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Cytotoxic effect of calcein acetoxymethyl ester on human tumor cell lines: drug delivery by intracellular trapping

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Calcein acetoxymethyl ester (calcein/AM) and some related cellular dyes with a cytoplasmic distribution were investigated with respect to cellular hydrolysis, accumulation, efflux and cytotoxicity in a panel of established human cell lines, including multidrug resistant (MDR) phenotypes. At 0.1–1 μg/ml, calcein/AM was highly cytotoxic against several cell lines, even after short-term exposure (30 min). Calcein/AM induced no immediate loss (3 h) of membrane integrity and the drug was more active against low compared with high density plated cells. In cell lines with the MDR phenotype and in the renal carcinoma cell line ACHN, the drug was considerably less active. Non-esterified calcein had no effect and calcein/AM was significantly more potent than other structurally related fluorescein analogs and AM esters tested. Although MDR cell lines showed a decreased cellular hydrolysis and accumulation of the dye, there was no strict relationship between cytoplasmic calcein exposure and cytotoxic activity. The rate of efflux was low in the two most sensitive cell lines, the human lymphoma U-937-GTB and its vincristine (vcr) resistant subline U-937/vcr10, while the remaining cell lines showed similar biphasic efflux patterns, including cell lines of the MDR phenotype. The results show that calcein/AM has cytotoxic activity against human tumor cell lines at low concentrations. The effect appears dependent on the intracellular trapping of the drug, although the specific cellular target remains unknown. Due to its cytotoxic efficacy and unique principle of cellular drug delivery, further investigation of calcein/AM and related compounds as potentially new anticancer agents seems warranted.

Key words: Calcein/AM, cytotoxicity, human tumor cell lines.

Introduction

4′5′-Bis(*N*,*N*-bis (carboxymethyl) aminomethyl fluorescein acetoxymethyl ester (calcein/AM) is a non-fluorescent fluorescein analog which can rapidly enter cells where it is hydrolyzed to free, strongly fluorescent calcein by cytoplasmic esterases. Due to its high negative charge, de-esterfied calcein is well retained by viable cells with intact plasma membranes.[1,2] Calcein/AM has been widely used as a cytoplasmic marker and viability probe.[2,3–5]

Recently we and others showed that the AM ester of calcein is probably a substrate for multidrug resistance (MDR) related efflux 170 kDa permeability glycoprotein (Pgp) and the dye may be used as a convenient functional fluorescent probe for this transport protein.[6–8] Due to its non-nuclear cellular localization and its unusual principle for cellular drug delivery (esterase-dependent trapping), this and related compounds seem worthy of further investigation of their antitumor properties and cellular pharmacology. The fact that calcium chelators administered as AM esters and delivered to neuronal cells by intracellular trapping were recently demonstrated to have neuroprotective effects in rats indicates a potential for *in vivo* application for this group of agents.[9]

Therefore, as part of an ongoing effort to identify new pharmacological principles for antitumor therapy, we have investigated the cytotoxic potential and cellular pharmacology of calcein/AM and some structurally related compounds on a panel of human tumor established cell lines. The results show that calcein/AM is accumulated inside cells by esterase-dependent intracellular trapping and it is cytotoxic against several cell lines at relatively low concentrations.

Materials and methods

Cells and cell culture

The human myeloma cell line RPMI 8226/S and its doxorubicin (dox) resistance subline RPMI 8226/dox40[10] were kindly provided by Dr WS Dalton

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(Department of Medicine, Cancer Center Division, University of Arizona, Tuscon, AZ). The human kidney carcinoma cell line ACHN[11] was obtained from ATCC (Rockville, MD). The human histiocytic lymphoma U-937 and erythroleukemia K562 cell lines were established as described previously.[12,13] The cell lines were cultured in 250 ml flasks (Falcon; Becton Dickinson, Plymouth, UK) in RPMI 1640 medium (Northumbria Biologicals, Cramlington, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Northumbria), 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin (Northumbria). The dox resistant subline RPMI 8226/dox40 was treated with 100 μg/ml dox (Adriamycin; Farmitalia Carlo Erba, Milano, Italy) once a month. The dox40 subline shows a high level of Pgp expression as determined by immunohistochemistry and Western blot.[10] The U-937/vcr10 and U-937/vcr100 sublines were continuously cultured in the presence of 10 and 100 ng/ml of vincristine (vcr), respectively. The former cell type lacks Pgp expression as recently described,[14] whereas the U-937/vcr100, on the other hand, shows high levels of Pgp expression and a cross-resistance pattern typical of classical MDR (not shown). All cultures were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2, with medium change and subcultivation two to three times a week. The general characteristics of the cell lines are shown in Table 1.

