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European Journal of Pharmacology 383 (1999) 321–329

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On the mechanism underlying calcein-induced cytotoxicity

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Received 1 July 1999; received in revised form 8 September 1999; accepted 9 September 1999

Abstract

The cellular pharmacology of calcein acetoxymethyl ester (calcein/AM)-induced cytotoxicity was investigated in human tumor cell lines in order to identify tentative mechanisms of action. The activity profile in 10 cell lines with known mechanisms of resistance was compared with the activity profiles of standard drugs and experimental substances. The activity of calcein correlated with that of different topo II inhibitor/intercalating compounds and mitochondrial accumulating compounds, such as Rhodamine 123, Mito Fluor Green and Acridine Orange-10. Using U-937 GTB as a model cell line, calcein was found to distribute throughout the whole cell, nuclei and mitochondria included. In addition, studies of mitochondrial dehydrogenase activity and extracellular acidification rate showed an almost complete lack of dehydrogenase activity and extracellular acidification at 12 and 24 h, respectively. The results indicate that calcein/AM may induce cytotoxicity through interference with both mitochondrial and nuclear DNA.

Keywords: Calcein/AM; Calcein; Cytotoxicity; Cytosensor®; U-937 GTB

1. Introduction

The acetoxymethyl ester of calcein (calcein/AM) has, beside its widespread use as a fluorescent viability probe, been increasingly used in functional studies of the membrane-bound efflux transporters *P*-glycoprotein 170 (Pgp) and Multidrug resistance-associated protein (MRP) (Homoloya et al., 1993; Liminga et al., 1994), (Versantvoort et al., 1995; Dhar et al., 1998). Also the functional status of the permeability pore of the mitochondria has been studied using this molecule. With this latter application contradictory results have been published with respect to calcein accumulation within the mitochondria. Thus, both efflux and influx of calcein/AM have been used as markers of the opening of a permeability pore and to identify collapse of the mitochondrial membrane potential (Nieminen et al., 1997; Hüser et al., 1998; Minamikawa et al., 1999).

In addition to the use of calcein/AM as a diagnostic tool in cellular research, it has been demonstrated that calcein/AM is cytotoxic both in human tumor cell lines (Liminga et al., 1995) and in primary cultures of human tumor cells (Jonsson et al., 1996). In the latter study, calcein/AM showed high activity against primary cultures of tumor cells from patients with solid tumors and had an activity profile somewhat similar to that of doxorubicin (Jonsson et al., 1996). Furthermore, recent studies have demonstrated that calcein/AM-induced cell death is the result of rapid inhibition of DNA synthesis, partial depolarization of the mitochondrial membrane followed by activation of the caspase cascade and nuclear fragmentation, a chain of events consistent with the induction of programmed cell death and classical apoptosis (unpublished data). Calcein/AM is readily cleaved by intracellular esterases into fluorescent calcein, but in this article we have chosen to refer to it as calcein/AM except in the confocal microscopy part.

In the present study we attempted to further delineate the mechanisms of action by using laser scanning confocal microscopy, Cytosensor® microphysiometry and measurements of mitochondrial dehydrogenase activity and by comparing calcein/AM activity with that of compounds with known mechanisms of action. The results suggest that calcein/AM may induce apoptosis and cell death by early

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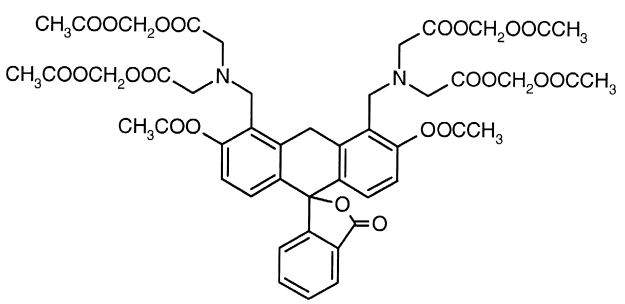
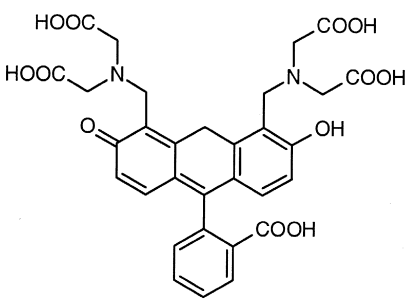
0014-2999/99/$ - see front matter
PII: S0014-2999(99)00655-X

Fig. 1.



