concentration. α is the free fraction of the compound in the intracellular medium (supposed constant).

This monocompartmental model describes the cell transmembrane passages to the interior and to the exterior of the cell, which are characterised respectively by k_+ and k_-, the rate constants. As it is the total transport which was considered here, k_+ is then a combination of diverse constants characterising the diffusion, the active transport and even a potential adsorption.

Putting, $K_+ = \frac{k_+ \cdot N \cdot S}{V_i}$, $K_- = \frac{k_- \cdot N \cdot S \cdot \alpha}{V_i}$ and $C = \frac{n}{V}$ (with C = molar concentration, n = number of moles, V = volume and indexes i for intracellular and e for extracellular; V_i = intracellular volume of N cells), we obtain

$$\frac{dCi(t)}{dt} = K_+ \cdot Ce(t) - K_- \cdot Ci(t) \quad (2)$$

As $n_e(t) + n_i(t) = \text{constant} = n_e(o) + n_i(o)$ then $n_e(t) = n_e(o) + n_i(o) - n_i(t)$

Let $\frac{V_i}{V_e} = \lambda$, we have

$$Ce(t) = Ce(o) - Ci(t) \cdot \lambda + Ci(o) \cdot \lambda \quad (3)$$

with λ known by an extra experiment.

Solving the differential equation

$$\frac{dCi(t)}{dt} = -[K_+ \cdot \lambda + K_-]Ci(t) + K_+ \cdot [Ce(o) + \lambda \cdot Ci(o)] \quad (4)$$

leads to:

$$Ci(t) = \frac{K_+ \cdot Ce(o) + K_+ \cdot \lambda \cdot Ci(o)}{K_+ \cdot \lambda + K_-} - \frac{K_+ \cdot Ce(o) - K_- \cdot Ci(o)}{K_+ \cdot \lambda + K_-} \cdot e^{-A \cdot t} \quad (5)$$

with $A = K_+ \cdot \lambda + K_-$

Eq. (5) can be transformed in order to express $Ci(t)$ as a linear function of $Ce(o)$:

$$Ci(t) = \left[\frac{Ci(o)}{K_+ \cdot \lambda + K_-} \cdot (K_- \cdot e^{-A \cdot t} + K_+ \cdot \lambda)\right] + \left[\frac{K_+}{K_+ \cdot \lambda + K_-} \cdot (1 - e^{-A \cdot t})\right] \cdot Ce(o) \quad (6)$$

3.2. Determination of $Ci(o)$, K_+ and K_-

3.2.1. Cost function

All the experiments were performed three times. For each polyamine studied in a particular cell line, all the results were treated simultaneously, by minimising a least squares cost function defining the distance between the experimental data and the points calculated by the model. Samples treated by this cost function are equiweighted.

$$J = \sum_{i=1}^{n} \sum_{k=1}^{N_i} [Ci(t_{ik}, Ce_i(o)) - f(t_{ik}, Ce_i(o); K_+, K_-, Ci_i(o))]^2 \quad (7)$$

where $Ci(t_{ik}, Ce_i(o))$ are measured, $(t_{ik}, Ce_i(o); K_+, K_-, C_i(o))$ is the model function [Eq. (6)] and t_{ik} stands for the measurement time of sample k in experiment i. Note that $Ce(t_{i1}) = Ce_i(o)$ and $Ci(t_{i1}) = Ci_i(o)$.

This cost function has to be minimised with respect to K_+, K_- and $Ci_i(o)$. The corresponding non-linear optimisation problem is solved by using the simplex algorithm [23]. The software used is Matlab (The MathWorks Inc., Natick, MA, USA).

K_+ and K_- are considered as constants related to the transport of one polyamine in one cell line. On the opposite, $Ci_i(o)$ which is the

polyamine intracellular concentration at time $t = 0$, depends on the experiment (i). This parameter has not the same meaning as the measure of blank; this latter represents the $Ci_i(t)$ when $Ce_i(o) = 0$.

3.2.2. Parameter initialisation

The previous observation allows the blank measures to be used as initial estimation for the $Ci(o)$ parameters.

The K_+ initialisation was performed thanks to the simplification of Eq. (2) following the Euler approximation:

$$\frac{Ci(t_1) - Ci(o)}{t_1} \cong \left.\frac{\mathrm{d}Ci(t)}{\mathrm{d}t}\right|_{t=0} = K_+ \cdot Ce(o) - K_- \cdot Ci(o) \qquad (8)$$

where t_1 is the time at which Ci is measured. This equation can then be written as

$$Ci(t_1) = Ci(o) \cdot [1 - K_- \cdot t_1] + K_+ \cdot t_1 \cdot Ce(o) \qquad (9)$$

K_+ is then extracted from the slope $(K_+ \cdot t_1)$. Finally, the estimation of the K_- initialisation is calculated by replacing K_+ and $Ci(o)$ by the previously obtained value, in the Euler approximation.

