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Biochimica et Biophysica Acta 1611 (2003) 161–170
BIOCHIMICA ET BIOPHYSICA ACTA
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Relevance of drug uptake, cellular distribution and cell membrane fluidity to the enhanced sensitivity of Down's syndrome fibroblasts to anticancer antibiotic—mitoxantrone

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Received 19 November 2002; received in revised form 17 January 2003; accepted 28 January 2003

Abstract

Sensitivity of human fibroblasts derived from Down's syndrome (DS) individuals (S-240, T-158, T-74, T-164) and normal donors (S-126, WA-1) to anticancer antibiotic—mitoxantrone (1,4-dihydroxy-5,8-bis((2-((2-hydroxy-ethyl)amino)ethyl)amino)-9,10-anthracenedione dihydrochloride; MIT) and its relationship to the transport rate, cellular distribution and interaction with cell membrane were studied. The survival assay showed that MIT was more toxic to trisomic fibroblast lines than to normal cells. Studies of transport kinetics indicated that the amount of drug taken up and extruded by DS cells was diminished, compared to control cells. In contrast, the cellular level of MIT associated with DNA was greater in trisomic than in normal cells. The fluorescence anisotropy measurements of TMA-DPH and 12-AS demonstrated that the fluidity of the polar region of the outer lipid monolayer of DS cell membrane was decreased in comparison with normal cells. MIT treatment decreased fluidity of the inner hydrophobic region of plasma membrane, but only slightly influenced the fluidity of the outer surface of the cell membrane. Finally, we concluded that lowered membrane fluidity, diminished amount of MIT extruded by cells and the enhanced level of the drug associated with DNA could be responsible for the enhanced sensitivity of DS fibroblasts to the MIT treatment.

Keywords: Down's syndrome; Mitoxantrone; Survival; Drug accumulation; Fluidity

1. Introduction

Down's syndrome (DS), caused by a complete or partial trisomy of chromosome 21 (21q22.1), is one of the most prevalent chromosomal aneuploidy in human liveborns [1]. In addition to mental retardation, DS is associated with plenty of serious pathological disorders [2]. Individuals with DS have pronounced risk of developing diabetes [3], Alzheimer's type dementia [4] and haematological neoplasms [5–7]. DS is, in particular, frequently associated with childhood cancer [8,9]. Affected children have a 10- to 30-fold enhanced risk of acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML), especially during the first 4 years of life [7]. Within the broad category of acute nonlymphoblastic leukemias (ANLL), transient megakaryocytic leukemia occurs almost exclusively in DS individuals and the other form, acute nonlymphocytic leukemia subtype M7 (ANLL-M7) accounts for 50% of DS leukemias and is extremely rare in the unaffected population [10].

The etiological role of chromosome 21-linked genes in leukemogenesis is still poorly understood. Well-documented biochemical defect that has been associated with DS is an overexpression of Cu–Zn superoxide dismutase (SOD-1) (EC: 1.15.1.1.), an enzyme catalysing the dismutation of superoxide anion radicals to H_2O_2 and O_2 [11]. Recently, several new genes from the DS critical region have been reported to be expressed at higher levels in the brain and heart; however, the precise consequences of this require further explanation [12,13].

The enhanced prevalence of malignant haematological disorders in patients with DS makes the elucidation of the specific response of trisomic cells to anticancer drugs of special interest. In our study, we selected mitoxantrone (1,4-dihydroxy-5,8-bis((2-((2-hydroxy-ethyl)amino)ethyl)amino)-9,10-anthracenedione dihydrochloride) (novantrone; MIT)—an antineoplastic drug, structurally related to anthracyclines. MIT is the first fully synthesised tetracyclic

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doi:10.1016/S0005-2736(03)00051-8

anthracenedione derivative with two symmetrical aminoalkyl side chains, introduced into cancer therapy in the late 1970s [14]. Since then, it has been commonly used for the treatment of many neoplastic diseases such as leukemias, sarcomas and breast cancers [15]. Generally, it is postulated that like the anthracyclines, MIT intercalates into DNA and causes DNA–protein cross-links and protein-associated single-stranded and double-stranded breaks in the DNA [16,17]. Moreover, MIT stabilises the DNA–topoisomerase II cleavable complex and inhibits DNA synthesis [18]. The ability of MIT to undergo one-electron reduction and generate free radicals in a manner similar to that of anthracyclines is considerably decreased [19]. Like anthracyclines, MIT sensitises cells to both hyperthermia and ionising radiation [20] and it can stimulate apoptosis in a variety of cells [21].

Considering diminished DNA-repair capacity [22–24], the disturbance of the reactive oxygen species metabolism in DS persons [25] and the fact that cytotoxic effect of MIT is related to DNA strand breaks and participation in free-radical generation [19], it may be expected that the response of trisomic (+21) cells to MIT treatment may be different than that of non-DS cells. Currently available data, for obvious reasons concern, first of all, different kinds of malignant cells, whereas the cancer chemotherapy remains still largely nonspecific and the majority of the antineoplastic drugs are toxic to normal cells as well [14,15,20,26,27]. In this context, the elucidation of the response of non-malignant cells to anticancer drugs is of special importance. For this reason, the purpose of this study was to compare the cytotoxicity, the uptake dynamics and the interaction of MIT with cell membranes of human normal (S-126 and WA-1) and trisomic, with respect to chromosome 21 (S-240, T-158, T-74 and T-164) fibroblast lines.