Drugs and reagents

Calcein/AM, calcein, calcein blue/AM, carboxyfluorescein diacetate and carboxyfluorescein diacetate/AM (Molecular Probes, Eugene, OR) were dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) to a stock solution of 1 mg/ml and kept at −20°C until use at indicated concentrations. Fluorescein diacetate (FDA; Sigma, St Louis, MO) was dissolved in DMSO and stored at −20°C. Structural formulas for the different dyes are depicted in Figure 1. The cyclosporin SDZ PSC 833 (PSC) with Pgp blocksing properties was a kind gift from Sandoz (Basel, Switzerland). All other reagents were of analytical grade. Microtiter plates (Nunc, Roskilde, Denmark) were prepared with test substances in advance and stored at −70°C until use.[15]

Measurement of cytotoxicity

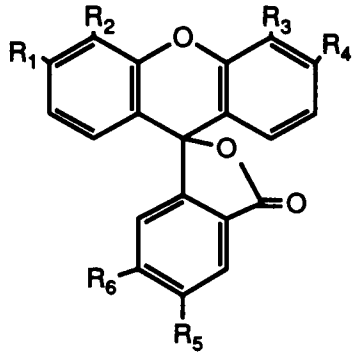
The principal steps of the fluorometric microculture cytotoxicity assay (FMCA) procedure have been described previously.[15,16] On day 1, 180 μl of the tumor cell preparation (0.5–2 × 10^5 cells/ml culture medium) was seeded into the wells of V-shaped 96-well experimental microtiter plates (Nunc) prepared as described above. Six blank wells received only culture medium and six wells with cells but without drugs served as control. The culture plates were then incubated at 37°C in humidified atmosphere containing 95% air and 5% CO_2. At the end of the incubation period, the plates were centrifuged (200 *g*, 7 min) and the medium removed by aspiration in a microtiter plate washer (Dynatech Laboratories, Chantilly, VA). After one wash with phosphate buffered saline (PBS), 200 μl of PBS containing FDA (10 μg/ml) was added columnwise to control, experimental and blank wells. Subsequently the plates were incubated for 1 h before reading the fluorescence (ex 480 nm, em 530 nm) in the microtiter plate fluorometer Fluoroscan II (Labsystems OY, Helsinki, Finland). The fluorometer was blanked against wells containing PBS including the fluorescent dye but without cells. The results obtained are presented as survival index (SI), defined as fluorescence in per cent of control cultures, with

Table 1. Characteristics of investigated cell lines

Cell line designation	Type	Selecting agent	Resistance factor (RF)	Pgp 170	Reference
RPMI 8226/S	myeloma	—	—	–	10
RPMI 8226/dox40	myeloma	doxorubicin	>40	+	10
ACHN	renal carcinoma	—	—	–	11
U-937 GTB	histiocytic lymphoma	—	—	–	12
U-937/vcr 10	histiocytic lymphoma	vincristine	150	–	14
U-937/vcr100	histiocytic lymphoma	vincristine	200	+	—[a]
K562	erythroleukemia	—	—[a]	–	13

RF = IC_{50} in resistant subline/IC_{50} in parental cell line for selecting agent.
[a] Data to be published

a.

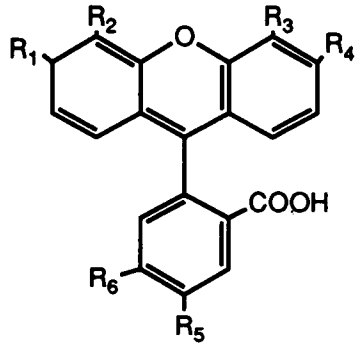


Calcein/AM
R_1,R_4 = -OOCCH$_3$
R_2,R_3 = -CH$_2$N(CH$_2$COOCH$_2$OOCCH$_3$)$_2$
R_5,R_6 = -H

CFDA
(5-and&6-carboxyfluorescein diacetate mixed isomers)
R_1,R_4 = -OOCH$_3$
R_2,R_3 = -H
R_5 = -COOH, R_6 = -H
or R_5 = -H, R_6 =-COOH

CFDA/AM
R_1,R_4 = -OOCH$_3$
R_2,R_3 = -H
R_5 = -COOCH$_2$OOCCH$_3$
R_6 = -H

b.