In order to avoid identified values of $Ci(o)$, K_+ and K_- without physiological meaning, these parameters have been constrained so as to exhibit positive values. These constraints have been implemented by identifying (with the simplex algorithm of Matlab) the logarithms of the physical parameters. The exponential of these identified values [which are used for computing the cost function Eq. (7)] are then necessarily positive.

3.3. Parametric error

On the basis of the cost function $J(\theta)$(7) development in Taylor series, limited to the second order, around the identified parameters $\hat{\theta}$:

$$\hat{\theta} = \begin{bmatrix} \hat{K}_+ \\ \hat{K}_- \\ \hat{Ci}_1(o) \\ \hat{Ci}_2(o) \\ \hat{Ci}_3(o) \end{bmatrix}, \quad \in \mathbb{R}^5 \qquad (10)$$

the variance–covariance matrix can be approximated by

$$\hat{E} = [\tilde{\theta}\tilde{\theta}^T] \cong \hat{\sigma}^2 P(\hat{\theta}) \quad \in \mathbb{R}^{5\times 5} \qquad (11)$$

where $\hat{E} = [\tilde{\theta}\tilde{\theta}^T]$ is the estimate of the mathematical expectation of the parametric error

$$\tilde{\theta} = \theta - \hat{\theta} \quad \in \mathbb{R}^5; \qquad (12)$$

$$\hat{\sigma}^2 = \frac{F(\hat{\theta})}{\sum_{i=1}^{n} N_i - (n+2)} \quad \in \mathbb{R} \qquad (13)$$

is the estimate of the measurement noise variance which is nothing but the minimised least squares cost function divided by the difference between the number of measurements and the number of parameters;

$$P^{-1}(\hat{\theta}) = \sum_{i=1}^{n} \sum_{k=1}^{N_i} F_{ik}^T(\hat{\theta}) \cdot F_{ik}(\hat{\theta}) \quad \in \mathbb{R}^{5\times 5} \qquad (14)$$

is the sensitivity matrix (or Fisher information matrix) of the model with respect to the parameters where

$$F_{ik}(\hat{\theta}) = \left.\frac{\mathrm{d}f(t_{ik}, Ce_i(o); \theta)}{\mathrm{d}\theta}\right|_{\theta=\hat{\theta}} \quad \in \mathbb{R}^{1\times 5} \qquad (15)$$

is the Jacobian of the model with respect to the parameters. These latter relations [Eqs. (11)–(15)] are given in order to provide details about the whole method. Some commercial softwares include the computation of this variance–covariance matrix. The reader who is interested in more theoretical details can refer to Seber and Wild [24].

4. Results

4.1. Determination of global parameters K_+ and K_-

Table 1 shows the values of K_+ and K_- obtained for the transport of the different polyamines in the three cell lines when the data of the three experiments are simultaneously treated. Fig. 1 represents the treatment of spermidine transport data in P388/adr-10 cells. Table 1 shows the identified values of K_+ and K_- for which, each determined value is considered as the mean value of a Gaussian distribution from which a standard deviation can be approximated (see Section 3.3). We observe from this table that if values obtained for K_+ are significant with low standard deviations, values of K_- are very small with high standard deviation such that 0 is included within the confidence interval. This observation shows that there is no polyamine efflux in our experimental conditions.

Values of the error root mean square (ERMS) presented in the same table give an idea of the fit

Table 1
Comparison of the polyamine transport constants in sensitive and multidrug P388 cells

	K_+ K_-	Global parameters ± S.D. of the parametric error (min^{-1})	ERMS (1)	ERMS (2)
Putrescine				
P388/s	K_+	0.203 ± 0.008	113	104
	K_-	0.00 ± 0.02	251	113
			208	52
P388/adr-1	K_+	0.071 ± 0.001	59	52
	K_-	0.0009 ± 0.004	34	30
P388/adr-10	K_+	0.164 ± 0.006	178	127
	K_-	0.002 ± 0.02	85	67
			255	170
Spermidine				
P388/s	K_+	0.19 ± 0.03	503	414
	K_-	0.003 ± 0.03	580	476
			366	275
P388/adr-1	K_+	0.12 ± 0.01	277	270
	K_-	0.001 ± 0.02	236	208
			277	234
P388/adr-10	K_+	0.21 ± 0.03	299	277
	K_-	0.01 ± 0.03	525	385
			486	128
Spermine				
P388/s	K_+	0.14 ± 0.01	285	281
	K_-	0.001 ± 0.01	152	120
			177	123
P388/adr-1	K_+	0.39 ± 0.03	555	281
	K_-	0.002 ± 0.03	223	118
			401	238
P388/adr-10	K_+	0.31 ± 0.01	272	148
	K_-	0.003 ± 0.02	327	144
			98	98