2. Materials and methods

2.1. Drug and chemicals

MIT (novantrone) (1,4-dihydroxy-5,8-bis((2-((2-hydroxy-ethyl)amino)ethyl)amino)-9,10-anthracenedione dihydrochloride) was purchased from Pharmaceutical Company Jelfa (Jelenia Góra, Poland); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorescence probes: 1-(4-trimethyl-ammoniumphenyl)-6-phenyl-1,3,5-hexatriene) (TMA-DPH) was from Molecular Probes (Eugene, OR, USA) and 12-(9-anthroyloxy)-stearic acid (12-AS) was from Sigma. The Eagle minimal essential medium was supplied by Sera and Vaccines Factory (Lublin, Poland) and Triton X-100 was obtained from POCh (Gliwice, Poland). The newborn calf serum and lactoalbumin hydrolysate were from Gibco BRL (Edinburgh, Scotland). Gentamycin was purchased from Biochemie G.m.b.H. (Vienna, Austria).

2.2. Cell lines and cell culture conditions

Human fibroblasts (Table 1) derived from DS persons (S-240, T-158, T-74 and T-164) and from non-trisomic volunteers (S-126 and WA-1) were purchased from tissue bank of the Centre of Child Health (Warsaw, Poland). Cell lines were regularly tested to ensure they were free of contamination or infections and re-established from frozen stocks at periods of 3–4 weeks. Fibroblasts were routinely grown as a monolayer in tissue culture flasks (Nunck; Naperville, IL, USA), in a humidified atmosphere of 5% CO_2 at 37 °C in Eagle minimal essential medium supplemented with 10% newborn calf serum, 10% lactoalbumin hydrolysate and gentamycin solution (50 mg/cm^3). For the experiments, exponentially growing fibroblasts between the 5th and 15th passage were used.

2.3. Cell survival assay

The antiproliferative efficacy of MIT was examined in 96-well flat-bottom microtiter plates (Costar, D. Dutscher, France) by using the standard MTT colorimetric procedure [28]. This indirect method is based on the ability of the mitochondrial dehydrogenases of metabolically viable cells to reduce the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to blue formazan product which can be measured spectrophotometrically. For this purpose, after trypsinization and cell counting, fibroblast suspension was diluted in culture medium to achieve a final cell density of 10^4 cells/cm^3. Subsequently, 0.2 cm^3 of this cell suspension was seeded into each well of 96-well microtiter plates. After an overnight incubation, 0.05 cm^3 of MIT solution (at concentrations ranging 0–150 μmol/dm^3 for normal cell lines and 0–100 μmol/dm^3 for DS cell lines) was added and cells were incubated for 2 h (37 °C, 5% CO_2). Wells containing no drug were used as control and wells containing no cells as blanks. After desired time, the MIT solutions (or PBS in the case of control samples) were discarded and replaced by fresh culture medium and cells were allowed to grow for 3 days (37 °C, 5% CO_2). Then, 0.05 cm^3 of MTT solution in PBS (6×10^{-3} mol/dm^3) was added to each well and incubation was continued for further 4 h. Subsequently, culture medium was removed and the resulting formazan crystals were solubilised in 0.1 cm^3 of DMSO. The extent of MTT reduction to formazan within cells was calculated by the measurement of the absorbance at 570

Table 1
Characteristics of the fibroblast lines

Cell line	Karyotype	Origin	Donor age
S-126	46, XY	skin	26 years
WA-1	46, XX	skin	16 years
S-240	47, XY, +21	skin	21 years
T-158	47, XX, +21	skin	18 years
T-74	47, XX, +21	fetus	–
T-164	47, XX, +21	fetus	–

nm, using a microplate reader (Awareness Technology Inc., Palm City, FL, USA). The percentage of survived cells was calculated by dividing the mean absorbance values of the drug-treated samples by that of untreated ones (control). The IC_{50} values, defined as the drug concentration that reduces cell proliferation to 50% compared with the untreated cells, were determined directly from semilogarithmic concentration–response curves. Each experiment was performed in 6-fold and was carried out independently six times.

2.4. Cellular drug accumulation and distribution

The studies on the dynamics of the MIT transport through the cell membrane and the cellular partition of this compound were carried out according to the modified method [29], previously described by Andreoni et al. [30,31]. For this purpose, the equal number of cells (10^6) in 3 cm^3 of culture medium was seeded into series of 35-mm Petri dishes. After an overnight incubation (37 °C, 5% CO_2), medium was removed and replaced by 3 cm^3 of the MIT solution (3×10^{-6} mol/dm^3) in culture medium. An equal volume of PBS was added to control samples. The MIT solution (3×10^{-6} mol/dm^3), without cells, was used as references for initial drug concentration. At a desired length of time of the MIT treatment, medium was aspirated from dishes, to remove the drug not associated with the cell monolayer and centrifuged (1500 rpm, 5 min). To estimate the amount of the drug in supernatants, absorbance of the samples at 610 nm, using a Cary 1 UV–Visible spectrophotometer (Varian, Australia Pty Ltd., Mulgrave, Australia) was measured. The intracellular partition of MIT taken up by cells between nucleus (DNA-bound drug fraction) and the rest of the cell volume (free drug fraction) was determined as follows. Cell monolayers were washed gently three times with 2 cm^3 of PBS. Subsequently, 3 cm^3 of culture medium supplemented with Triton X-100 solution (0.05%) was added to each dish, to permeabilize cell membranes. After 15 min, medium with Triton X-100 was transferred to a cuvette and absorbance at 610 nm was measured. The quantity of drug in extracellular medium and associated with cells were calculated from standard curves, prepared by measuring the absorbance of known MIT concentrations. All experiments were repeated five times.