Calcein Acetoxymethyl Ester
Calcein/AM



Calcein (Free acid)

Fig. 1. Chemical structure of Calcein/AM and Calcein.

interference with mitochondrial function and DNA synthesis.

2. Materials and methods

2.1. Drugs and reagents

Calcein/AM (Fig. 1), Rhodamine 123, JC-1, Mito Fluor Green and Acridine Orange were purchased from Molecular Probes (Leiden, The Netherlands) and were dissolved and stored according to instructions from the manufacturer. Cycloheximide and aphidicholin were from Sigma (St Louis, MO). The standard cytotoxic drugs cytarabine, cisplatinum, doxorubicin, etoposide, vincristine, paclitaxel (taxol) and topotecan were purchased from the local hospital pharmacy and were dissolved and stored according to instructions from the manufacturers. For the Cytosensor® experiments modified RPMI 1640 (both low buffering glucose and low buffering non-glucose, pyruvate-containing) medium was used (National Veterinary Institute, Uppsala, Sweden). Alamar Blue™ was purchased from Alamar Bioscience (Sacramento, CA) and stored at +4°C. All other chemicals used were of analytical grade.

2.2. Cell line panel

The cell line panel represents defined mechanisms of resistance and its maintenance was described earlier (Dhar et al., 1996). Table 1 shows the cell lines, origin, mechanism of resistance and reference.

Calcein/AM was serially diluted with phosphate-buffered saline (PBS) to 10 times the desired final concentrations and 20 μl was plated in triplicate into V-shaped 96-well microtiter plates (Nunc, Roskilde, Denmark), with control wells receiving only PBS, using an automated robot, Propette II (Perkin Elmer, Norwalk, CT). Then 180 μl of cell suspension (110 000 cell /ml) was added and the plates were incubated at 37°C, 5% CO_2 for 72 h in a standard incubator.

Drug activity was determined using the Fluorometric Microculture Cytotoxicity Assay (FMCA) (Larsson et al., 1992). IC_{50}, the concentration allowing survival of 50% of the cells of each cell line, was calculated using a custom made program in Excel (Microsoft). The overall mean $^{10}\log IC_{50}$ for all cell lines was determined and defined as the mean of all $^{10}\log$ values. Then the mean $^{10}\log IC_{50}$ was subtracted from the $^{10}\log$ of each cell line, yielding a value defined as delta. Negative values indicate cell lines more sensitive than the average and positive values indicate more resistant cell lines.

A procedure similar to the COMPARE analysis (Paull et al., 1989) using Pearson's correlation coefficient was used to compare the different delta values with those for

Table 1
The cell lines, origin, mechanisms of resistance and reference

Cell line	Origin	Mechanism of resistance	Reference
U-937 GTB	Histiocytic lymphoma	parental	Sundström and Nilsson, 1976
U-937 vcr	Histiocytic lymphoma	tubulin-associated	Botling et al., 1994
CCRF-CEM	Leukemia	parental	Beck et al., 1979
CEM/VM-1	Leukemia	topo II-associated	Danks et al., 1987
ACHN	Renal	primary resistant	Borden et al., 1982
NCI-H69	Small Cell Lung Cancer	parental	Mirski et al., 1987
H69AR	Small Cell Lung Cancer	MRP-associated	Cole et al., 1992
8226/S	Myeloma	parental	Dalton et al., 1986
8226/dox40	Myeloma	Pgp-associated	Dalton et al., 1986
8226/LR-5	Myeloma	GSH-associated	Mulcahy et al., 1994

Fig. 2.

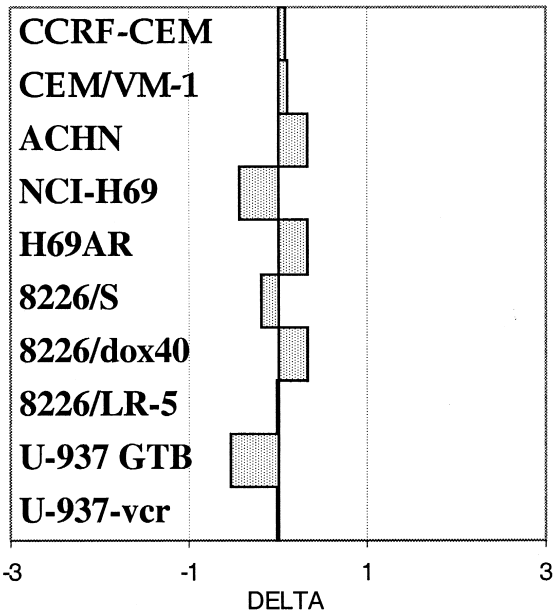


Fig. 2. The mean graph delta generated for calcein/AM in the cell line panel. Compared to the mean graph delta, more sensitive cell lines have bars to the left and more resistant cell lines have bars to the right.

compounds previously analysed in this way (at present approximately 160 compounds).