Notes. Global transport rate constants [K_+ and K_-, (min^{-1})], determined for putrescine, spermidine and spermine transport in sensitive P388 cells and multidrug resistant (P388/adr-1 and P388/adr-10 cells) (means ± S.D.). ERMS represents the error root mean square. ERMS calculated for the results of the model applied simultaneously to the three experiments (1) are compared to those calculated for the results of each linear regression (2).

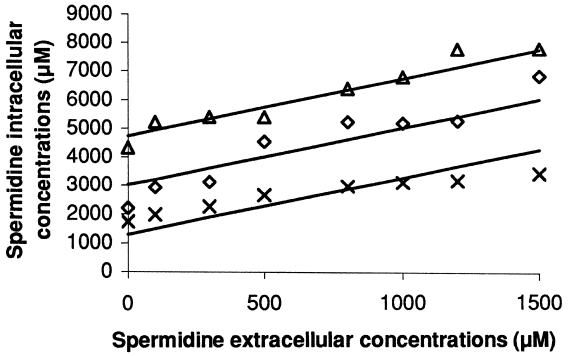

Fig. 1. Spermidine intracellular concentrations evolution as a function of spermidine extracellular concentrations. Measures taken after 10 min contact in classical culture conditions. Points correspond to experimental data and lines to the identified model. The three experiments on P388/adr-10 are treated together.

between measured $Ci(t)$ and the calculated ones by the model, for each experiment.

$$\mathrm{ERMS} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(Ci_{\mathrm{measured}}(t_i) - Ci_{\mathrm{identified}}(t_i)\right)^2} \tag{16}$$

The ERMS (1) values in Table 1 correspond to the global identification of $\{K_+,\ K_-,\ Ci_1(o),\ Ci_2(o),\ Ci_3(o)\}$ in the model of Eq. (6) on the basis of three experiments, whereas the ERMS (2) values correspond to three different identifications of $\{K_+,\ K_-,\ Ci(o)\}$ in the model of Eq. (6).

The comparison of ERMS shows that for most of the experiments, values of ERMS are not significantly higher for the global treatment than that of the separated treatment. This means that our model well describes the phenomenon of polyamine transport. These results are confirmed by cross-validation (see Section 4.2). The difference between ERMS (1) and (2) can be explained by the number of parameters identified in each case. Actually, the global treatment needs the identification of five parameters with 24 experimental points, whereas the separated treatments need the identification of three parameters with each set of eight experimental points (which means globally nine parameters with 24 experimental points).

4.2. Model cross-validation

The cross-validation consists in testing the model accuracy with data which have not been used for the parameter identification. In this case, one has to determine the value of the constants K_+ and K_- by treatment of two experiments out of three and then injecting them in the third experiment. The model is valid when the $Ci(o)$ for the third experiment is significant from a

Table 2
Results of cross-validation for two sets of experiments

		Identified *Ci(o)*	Measured *Ci(o)*	K_+	K_-	ERMS[a]	Global ERMS[b]
Putrescine P388/s	1	368 ± 31	169	0.167 ± 0.007	4.10^{-6} ± 0.02	176	113
	2	75 ± 6	86	0.167 ± 0.007	4.10^{-6} ± 0.02	174	251
	3	492 ± 65	178	0.167 ± 0.007	4.10^{-6} ± 0.02	297	208
Cross-validation					Sum	647	572
Spermine P388/adr-10	1	1659 ± 340	2005	0.31 ± 0.03	0.005 ± 0.04	353	272
	2	2598 ± 533	1952	0.31 ± 0.03	0.005 ± 0.04	331	327
	3	1343 ± 73	1153	0.31 ± 0.03	0.005 ± 0.04	98	98
Cross-validation					Sum	782	697

Notes. Cross-validation of the model consists in determining K_+ and K_- values by treatment of two experiments together and then in injecting these values in the third experiment. The evaluation of the identified *Ci(o)* and ERMS quantifies the fitting of the global model to the points of the three experiments.

[a] ERMS is calculated on the basis of identification of K_+, K_- with exp. 1 and 2 and values injected in 3.