To determine the parameters of MIT transport through cellular membrane, a model of transport kinetics, involving three compartments: (M)—extracellular medium containing the M amount of drug; (F)—cellular compartment containing the F amount of relatively free drug; and (B)—containing the B amount of MIT associated with DNA, was proposed. The cellular amount of drug ($C=B+F$) was calculated from the following equation [31]:

$$C = M_{tot} - M_t \quad (1)$$

where: M_{tot} symbolises the total content of drug to which fibroblasts were initially submitted and M_t is the amount of MIT in external medium at various times of incubation.

Data representing the cellular content of the drug were fitted to a third-order polynomial [determination coefficients (R^2) for plots were always greater than 0.95]. The initial velocity of MIT uptake ($I_{t=0}$) was calculated as a value of a first derivative of the curve representing time dependence of MIT amount in external medium. Uptake rate constants of MIT transport were calculated according to the assumption that MIT influx follows a first order equation [32,33]:

$$I_{t=0} = k_{in} M_{tot} \quad (2)$$

where: k_{in} is the influx rate constant.

The amount of drug taken up by cells (U) was next calculated from the rate equation:

$$U = M_{tot}(1 - e^{-k_{in}t}) \quad (3)$$

The kinetic parameters for drug extruded by fibroblasts (k_{out} and $E_{t=0}$) were calculated in a similar manner from curves representing the time dependence of the subtraction of the intracellular amount of drug (C) from the amount of drug taken up by cells (U) within the same time of MIT treatment.

2.5. Fluorescence anisotropy measurement

To estimate the cell membrane fluidity of normal and DS fibroblasts submitted to MIT treatment, the steady-state fluorescence anisotropy (r) of fluorescence probes TMA-DPH and 12-AS was measured. For this purpose, the same number of fibroblasts (3×10^5 cells/cm^3) suspended in 1.8 cm^3 of culture medium was disposed into the set of test tubes. Then, 0.05 cm^3 of MIT solutions of various concentrations ($2–100 \times 10^{-6}$ mol/dm^3) was added. Samples containing cell suspensions in medium without drug were

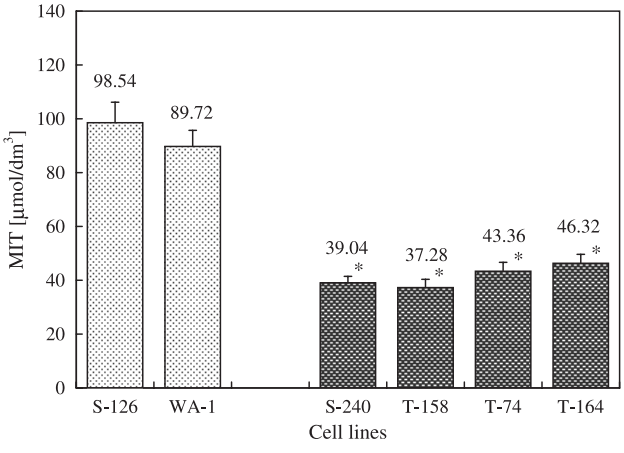

Fig. 1. IC_{50} parameters (drug concentration reducing cell growth to 50% as compared with untreated cells) for normal (S-126 and WA-1) and trisomic (S-240, T-158, T-74 and T-164) human fibroblasts treated with MIT. Data are expressed as means ± S.D. for six independent experiments. * Significantly different when compared to the mean value obtained for normal cell lines ($P<0.001$).