2.3. Confocal microscopy

U-937 GTB cells were resuspended in PBS containing 5 mM glucose. Three milliliters of cell suspension (300 000 cells/ml) were placed in a live cell POC chamber (Carl Zeiss Jena GmbH., Jena, Germany), the bottom glass of which was coated with poly-L-lysine, MW > 300 000 (Sigma) to enhance cell adhesion and to avoid cell movements during repeat scanning. The following concentrations and preincubation times were used: calcein/AM 0.1, 0.5 and 2.5 μg/ml, no preincubation; JC-1 0.5 μg/ml preincubation for 10 min; and Rhodamine 123 0.2 μg/ml, Mito Fluor Green 0.06 μg/ml and Acridine Orange 1.5 μg/ml preincubation for 20 min. The preincubations were followed by a wash step and resuspension in fresh PBS–glucose buffer.

Microscopy was performed using a computer controlled Zeiss LSM 410 inverted laser scan microscope (Carl Zeiss) equipped with a 40× water immersion lens. Laser excitation was at 488 nm and the emission filter was set to 515–525 nm for calcein and Mito Fluor Green. For Rhodamine 123, JC-1 and Acridine Orange, excitation at both 488 and 543 nm and emission filters at 510–540 nm and 570 nm (longpass-filter) for Rhodamine 123 and JC-1, and for Acridine Orange 510–540 and 665 nm (long-pass filter) filters were used. Attenuation was set to avoid photobleaching and 16 scans were usually performed to acquire one picture. Digital images were imported into a personal computer and printed using Microsoft Powerpoint (Microsoft).

2.4. Extracellular pH-measurements

The Cytosensor® microphysiometer (Molecular Devices, Sunnyvale, CA) was used to study the effect of calcein/AM on cellular metabolism. Cells were suspended in an agarose-matrix and placed in a cell cup. The cell cup was perfused with low buffered RPMI 1640 medium and every 90 s the flow was stopped for 30 s and the extracellular acidification rate was measured with a light-sensitive potentiometric sensor (McConnell et al., 1992). Acidification originates from the excreted acidic byproducts of cellular respiration. The Cytosensor® offers a continuos ''on-line'' measurement of the metabolic response to the compounds of interest and it contains two four-channel workstations, allowing eight separate test conditions to be run in parallel (Ekelund et al., 1998).

In order to establish a baseline for the experiment, cells were only exposed to medium during the first hour of assay time. Cells in 1 or 2 of the channels in each experiment were kept unexposed and served as control(s). Each experiment was run for approximately 24 h with continuous or short-term (1 h) initial calcein/AM exposure. In some experiments aiming to investigate the calcein/AM effect under non-glycolytic conditions, a pyruvate-supplemented no-glucose-containing medium was used.

2.5. Measurement of mitochondrial dehydrogenase activity

Alamar Blue™ is a fluorometric/colorimetric probe which, when reduced, changes color and absorbance spectra and becomes fluorescent (Ansar Ahmed et al., 1994). Alamar Blue™ is proposed to be reduced by mitochondrial dehydrogenases and has been used as a probe for viability and proliferation (Ansar Ahmed et al., 1994; Teicher, 1997a). In our experience fluorescence is a more sensitive endpoint than absorbance.

Table 2
Correlation between the cytotoxic activity of calcein/AM and that of the ten highest ranked compounds from the drug data base. [10] log IC_{50} values for ACHN, H69AR and 8226/dox40 were set to 2.5 μg/ml
R = Pearsons coefficient of correlation.