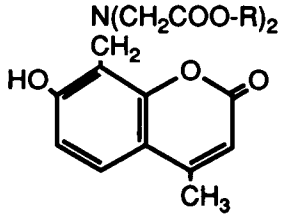
Fluorescein
R_1 = =O
R_2,R_3 = -H
R_4 = -OH
R_5,R_6 = -H

FDA
R_1,R_4 = -OOCCH$_3$
R_2,R_3,R_5,R_6 = -H

Calcein
R_1 = =O
R_2,R_3 = -CH$_2$N(CH$_2$COOH)$_2$

Carboxyfluorescein
R_1 = =O
R_2,R_3,R_6 = -H
R_4 = -OH
R_5 = -COOH

c.



Calcein Blue/AM R = -CH$_2$OOCCH$_3$
Calcein Blue R = -H

Figure 1. Molecular structure of calcein/AM and the structurally related dyes tested.

blank values subtracted. Senzitization ratio (SR) was defined as IC_{50} for calcein/AM in the absence of SDZ PSC 833/IC_{50} for calcein/AM in the presence of PSC. In separate experiments it was verified that the influence of calcein/AM on the FDA signal at the highest calcein/AM concentration was less than 5%. In addition, wells containing calcein/AM were routinely evaluated for morphological evidence of cytotoxicity using the differential staining cytotoxicity (DiSC) assay[17,18] to verify the accuracy of the fluorescent readings.

Measurement of hydrolysis and cellular accumulation

Measurement of calcein/AM hydrolysis and uptake was performed as described previously.[8] A 96-well microtiter plate (Nunc) was prepared with quadriplicate wells of 20 μl of 5 mM glucose in PBS with or without 10 μg/ml of PSC. Cells were washed and diluted in PBS containing 5 mM glucose to a final concentration of 390 000/ml and 160 μl of the cell suspension was dispensed into each well of the microtiter plate. Subsequently, calcein/AM was dispensed to the wells to a final concentration of 2.5 μg/ml. The fluorescence (ex 480 nm, em 530 nm) was immediately read in the Fluoroscan II (Labsystems), with the temperature mode set to 37°C. The fluorescent signal generated was then measured every 5 min up to 30 min, and subsequently every 15 min up to 120 min. Finally the fluorescence accumulated was also measured at approximately 2 h after two washes with PBS to remove all extracellular dye.

Measurement of cellular efflux

Cell suspensions of the investigated cell lines were diluted in PBS to 0.6–1.2 × 10^5 cells/ml. Calcein/AM at a final concentration of 2.5 μg/ml was added to each cell suspension. The suspensions were incubated for 2 h at 37°C and 5% CO_2. After the incubation the suspensions were centrifuged for 5 min at 200 *g* and then resuspended in fresh RPMI 1640 medium. The cells were then dispensed as quadriplicates into 10 replicate V-shaped microtiter plates (Nunc). One plate was immediately washed twice with PBS and fluoresence was measured in the Fluoroscan II. The other plates were incubated at 37°C and 5% CO_2 and were measured the same way at 1, 3, 6, 12, 24, 30, 36, 48 and 72 h.

Results

Cytotoxicity

The IC_{50} for calcein/AM in all cell lines after 72 h exposure is shown in Table 2. The U-937 GTB cell line was the most sensitive one followed by U-937/vcr10, RPMI 8226/S and K562 cells, and with the MDR sublines and ACHN being the least sensitive. Compared with calcein/AM, carboxyfluorescein diacetate and its AM ester as well as calcein blue and FDA showed considerably less activity and non-esterified calcein lacked cytotoxic effects in U-937 GTB cells (Figure 2). A similar pattern of cytotoxic activity was found for those compounds also in the other calcein sensitive cell lines (not shown). Adding the Pgp-blocking cyclosporin PSC (3 μg/ml) to

Table 2. IC_{50}, sensitization ratio (SR) and area under the curve (AUC) for calcein/AM in different cell lines

Cell type	IC_{50}[a] (SEM)	SR[b]	AUC[c] ($F \times h^{-1}$)
RPMI 8226/S	0.77 (0.20)	1.1	913
RPMI 8226/dox40	9.5 (0.68)	5.6	61
ACHN	11.3 (0.90)	1.5	813
U-937 GTB	0.25 (0.02)	0.9	608
U-937/vcr10	0.32 (0.03)	1.2	420
U-937/vcr100	2.2 (0.33)	18.8	51
K562	1.1 (0.22)	1.0	876

[a] Inhibitory concentration 50% (IC_{50}) was determined in five to seven individual experiments after 72 h of incubation.
[b] SR was defined as the ratio of IC_{50} in the absence and in the presence of 3 μg/ml PSC 833 and expressed as mean values from at least two separate experiments.
[c] AUC was calculated after exposure to 2.5 μg/ml calcein/AM for 2 h from 0–12 h post-washing using the trapezoidal method, see Figure 3. *F* = fluorescence intensity.