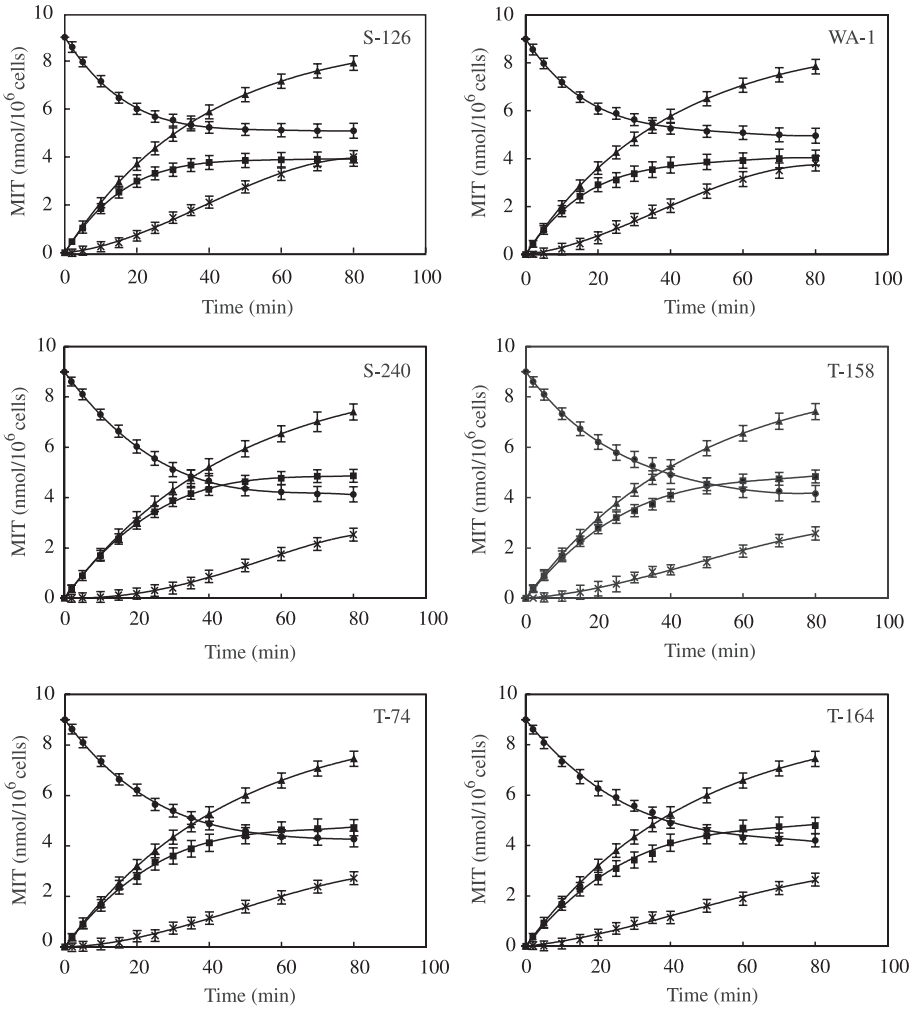

Fig. 2. Time dependence of MIT taken up (▲) and MIT removed (×) by human normal (S-126 and WA-1) and trisomic (S-240, T-158, T-74 and T-164) fibroblasts. (●)—amount of drug in external medium. (■)—amount of drug associated with cells. Data are expressed as means ± S.D. for five independent experiments.

used as controls. After incubation (2 h in humidified atmosphere of 5% CO_2, at 37 °C), samples were centrifuged (1500 rpm, 5 min), the supernatant was aspirated and the cell pellet was resuspended in 1.8 cm^3 of PBS. To determine cell membrane fluidity, fibroblast suspensions were incubated either with TMA-DPH or 12-AS (final concentration in samples—10^{-6} mol/dm^3) at room temperature for 5 or 10 min, respectively. The measurements were carried out with a Perkin Elmer spectrofluorometer model LS-5 B (Beaconsfield, Buckinghamshire, England). The excitation and the emission wavelengths were $\lambda_{ex}=358$ nm and $\lambda_{em}=428$ nm for TMA-DPH and $\lambda_{ex}=360$ nm and $\lambda_{em}=471$ nm for 12-AS. Fluorescence anisotropy was calculated according to the equation [34]:

$$r = (I_{vv} - I_{vh}G)/(I_{vv} + 2I_{vh}G) \quad (4)$$

where: I_{vv} and I_{vh} are the intensities of the light emitted, respectively, parallel and perpendicular to the direction of the vertically polarised excitation light and G (Eq. (5)) is the correction factor for the optical system, given by the ratio of the vertically to the horizontally polarised emission components when the excitation light is polarised in the horizontal direction:

$$(G = I_{hv}/I_{hh}) \quad (5)$$

According to Shinitzky and Barenholz [34], the fluorescence anisotropy values are inversely proportional to cell membrane fluidity. A high degree of fluorescence anisotropy represents a high structural order or low cell membrane fluidity. Studies on membrane fluidity performed with two fluorescent probes TMA-DPH and 12-AS which locate in different regions of the bilayer yield information about the rigidity of the cell membrane near the lipid polar heads (TMA-DPH) [35] and inside the hydrophobic lipid core (12-AS) [36]. All the fluorescence measurements were corrected

Table 2
Transport parameters of MIT in human normal (S-126 and WA-1) and trisomic (T-164, S-240, T-158 and T-74) fibroblasts

Cell lines		$K_{in} \times 10^2$ [min^{-1}]	$I_{t=0}$ [nmol $min^{-1}/10^6$cells]	$U_{t=60}$ [nmol/10^6cells]	$K_{out} \times 10^5$ [min^{-1}]	$E_{t=0} \times 10^4$ [nmol $min^{-1}/10^6$cells]	$E_{t=60}$ [nmol/10^6cells]
Normal cells							
A	S-126	2.65 ± 0.11	0.238 ± 0.010	7.17 ± 0.14	9.60 ± 0.81	8.64 ± 0.73	3.28 ± 0.11
B	WA-1	2.57 ± 0.12	0.231 ± 0.011	7.08 ± 0.16	7.94 ± 0.86	7.14 ± 0.78	3.15 ± 0.10
DS cells							
C	S-240	2.15 ± 0.98	0.194 ± 0.009	6.53 ± 0.12	2.28 ± 0.16	2.05 ± 0.14	1.76 ± 0.12
D	T-158	2.17 ± 0.96	0.195 ± 0.008	6.55 ± 0.13	2.61 ± 0.15	2.35 ± 0.13	1.87 ± 0.13
E	T-74	2.19 ± 0.13	0.197 ± 0.011	6.58 ± 0.16	2.93 ± 0.20	2.63 ± 0.18	1.96 ± 0.14
F	T-164	2.19 ± 0.12	0.197 ± 0.011	6.57 ± 0.16	2.74 ± 0.18	2.46 ± 0.16	1.89 ± 0.15
ANOVA I							
F		21.71	21.71	21.82	241.15	241.15	178.52
df		5; 30	5; 30	5; 30	5; 30	5; 30	5; 30
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Tukey's test		*AB CDEF*	*AB CDEF*	*AB CDEF*	*A B CDEF*	*A B CDEF*	*AB CDEF*

K_{in}—influx rate constant; $I_{t=0}$—initial influx; $U_{t=60}$—drug taken up by cells within 60 min; K_{out}—efflux rate constant; $E_{t=0}$—initial efflux; $E_{t=60}$—drug removed by cells within 60 min. Data are expressed as means ± S.D. for six independent experiments.

for the contribution of light scattering by performing control experiments on cells without fluorescent probes added and were repeated six times.