Rank	Drug	Group	R
1	Mitoxantrone	Topo II inhibitor	0.77
2	Mito Fluor Green	Mitochondrial stain	0.77
3	Rhodamine 123	Mitochondrial stain	0.75
4	Etoposide	Topo II inhibitor	0.73
5	Epirubicin	Topo II inhibitor	0.72
6	Zinostatin	Other	0.71
7	Doxorubicin	Topo II inhibitor	0.69
8	Acridine Orange-10	Mitochondrial stain	0.67
9	Idarubicin	Topo II inhibitor	0.64
10	Daunorubicin	Topo II inhibitor	0.64

Table 3
Resistance factors for calcein/AM

Mechanism of Resistance	RF[a]
Pgp-associated	> 3.33
topo II-associated	1.01
tubulin-associated	3.26
GSH-associated	1.52
MRP-associated	> 5.81
Primary resistant	> 3.33

[a]RF = Resistance Factor is generated by dividing the IC_{50} of the resistant cell by the IC_{50} of the parental cell line. For ACHN, which is primary resistant, the resistance factor was defined as IC_{50} of ACHN divided by mean IC_{50} of all parental cell lines. > indicates a resistance factor greater than, since no true IC_{50} was generated in the resistant cell lines.

U-937 GTB cells (160 μl), suspended in ordinary supplemented RPMI 1640 medium (125 000 cells/ml) were seeded into wells of a flat-bottomed microtiter plate (Nunc, Roskilde, Denmark). Then 20 μl of drug solution and 20 μl Alamar Blue™ were added in duplicate for each drug and concentration. Standard cytotoxic drugs at concentrations resulting in less than 10% viability after 72 h as measured in the FMCA (= Fluorometric Microculture Cytotoxicity Assay) were used as comparisons. After cell seeding and drug administration, the plate was read in a microtiter plate spectrofluorometer, Fluoroscan II, (Labsystems OY., Helsinki, Finland), with excitation and emission filters set to 544 and 590 nm, respectively, at different time points for up to 72 h.

3. Results

3.1. Mechanistic classification using the cell line panel

For ACHN, H69-AR and 8226/dox40 no true IC_{50} value was detected and these IC_{50} values were therefore set to 2.5 μg/ml, the highest concentration used (Boyd and Paull, 1995). Fig. 2 shows the mean graph delta generated for calcein/AM. Six out of the 10 correlations were observed for topo II inhibitors or DNA intercalating compounds with coefficents between 0.77 for mitox-

Fig. 3A.

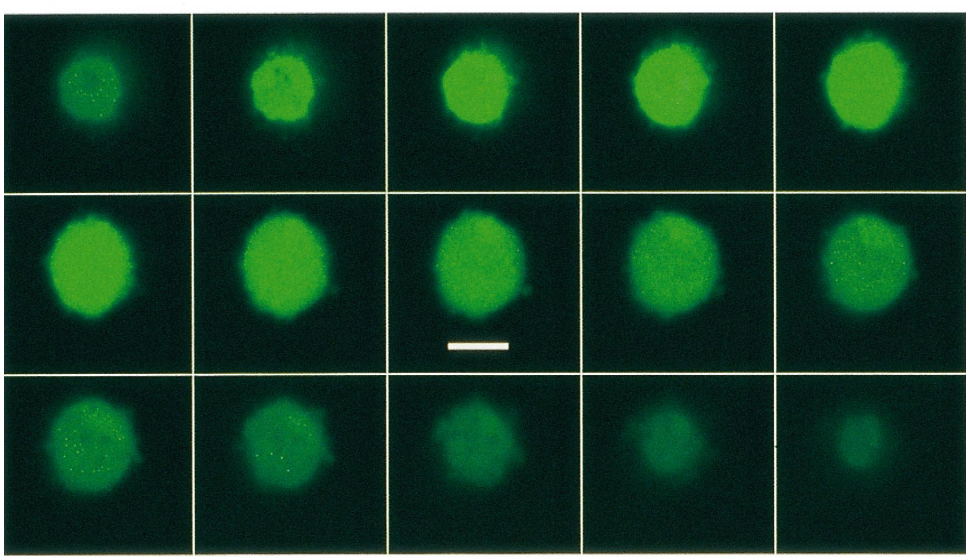


Fig. 3B.