[b] ERMS is calculated on the basis of identification of K_+, K_- with experiments 1, 2 and 3.

physiological point of view and the ERMS calculated for this experiment is not significantly higher than that of the two other experiments. Table 2 presents the results for cross-validation made with two sets of three experiments. Similar results were obtained with other sets of experiments. From this table it can be concluded that the global model well fits the three experiments. The total ERMS for the three experiments are very close in spite of the differences between separated values of ERMS.

4.3. Determination of $Ci(o)$

Polyamine intracellular concentrations at time $t = 0$ was the third parameter to be identified (see above). The values of $Ci(o)$ identified for each experiment after a global treatment by the model, are presented in Table 3 with their standard deviation and compared to the intracellular concentrations measured when $Ce(o) = 0$.

This table shows that for 53% of the experiments, the measured $Ci(t)$ for $Ce(o) = 0$ is included in the 95% confidence interval of the identified $Ci(o)$. This result confirms that those two notions are slightly different and cannot be assimilated.

4.4. Determination of k_+

As previously described, k_+ is given by

$$k_+ = \frac{K_+ \cdot V_i}{N \cdot S} \tag{17}$$

which can be summarised to

$$k_+ = K_+ \cdot r/3 \tag{18}$$

as V_i is the volume of N cells with a membrane surface S, r is the cell radius, determined from the cellular volume. Table 4 contains the k_+ constant values for putrescine, spermidine and spermine uptake by P388/s, P388/adr-1 and P388/adr-10 cells.

This table allows statistical comparisons between cell lines and shows that putrescine as well as spermidine enters significantly more slowly

Table 3
Values of intracellular concentrations (Ci) measured when $Ce(o) = 0$ and identified for $t = 0$ [$Ci(o)$]

	Intracellular concentrations when $Ce(o) = 0$ (μM)	$Ci(o)$ identified $\pm$S.D. (μM)
Putrescine		
P388/s	169	268 ± 20
	86	0 ± 0
	178	395 ± 30
P388/adr-1	150	199 ± 4
	95	145 ± 3
P388/adr-10	87	177 ± 14
	179	145 ± 11
	244	388 ± 30
Spermidine		
P388/s	4682[a]	5884 ± 2474
	8369[a]	8185 ± 3442
	4817[a]	5064 ± 2129
P388/adr-1	5216[a]	5687 ± 1045
	4471[a]	4594 ± 844
	3824[a]	4191 ± 770
P388/adr-10	4326[a]	5064 ± 1339
	2191	3229 ± 854
	1735	1366 ± 361
Spermine		
P388/s	2600[a]	2983 ± 414
	1868[a]	1959 ± 272
	2146[a]	2507 ± 348
P388/adr-1	2798[a]	3815 ± 529
	3427[a]	3296 ± 475
	2227[a]	1992 ± 276
P388/adr-10	2005	1643 ± 150
	1952	2577 ± 235
	1153[a]	1330 ± 121

Values of $Ci(o)$ [$Ci(t)$ when $t = 0$] were identified for each experiment by the global model and compared to the $Ci(t)$ measured for $Ce(o)$ equal to 0 (mean of two determinations).
[a] $Ci(t)$ for $Ce(o) = 0$ included in the 95% confidence interval of $Ci(o)$ ($t = 0$).

P388/adr-1 cells than the two other cell lines. Spermine presents totally different results, in fact, multidrug resistant P388 cells present k_+ constants for spermine, more than twice higher than that of the sensitive line.

When polyamines are compared, a significant difference is seen between the three k_+ obtained for multidrug resistant P388 cells. Putrescine has the lowest k_+ and spermine, the highest. In

Table 4
Values of kinetic parameter k_+

k_+ (μm/min)	P388/s[a] Mean ± S.D.	P388/adr-1[b] Mean ± S.D.	P388/adr-10[c] Mean ± S.D.
Putrescine*	0.33 ± 0.01	0.125 ± 0.002	0.30 ± 0.01
Spermidine**	0.30 ± 0.05	0.21 ± 0.02	0.38 ± 0.05
Spermine***	0.22 ± 0.02	0.69 ± 0.05	0.56 ± 0.02

Notes. Kinetic parameter k_+ (means ± S.D.) for putrescine, spermidine and spermine transport in P388/s, P388/adr-1 and P388/adr-10 cell lines ($N = 24$).
* Significant difference between P388/adr-1 and the two other cell lines ($\alpha < 0.1\%$); ** significant difference between P388/adr-1 and P388/adr-10 ($\alpha < 1\%$); *** significant difference between the three cell lines ($\alpha < 5\%$) and P388/s significantly different from the two multidrug resistant cell lines with $\alpha < 0.1\%$.
[a] Significant difference between putrescine and spermine ($\alpha < 1\%$).
[b] Significant difference between spermine and the two other polyamines ($\alpha < 0.1\%$).
[c] Significant difference between putrescine and spermine ($\alpha < 0.1\%$).

sensitive P388 cells, k_+ obtained for putrescine and spermidine are significantly higher than that of spermine. These results could partly explain the toxicity of these natural polyamines observed previously on the same cell lines [25].