2.6. Statistical analysis

All data were expressed as mean and standard deviation of five or more separate experiments. One-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey test) were used to assess statistical differences in this study. A *P*-value of less than 0.01 was considered statistically significant. All statistical analyses were done using STATISTICA software (2000) (StatSoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Cellular sensitivity to MIT

The cell survival assay showed that the exposure of human fibroblasts to MIT resulted in a successive decrease of cell survival. Comparison of the IC_{50} values, determined directly from dose–response curves (data not presented), for normal (S-126, WA-1) and trisomic (S-240, T-158, T-74, T-164) cell lines (Fig. 1), indicated that antiproliferative efficacy of MIT was significantly higher for fibroblasts derived from DS individuals (average: 41.50 ± 4.60 μmol/dm^3) than for normal (average: 94.13 ± 7.96 μmol/dm^3) cell lines ($P < 0.01$).

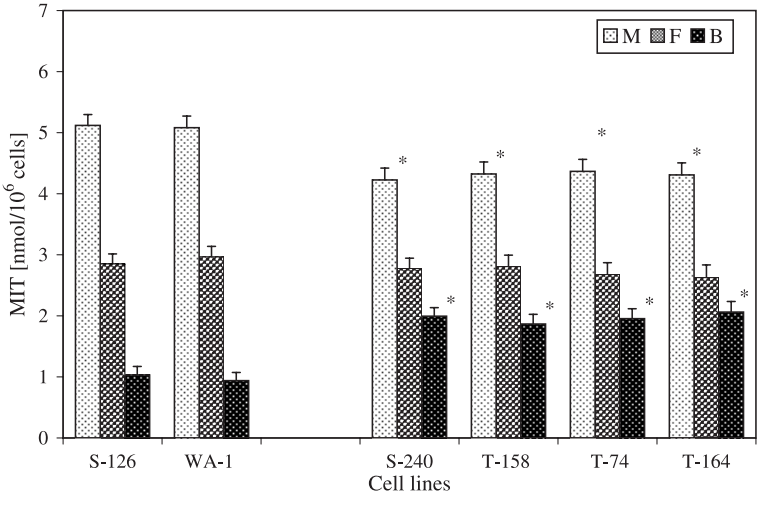

Fig. 3. Cellular distribution of MIT in normal (S-126 and WA-1) and trisomic (S-240, T-158, T-74 and T-164) human fibroblasts. M—amount of MIT in external medium; F—amount of cellular free MIT; B—amount of MIT bound to DNA. Data are expressed as means ± S.D. for five independent experiments. * Significantly different when compared to the mean value obtained for normal cell lines ($P < 0.001$).

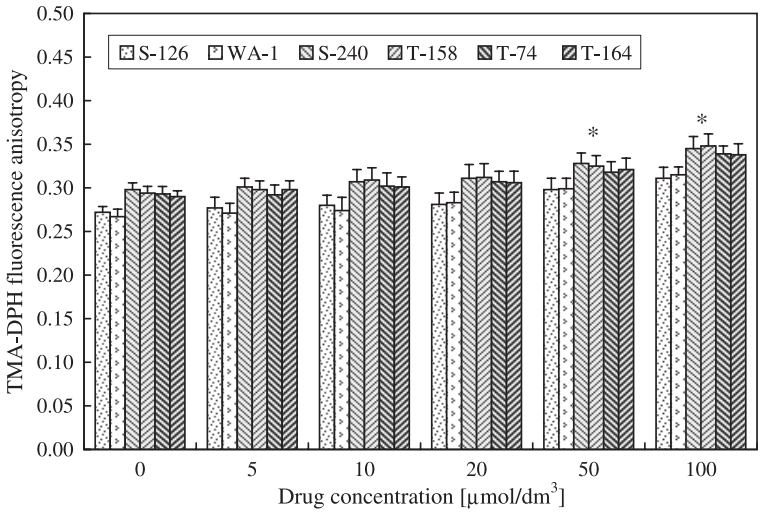

Fig. 4. Influence of MIT on fluorescence anisotropy of TMA-DPH in normal (S-126 and WA-1) and trisomic (S-240, T-158, T-74 and T-164) human fibroblasts. Data are expressed as means ± S.D. for six independent experiments. * Significantly different when compared to value obtained for non-treated cells ($P < 0.01$).