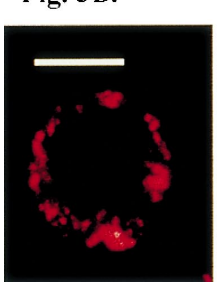


Fig. 3. (A) Laser scanning confocal picture of a U-937 GTB cell incubated with calcein/AM 0.5 μg/ml for 30 min. The cell is sectioned into 1-μm-thick sections, with an overlap of 0.05 μm. Bar equals 10 μm. (B) U-937 GTB cells stained with Rhodamine 123, approximately 30 min after the preincubation. Bar equals 10 μm.

antrone and 0.64 for daunorubicin and idarubicin (Table 2). The mitochondrial accumulating probes mito fluor green, Rhodamine 123 and Acridine Orange-10 with coefficients of 0.77, 0.75 and 0.67 ranked as numbers 2, 3 and 8. Zinostatin, an antitumor antibiotic with a coefficient of 0.71, ranked as number 6. Vincristine (tubulin active), topotecan (topo I inhibitor) and cytarabine, (antimetabolite) are examples of compounds with low coefficients of correlation (0.38, 0.41 and 0.18, respectively).

The cytotoxicity of calcein/AM put in relation to mechanisms of resistance is presented in Table 3. Interestingly, the IC_{50} in CEM/VM-1 was not higher than that in the parental cell line CCRF-CEM, indicating a lack of calcein/AM sensitivity to topo II-associated resistance. Calcein/AM was sensitive to the other types of resistance except for GSH-associated resistance.

3.2. Intracellular localization

There was a rapid increase in fluorescence after addition of calcein/AM. Within minutes the whole cell became fluorescent, including the nucleus and other intracellular organelles. The fluorescence was at its maximum after approximately 1 h. Fig. 3A shows a single cell sectioned into 1-μm-thick sections by laser scanning confocal microscopy. Cells were also incubated with calcein/AM 2.5 μg/ml for 3 and 6 h and thereafter microscopy was performed. At 3 and 6 h of exposure, an increasing number of small ovoid spheres judged as apoptotic bodies were observed (not shown). The mitochondrial accumulating probes, Rhodamine 123, Mito Fluor Green or JC-1, all gave rise to punctuate staining around the nuclei. Fig. 3B shows a typical cell stained with Rhodamine 123. For Acridine Orange green fluorescence were localized in the nuclei and red fluorescence (665 nm) was detected in differently sized spheres in the cytoplasm which were considered to be lysosomes (not shown).

3.3. Extracellular acidification

In the presence of glucose, calcein/AM 2.5 μg/ml generated a steady decline in metabolic activity with maximum inhibition after 20 h (Fig. 4A). Calcein/AM at 0.5 μg/ml showed a biphasic inhibition of metabolic activity. There was an initial rapid decrease in metabolic activity to a plateau at 80% of baseline that lasted up to approximately 8 h, followed by a rapid decrease in metabolism, with maximum inhibition after 20–25 h. Calcein/AM at 0.1 μg/ml had a varying effect. In some experiments extracellular acidification was absent at 24 h whereas in some, low degree inhibition of metabolism was observed (not shown). After the experiment was stopped, cytospin slides were prepared and stained with May-Grünewald Giemsa for morphological control. The viability at 24 h was close to zero at concentrations where acidification maximally decreased, whereas in the untreated control viability was close to 100%.

Fig. 4 A.

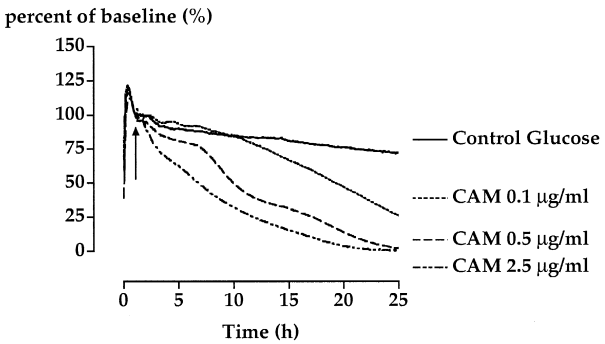


Fig. 4 B.

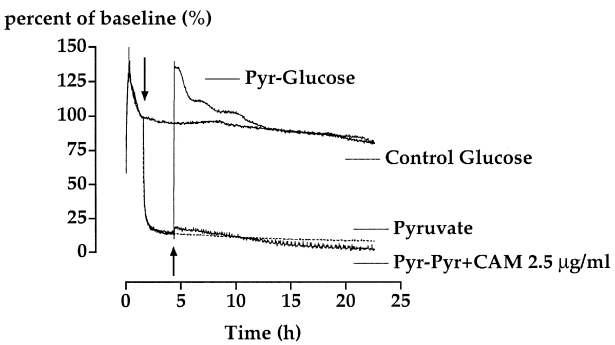


Fig. 4 C.