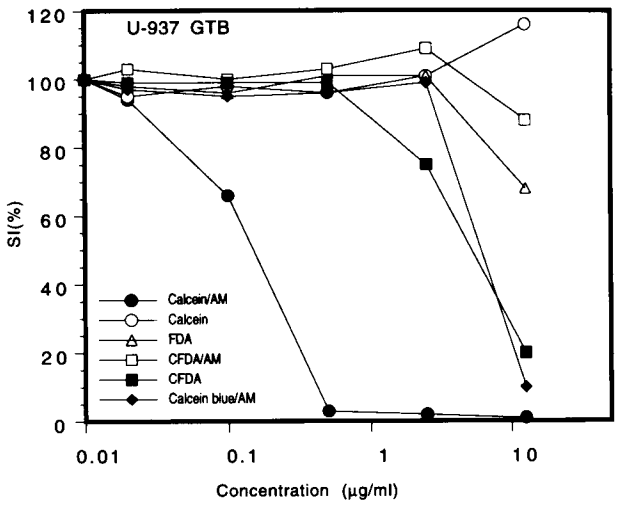


Figure 2. Effect of calcein/AM, free calcein, calcein blue/AM, FDA, carboxyfluorescein diacetate and carboxyfluorescein diacetate/AM on SI in U-937 GTB after 72 h incubation. One typical experiment is presented.

the cultures potentiated the effect of calcein/AM only in the two MDR cell lines and marginally in ACHN (Table 2). When the time dependency of the effect was investigated, little or no effect was observed after 3 h exposure, whereas profound concentration-dependent cytotoxicity was observed at 72 h (Figure 3A). When exposing U-937 GTB cells to calcein/AM for different periods of time (30 min–72 h) with a total assay time of 72 h there were no major differences in the concentration–response relationship (Figure 3B). Similar results was observed also in RPMI 8226/S (not shown). Finally, when U-937 GTB cells were incubated with different concentrations of calcein/AM for 30 min at the same cell density and plated in microtiter plates at high (10^6 cells/ml) and low (10^5 cells/ml) density, an increased cytotoxic activity was observed at the lower seeding density (Figure 3C).

Calcein hydrolysis and cellular accumulation

There was a time-dependent increase in the generation of fluorescent calcein with the highest values reached for U-937 GTB, U937/vcr10 and RPMI 8226/S cells (Figure 4A). The Pgp 170 overexpressing RPMI 8226/dox40 and U-937/vcr100 cells, on the other hand, showed a low rate of calcein/AM hydrolysis. After washing away extracellular calcein and calcein/AM, the cellular calcein