3.2. Transport kinetics and cellular distribution

The transport kinetics of MIT through cellular membrane, estimated indirectly from the measurement of the drug absorbance in external medium, indicated the substantial differences in cellular uptake of this compound by normal and DS cells. The analysis of curves representing the time dependence of the amount of drug taken up and extruded by cells, during the same time (Fig. 2), and the kinetic parameters, calculated from them (Table 2), showed that the amount of drug taken up for 60 min of incubation was greater for normal (average: 7.13 ± 0.15 nmol/10^6 cells) than for DS cells (average: 6.56 ± 0.15 nmol/10^6 cells). Comparison of the values representing the amount of MIT removed by fibroblasts during 60 min of incubation (average: 3.22 ± 0.12 nmol/10^6 cells—normal cells; 1.87 ± 0.15 nmol/10^6 cells—trisomic cells) suggests that efflux of MIT from cells was markedly lowered in DS fibroblast lines as compared to non-trisomic ones (Table 2).

Analysis of the cellular distribution of MIT (Fig. 3) showed that the amount of MIT bound to DNA is greater for DS fibroblast lines (average: 1.97 ± 0.11 nmol/10^6 cells) than for normal cells (average: 0.98 ± 0.08 nmol/10^6 cells). In contrast, no statistically significant differences in the amount of cellular free MIT, between normal and trisomic fibroblasts (average: 2.92 ± 0.17 nmol/10^6 cells—normal cells; 2.73 ± 0.14 nmol/10^6 cells—trisomic cells) were observed.

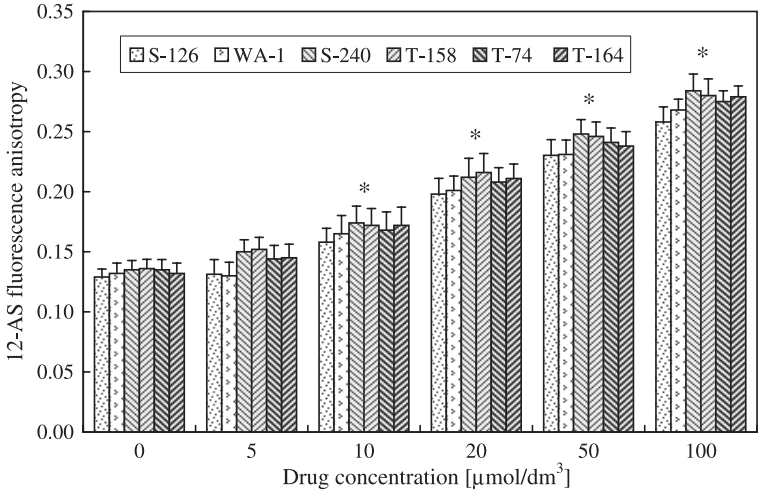

Fig. 5. Effect of MIT on fluorescence anisotropy of 12-AS in normal (S-126 and WA-1) and trisomic (S-240, T-158, T-74 and T-164) human fibroblasts. Data are expressed as means ± S.D. for six independent experiments. * Significantly different when compared to value obtained for non-treated cells ($P < 0.01$).

3.3. Cell membrane fluidity

The fluorescence anisotropy values of fluorescent dyes TMA-DPH and 12-AS, calculated for untreated cells, showed that the DS fibroblasts had ($P<0.01$) lower fluidity near the outer polar surface of cell membrane (average: $r=0.296\pm0.007$) than control cells (average: $r=0.268\pm0.007$) ($P<0.01$) (Fig. 4). Contrary to this, the fluidity of the hydrophobic lipid region of the cell membrane was nearly the same for both cell types (average: $r=0.131\pm0.005$—normal cells; $r=0.135\pm0.006$—trisomic cells) (Fig. 5).

The comparison of anisotropy fluorescence values of 12-AS indicated that association of MIT molecules with plasma membrane decreased their fluidity in the hydrophobic region of normal as well as trisomic cells, especially for drug concentrations above 5 μmol/dm^3 (Fig. 5). For the highest MIT concentration applied (100 μmol/dm^3), the fluorescence anisotropy of 12-AS increased, in average, from 0.135 to 0.280 in DS fibroblasts and from 0.131 to 0.263 for normal cells. In contrast, MIT treatment only slightly influenced the fluidity of the outer surface of the cell membrane. The fluorescence anisotropy values of TMA-DPH demonstrated statistically significant differences ($P<0.01$) only at the highest doses tested (Fig. 4).

4. Discussion

Biochemical disturbances of the reactive oxygen species metabolism revealed in subjects with DS make trisomic cells a convenient model system with the potentially altered resistance both to internal as well as external sources of free radicals [25,37]. Increased level of Cu–Zn superoxide dismutase (SOD-1) (EC: 1.15.1.1.), an enzyme coded on distal half of the long arm of 21 chromosome, is considered as a cause of an excessive production of H_2O_2 and consequently for a constant elevation of oxidative stress in trisomic cells [11,25]. Under such conditions, the balance between the production of free radicals and the ability of the cell antioxidant defence system may be disturbed [38,39].

Reports comparing in vitro as well as in vivo sensitivity of normal and DS cells of various origins to MIT treatment have resulted in different conclusions depending on the tested cells [40,42]. Our in vitro investigations of the influence of MIT on the survival of human normal (S-126 and WA-1) and trisomic (S-240, T-158, T-74 and T-164) fibroblast lines (Table 1) indicated that the sensitivity of DS cells to MIT treatment was significantly elevated ($P<0.01$), in comparison to diploid cells (Fig. 1). Greater in vitro sensitivity of cell lines derived from DS individuals was confirmed by results obtained by several oncological pediatric groups which reported that AML in children with DS has a significantly better outcome than AML in non-DS children [42,43]. Our results are as well in accord with data obtained by Kaspers et al. [41], who found the 26.2-fold growth inhibition of ANLL cells of DS patients submitted to in vitro MIT treatment as compared to those, derived from non-trisomic ANLL individuals. Moreover, trisomy of 21 chromosome has been reported as one of the largest risk factor for the development of MIT-associated cardiotoxicity, greatly decreasing a survival rate of DS patients [44].