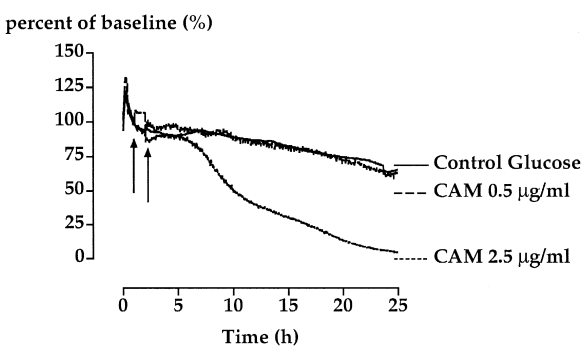
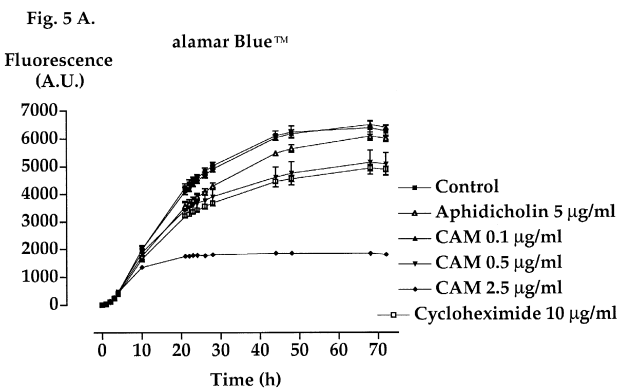


Fig. 4. Cytosensor® experiments. (A) Effect of calcein/AM (CAM)(2.5, 0.5 and 0.1 μg/ml) on extracellular acidification. The arrow denotes where cells are switched to drug-containing medium. (B) Extracellular acidification in cells receiving only pyruvate quickly dropped to approximately 20% of baseline value (first arrow). Cells perfused with pyruvate medium without calcein/AM showed the same acidification rate when switched back to glucose-containing medium as cells continuously perfused to glucose medium. Second arrow denotes either switching from pyruvate to glucose medium or switching to calcein/AM-containing pyruvate medium. (C) Extracellular acidification after a 1-h incubation of calcein/AM in glucose medium (between the arrows). Only 2.5 μg/ml had an effect. One typical example is shown in each figure.

When pyruvate was used as the main energy source, a rapid decline to approximately 20% of the initial baseline value was observed when cells were switched from glucose to pyruvate-containing medium (Fig. 4B). When pyruvate medium containing calcein/AM was added a small increase in acidification rate was immediately and consistently noticed, but thereafter there was a slow decline of the curve and maximum inhibition was reached after approximately 20 h for calcein/AM concentrations of 2.5 and 0.5 μg/ml. At this point of time viability was less than 10% as judged by light microscopy of May-Grünewald Giemsa-stained cytospin slides. The viability of cells receiving pyruvate only was > 90% at the end of the experiment. Cells perfused with pyruvate medium and then switched back to glucose-containing medium showed the same acidification rate and viability as cells only perfused with glucose-containing medium. When cells were exposed to calcein/AM for 1 h, only cells exposed to 2.5 μg/ml were affected with maximum activity approximately 20 h, and with a biphasic curve (Fig. 4C).



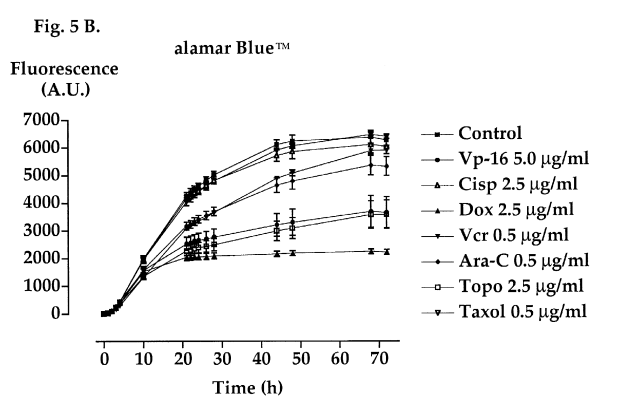


Fig. 5. (A) Effect on mitochondrial dehydrogenase activity of calcein/AM at the indicated concentrations and of the DNA and protein synthesis inhibitors aphidicholin and cycloheximide. At 10 h an almost complete inhibition was noted for calcein/AM 2.5 μg/ml. For calcein/AM 0.5 and 0.1 μg/ml the curves were similar to those of the untreated control, which was also observed for aphidicholin and cycloheximide. (B) Curves generated for standard cytotoxic drugs. Doxorubicin induced an almost complete inhibition of dehydrogenase activity after 10 h in a similar manner as calcein/AM 2.5 μg/ml. Both figures are presented as the means of 4 consecutive experiments ± S.E.M.