4.5. Polyamines endogenous content of P388 cell lines

Nine blank measures were determined for each cell line (three experiments for each of the three polyamines) allowing the calculation of the mean putrescine, spermidine and spermine endogenous content of P388/s, P388/adr-1 and P388/adr-10 cell lines. Results are presented in Table 5.

Table 5 shows that the P388/adr-1 putrescine endogenous content is significantly different from the two other cell line's contents. This observation could explain the lower putrescine uptake by this cell line. No significant difference is observed for spermidine and spermine, between the three cell lines. It can be deduced from these results that the differences observed between P388 phenotypes for polyamines transport are not only due to potentially different polyamines endogenous contents.

Table 5
Polyamines endogenous contents

Cell lines/ polyamines	Putrescine (μM)	Spermidine (μM)	Spermine (μM)
P388/s	171 ± 131	5111 ± 1113	2296 ± 588
P388/adr-1	310 ± 153*	4807 ± 1217	1807 ± 774
P388/adr-10	128 ± 53	3748 ± 1785	1769 ± 646

Notes. Polyamines endogenous contents expressed in μM (means ± S.D.) of P388/s, P388/adr-1 and P388/adr-10 cells ($n = 9$).
* Significantly different from the two other cell lines ($\alpha = 2\%$).

5. Discussion

The importance of polyamines for cell proliferation has been established for many years. One or two polyamine carriers have been characterised in different cell lines. Nevertheless, until now, the influence of multidrug resistance on polyamine uptake, has not been studied. The aim of the present work is therefore to compare putrescine, spermidine and spermine transport by sensitive and two multidrug resistant P388 cell lines.

As explained in the introduction, the Michaëlis–Menten model is not applicable to every experimental condition. For this reason, an experimental model has been developed to describe uptake and efflux in a sole equation, each characterised by a rate constant (K_+ and K_-). The proposed methodology allows to identify these global constants for a given cell line and a given polyamine, and this despite the variations of cell conditions. The effect of these latter is taken into account thanks to the identification of the initial polyamine concentration $Ci(o)$ for each experiment.

Identified parameters for the transport of putrescine, spermidine and spermine in sensitive and multidrug resistant P388 cells show that there is no efflux during the 5 or 10 min of the experiment. This suggests that P-gp170 [15–17] which was demonstrated to be present in our multidrug resistant P388 cell lines, does not efflux

polyamines. This result was also indirectly demonstrated when addition of verapamil, a well known P-gp170 revertant, did not increase the putrescine and spermidine uptake in multidrug resistant K562 cells [18].

This comparison also shows that multidrug resistant P388/adr-1 cells present a decreased uptake for putrescine which is the first polyamine of the metabolic cycle. This same cell line shows also the highest putrescine endogenous content. On the opposite, the uptake constant k_+ determined for spermine in both multidrug resistant P388 cells is approximately triple that of the sensitive.

The increased spermine transport might be a consequence of the decreased putrescine uptake as it is one of the spermine precursors. However, different theories could explain these polyamine transport modifications. Among them the fact that multidrug resistance phenomenon induces membrane fluidity modifications [26–28] and variations of the membrane protein content [29–31]. Finally, multidrug resistance might simply have selected mutant phenotypes for polyamine need or transport. Nevertheless, no difference between the three cell lines endogenous spermidine and spermine contents might explain these modifications.

The uptake constants identified in this study could partly explain the differences of cytotoxicity showed by putrescine, spermidine and spermine between sensitive and multidrug resistant P388 cells [25]. Indeed, for each polyamine, toxicity was more important in P388 cell line presenting the highest k_+.

In conclusion, on the one hand, the use of this original model allows the identification of global uptake and global efflux parameters while taking the experimental variations into account by the identification of the initial intracellular polyamine concentrations. On the other hand, results show that multidrug resistance induces or is accompanied by important modifications of the polyamine uptake, principally for putrescine and spermine but that none of the three polyamines studied is excreted by the P-gp170. This observation added to the fact that spermine enters more rapidly in multidrug resistant P388 cells suggests that spermine could be an interesting investigation way in the anti-proliferative polyamine analogues research [32].

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