The IC_{50} values obtained in our examinations for normal as well as DS fibroblast lines (Fig. 1) were at least 10 to 200 times greater compared with the most currently available data reported for various kinds of malignant cells [45,46]. The above findings might imply that non-malignant cells were more resistant to MIT antiproliferative activity than tumour cells. Our results agree with the recent finding published by Andersson et al. [47], who obtained relatively high IC_{50} values varying from 57 to 72 μmol/dm^3 for isolated rat cardiomyocytes. Similar results were reported by Dorr et al. [46], who using the in vitro cytotoxicity assays in human 8226 myeloma cells and neonatal rat heart myocytes, obtained IC_{50} values for MIT in rat heart cells approximately 500-fold higher than that calculated for tumour cells.

Sensitivity of DS cells to various anticancer drugs, as compared to normal ones, is also related to the kind of the examined compound. In our previous studies, we examined cytotoxic effect of daunorubicin [29] and aclacinomycin (data submitted to publication)—anthracycline antibiotics closely related to MIT and we found that DS cells were considerably less sensitive to damage mediated by those two drugs. Similar results were reported by Rózga and Duong Thi Bach [48] who, on the basis of the kinetics of the ^{51}Cr release and by MTT and colony forming ability assays, showed significantly decreased sensitivity of trisomic DS fibroblast cell lines to carminomycin compared to diploid non-DS cells. Nevertheless, the same trisomic cell lines appeared to be more sensitive to γ-radiation which presumably indicated the enhanced number of unrepaired DNA double-strand breaks, leading to reproductive death of these cells.

Explanation for the mechanism of greater or lower susceptibility of cells derived from DS individuals to various chemical and physical agents compared to non-trisomic cells is still open to question. It may be postulated that DS cells are, in general, more sensitive to the anticancer therapeutic agents whose molecular efficacy may be exerted mostly by the direct or indirect interaction with DNA or with the various DNA-bound proteins, leading to the blocking of the transcription or mediated DNA strand break. In this case, the increased sensitivity of DS cells appears to be caused by the diminished DNA-repair capacity [23,24].

The finding [49] that oxidation of MIT was enhanced by SOD-1 might be a crucial point for the explanation of increased susceptibility of DS cells to this compound. A number of studies demonstrated that in individuals with DS, the activity of SOD-1 and GPX were markedly elevated while catalase and glutathione reductase activities were normal in variety of cell types [38,50]. It might therefore

be speculated that, although the cellular level of superoxide radicals in DS tissues was decreased, the production of H_2O_2 was enhanced.

There is common agreement that the antiproliferative efficacy of MIT, likewise many other anticancer drugs, depends on the concentration achieved in compartments where its cellular targets are located. The final cellular steady state of MIT is the result of the numerous individual processes such as, uptake and efflux, distribution among cellular organelles and binding to various intracellular sites. The currently available data confirm the assumption that uptake of MIT, similarly to anthracycline antibiotics, occurs by passive diffusion [32,33]. The mechanism according to which cells extrude this compound still remains unknown [32]. Burns et al. [32], who investigated transport of radiolabeled MIT by cultured mouse L1210 leukemia cells, found that the efflux of this compound was not affected by KCN or verapamil and was not energy requiring. In contrast, the results published by Coloma et al. [33] showed that MIT transport might also involve an energy-dependent drug efflux pump similar to P-glycoprotein and multidrug resistance-associated protein carrier.

In our investigations, we found that MIT was rapidly taken up and distributed in the cellular compartments of all cell lines studied; however, the kinetics of drug transport through cell membranes were different in DS and non-DS fibroblasts (Fig. 2). Comparison of the influx and efflux rate constants as well as initial influx and initial efflux values (Table 2) demonstrated that the amount of drug taken up and removed, within the same time, by DS fibroblasts, was substantially lower than by normal cells. These data are confirmed by results reported by Koter et al. [51]. They indicated that the transport of a hydrophobic spin label probe-TEMPO across the erythrocyte membrane, studied by ESR method, was significantly slower in DS cells than in non-trisomic ones.

Further analysis of our data showed that the level of intracellular MIT content, being a function of the amount of drug transported inside the cell (influx) and the amount of drug expelled out of cell (efflux), was greater in trisomic than in normal cells (Fig. 3). Enhanced accumulation of MIT by DS cells probably resulted from substantially decreased drug efflux (Table 2). Comparison of cellular distribution of MIT showed that the fraction of MIT molecules considered as DNA-bound MIT was greater in trisomic cells as well. At the same time, no differences between normal and DS cells in the amount of the cellular free drug, associated with other than nucleus cellular structures, were found.

Different patterns of intracellular MIT distribution in trisomic and normal cells, provided by our study, corroborated results reported by Davies and Doroshow [52], who found predominant nuclear localization of this drug in cells more sensitive to MIT. Moreover, Feofanov et al. [53], using confocal spectral imaging technique, for quantitative analysis of the uptake, subcellular localization, and characteristics of localised binding and retention of MIT within human K562 erythroleukemia cells, found growing accumulation of MIT bound to nucleic acids within the nucleus in the cells treated with a high concentration of this drug.