3.4. Alamar Blue™

Alamar Blue™ fluorescence of untreated controls increased with the steepest slope during the first 24 h, thereafter it gradually declined during day 2 to reach a plateau during the last 24-h period (Fig. 5A and B). For calcein/AM 2.5 μg/ml and doxorubicin no increase in fluorescence was observed after 10–20 h, indicating an almost complete inhibition of dehydrogenase activity (Fig. 5A). Calcein/AM at 0.5 μg/ml decreased the fluorescence to approximately 80% of that of untreated control, whereas 0.1 μg/ml was without effect. There was very little decrease in dehydrogenase activity in cells treated with the DNA synthesis inhibitor aphidicholin at 5 μg/ml after the 72-h period. The response of cycloheximide-treated cells (10 μg/ml) resembled that of cells treated with calcein/AM (0.5 μg/ml). Cisplatinum and taxol did not significantly decrease the dehydrogenase activity compared to the control (Fig. 5B). Etoposide, topotecan, vincristine and cytarabine exhibited intermediate activity.

4. Discussion

In 1985 the National Cancer Institute introduced a new anticancer drug screening strategy based on automated drug response analysis in a panel of 60 cell lines representing the major forms of human cancer (Alley et al., 1988; Boyd and Paull, 1995). The differential drug activity information provided by the panel has been shown to reveal specific patterns of in vitro response to drugs with similar mechanisms of action which can be quantified by simple correlation analysis (Paull et al., 1989; Boyd and Paull, 1995). We previously described the use of a panel of only 10 cell lines representing different types of drug resistance for this purpose (Dhar et al., 1996, 1998). The differential activity of drugs across these 10 cell lines provided a pattern which could be used to describe the degree of similarity among standard drugs (Dhar et al., 1996). In this study, high correlations were obtained between drugs sharing a common mechanism of action, allowing a good discrimination between different mechanistic groups (Dhar et al., 1996). As a crude rule of thumb, correlations between 0.5 and 0.75 may be considered as moderate to high and above 0.75 as very high to excellent (Colton, 1974). For example, coefficients of correlation between different topo II inhibitors are generally above 0.90 and similar degrees of correlation have been reported for other mechanistic groups such as alkylating agents and tubulin active agents (Paull et al., 1989; Dhar et al., 1996).

In the present study seven of the 10 compounds with the highest correlation to calcein/AM belonged to the

group of DNA-interacting drugs, topo II inhibitors or intercalating agents whereas three were mitochondria-targeting agents.

The anthracyclines have been proposed to have more than one mechanism of action including DNA intercalation, topo II inhibition and generation of free radicals in the cytoplasmic/mitochondria compartments (Teicher, 1997b). The intracellular localization of anthracylines appears to depend on charge and lipophilicity. Lipophilic and uncharged anthracylines have a tendency to accumulate more in cytoplasmic and mitochondrial compartments and less in the nucleus (Lampidis et al., 1997). This may also to some extent explain why different mechanisms of action have been proposed. For mitoxantrone there have also been reports of localization not only in the nuclei but also in the cytoplasmic compartment (Smith et al., 1992). Zinostatin, which consists of a carrier peptide of 10.7 kDa and a fluorescent polycyclic apoprotein (Napier and Goldberg, 1983), also intercalates and binds to the carbon at the 5′ position of the deoxyribose sugar, inducing single or double stranded breaks in the DNA (Goldberg et al., 1986).