Little is known about the interaction of MIT with different components of cell membranes and particularly about its influence on cellular drug uptake. Burns et al. [32], who studied the effect of membrane structural alteration on the cellular association of the MIT in L1210 cells, modified by incubating them with the highly unsaturated docosahexaenoic acid (22:6) or with the monounsaturated oleic acid (18:1), have found that the rate of uptake by 22:6-enriched cells during the first minutes was 62% greater than by those enriched with 18:1 acid. The difference in cell-associated drug was apparently not due simply to a change in MIT solubility as measured by partitioning of the drug in lipophilic–hydrophilic systems containing lipids from the fatty-acid altered cells. They concluded that the type of fatty acids contained in L1210 cell membranes might affect the cell association of MIT. This effect could be on transmembrane flux or be due to differences in binding of the drug to intracellular structures [54].

We have also studied the relationship between sensitivity of normal and trisomic fibroblast lines to MIT injury and fluidity of the cell membrane, a parameter commonly considered as an important aspect of cellular physiology and an essential factor for cell integrity and survival and as well responses for numerous cancer cells properties, such as invasiveness and growth features [55].

For this purpose, we selected spectrofluorometric method based on the measurement of the fluorescence anisotropy of the lipid probes TMA-DPH and 12-AS. A major advantage of choosing TMA-DPH and 12-AS is that these fluorescent dyes are confined primarily to the plasma membrane [34] and yield information about the rigidity of the cell membrane near the lipid polar heads [35] or inside the hydrophobic lipid core, respectively [36].

Anisotropy values obtained for the fluorescent probe-TMA-DPH, presented in Fig. 4, show that untreated DS fibroblasts have substantially lower fluidity at the outer surface of the lipid monolayer of the cell membrane (higher anisotropy) (0.296 ± 0.007) than control cells (0.268 ± 0.007) ($P < 0.01$). In contrast, no statistically significant difference in rigidity at the inner, hydrophobic lipid region of the cell membrane, between normal and DS fibroblasts (0.131 ± 0.005—normal cells; 0.135 ± 0.006—trisomic cells) (Fig. 5), was observed. A high fluorescence anisotropy of TMA-DPH, related to low cell membrane fluidity, suggests an increase of structural order at the lipid–water interface region of DS cell membrane.

The relationship between the decreased cell membrane fluidity of DS individuals and cell membrane composition has still been under discussion. Data obtained by Kantar et al. [56] suggested that the critical factors influencing cell membrane fluidity, that is, phospholipid and cholesterol capacity and the cholesterol to phospholipid molar ratio,

are almost equal in cell membranes from DS persons and normal non-trisomic donors. The analysis of the content of polyunsaturated fatty acids in cell membranes [38] indicated significant alterations of the relative proportion of various groups of these compounds. The linoleic acid content as well as elongated products of arachidonic acid (adrenic acid, docosatetraenoic acid and docosapentaenoic acid) were markedly diminished in trisomic individuals. On the other hand, the amount of long-chain polyunsaturated fatty acids (dihomo-γ-linolenic acid and arachidonic acid) and docosahexenoic acid were enhanced in DS cells, compared with the control group. Moreover, data obtained by Kêdziora et al. [57,58] as well as Chapman et al. [59] indicated the existence of structural modifications of trisomic cells suggesting the loss of membrane surface. They concluded that protein conformational changes, revealed by scanning electron microscopy examinations, were rather responsible for membrane structural alterations of DS cells, than the cell membrane lipid composition or the degree of the acyl-chain unsaturation themselves.

We also examined the effect of MIT treatment on cell membrane fluidity. Association of MIT molecules with plasma membrane markedly decreased their fluidity in the hydrophobic region of normal as well as trisomic cells in the drug concentration-manner (Figs. 4 and 5). Contrary to this, MIT treatment influences the fluidity of the outer surface of the cell membrane only at higher drug doses applied. Moreover, we indicated that the extent of the rigidization of outer as well as inner domains of cell membrane was greater for all trisomic fibroblast lines compared to normal ones. These facts suggest that MIT molecules were preferentially accumulated in the inner hydrophobic core of cell membrane and that the association of MIT with trisomic cell membranes was altered, as compared to normal fibroblast lines.

In summary, the data presented in this paper suggest that the cellular mechanism of antiproliferative action of MIT in DS cells appears to be different than that of anthracyclines such as daunorubicin and aclarubicin. Lower membrane fluidity observed in trisomic fibroblasts might diminish influx and efflux rates and finally increase drug accumulation level. Alterations of cell membrane fluidity and differences in drug transport suggest that the plasma membrane is an important site of MIT accumulation and action. The greater cellular accumulation of MIT in trisomic cells, especially the drug fraction interacting with DNA, could be responsible for the decreased survival of cells derived from DS patients.

Acknowledgements

The authors are grateful to Joanna Lipecka, Anna Bogusławska and Sylwia Dragojew for helping to carry out this work, Aneta Koceva-Chyła for her helpful suggestion and constant encouragement, Mirosław Przybylski for his valuable advice and help in statistical analysis of data and Krzysztof Ilnicki for storage of the fibroblast lines in Tissue Bank of the Centre of Child Health (Warsaw, Poland).

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