In a recent article from our laboratory, we investigated how different classes of drugs affected extracellular acidification by using Cytosensor® microphysiometry and it appears that the curve generated for various compounds is cytotoxic drug class specific. (Ekelund et al., 1998). The cytosensor curves for calcein/AM in this paper and for doxorubicin (Ekelund et al., 1998) were very similar. In the present study calcein/AM and doxorubicin also showed very similar inhibition kinetics with respect to inhibition of mitochondrial dehydrogenase activity. Given these observations, it is tempting to propose that calcein is another DNA-interacting compound which either by intercalation or topoisomerase II inhibition generates nuclear DNA strand breaks as the trigger of apoptosis. Indeed, calcein possesses a planar polycyclic ring structure, permitting its intercalation into DNA. In addition, calcein/AM has been shown to induce early inhibition of DNA synthesis with similar kinetics as doxorubicin, further supporting this notion (unpublished data). The cytotoxic activity patterns of calcein/AM and doxorubicin were also strongly correlated in primary cultures of tumor cells from patients (Jonsson et al., 1996). Finally, confocal microscopy showed the nucleus to accumulate calcein/AM within minutes of exposure, compatible with a DNA-interacting mode of action.

The mitochondrion is a cellular organelle that has enjoyed a great deal of interest during the last years in the field of apoptosis and programmed cell death (Kroemer et al., 1998). It is an important organelle, contributing to cancer cell physiology (Dorward et al., 1997) and there are also reports of diminished tumorogenic phenotype after depletion of mitochondrial DNA (Cavalli et al., 1997).

However, mitochondrial-accumulating compounds such as Mito Fluor Green, Rhodamine 123 and Acridine Orange-10 also generated fairly high correlation coefficients. Rhodamine 123, a lipophilic cation, is proposed to accumulate in mitochondria depending on the mitochondrial membrane potential (Johnson et al., 1980; Davis et al., 1985), whereas Mito Fluor Green and Acridine Orange-10 preferentially accumulate in mitochondria regardless of membrane potential (Maftah et al., 1989; Haugland, 1997). Various reports of how Rhodamine 123 induces cell death have been published through the years, most of them suggesting inhibition of the metabolic function of mitochondria as the mechanism of action (Modica-Napolitano and Aprille, 1987; Sun et al., 1994). Rhodamine 123 has also been reported to be more cytotoxic in tumor cells than in normal cells (Bernal et al., 1982; Lampidis et al., 1983). The underlying reason could be the higher potentials detected in mitochondria and plasma membrane of tumor cells (Davis et al., 1985).Judging from the present results obtained with confocal microscopy, calcein also appears to rapidly localize to the mitochondria, as also reported by other groups (Hüser et al., 1998; Minamikawa et al., 1999). Calcein is negatively charged and does not enter the mitochondria as a free acid but since calcein/AM is highly lipophilic, it might undergo esterase-dependent trapping in this organelle. However, calcein/AM has also been claimed not to distribute to the mitochondria (Nieminen et al., 1995). In the study of Nieminen et al., calcein/AM was incubated together with the methylester of tetramethylrhodamine, which quenches the fluorescence generated from calcein (Petronilli et al., 1997; Hüser et al., 1998). In the present study a rapid (within minutes) increase in extracellular acidification rate was observed in response to calcein/AM in glucose-depleted medium. The acidification rate under these conditions subsequently declined and was below that of control after approximately 5–6 h. This indicates that an early interference with metabolism, such as inhibition of the respiratory chain enzymes, is less likely, which is supported also by the retained mitochondrial dehydrogenase activity observed during the first 4 h of calcein/AM exposure. However, we observed calcein/AM to induce partial depolarization of mitochondria within 1 h (unpublished data) and a direct interaction with mitochondrial DNA remains an alternative mitochondrial target for calcein/AM. Given that no resistance of calcein/AM was noted in CEM/VM-1, which is resistant to topo II-inhibiting drugs (Danks et al., 1987), intercalation of DNA appears a more likely mechanism of action than inhibition of topo II isomerase, although to our knowledge it is not known if the topo II resistance in CEM/VM-1 is only located in the nucleus.

Interestingly, topo II inhibitors including the anthracyclines have also been reported to exert cytotoxic activity by interaction with mitochondrial structures (Lin and Castora, 1991; Lampidis et al., 1999) and topoisomerase II isolated from mitochondria also appears more sensitive to inhibition by these compounds (Lin and Castora, 1991). Recently it was also reported that a novel anthracycline (AD 32) was less potent in a cell line lacking mitochon-

drial DNA, than in the parental cell line, indicating the importance of mitochondrial DNA for the cytotoxicity induced by this group of compounds (Lampidis et al., 1999).

In conclusion, the present results suggest that calcein may interact with nuclear and/or mitochondrial DNA to induce cytotoxicity and apoptosis.

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