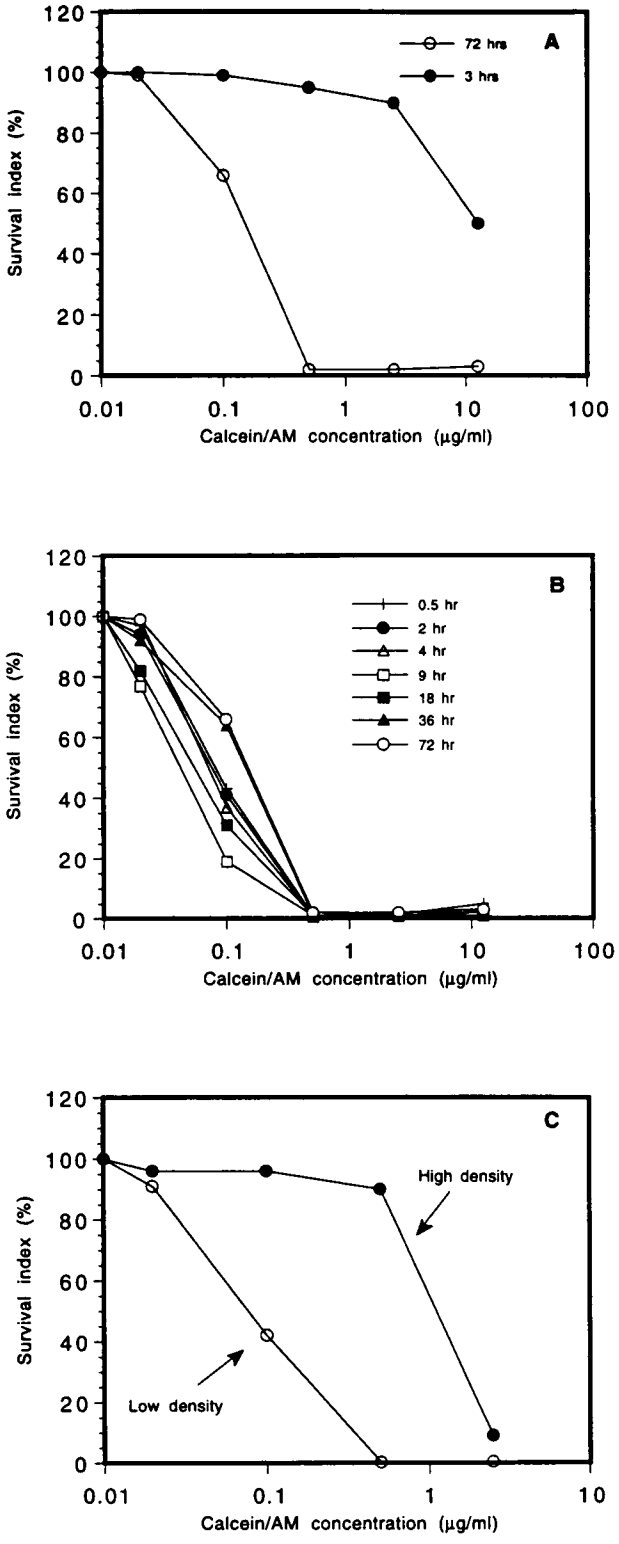


Figure 3. Effect of calcein/AM on SI in U937-GTB cells after 3 or 72 h of incubation (A), after indicated exposure times and 72 h of incubation (B), and after exposure for 30 min at 10^6 cells/ml followed by plating at high (10^6 cells/ml) and low (10^5 cells/ml) cell density (C). The results are presented as one representative experiment.

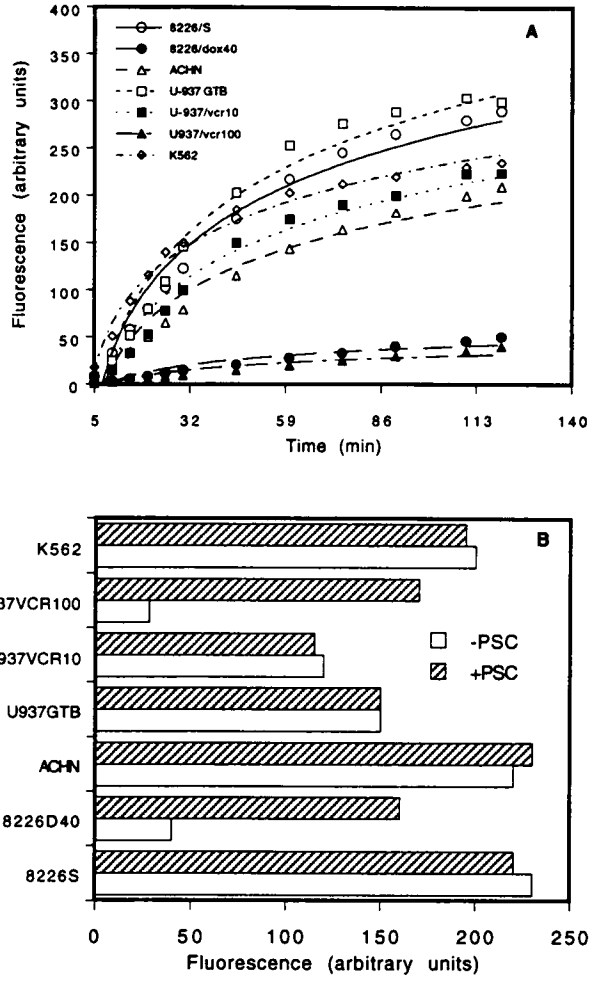


Figure 4. Time-dependent calcein/AM hydrolysis (A) and cellular accumulation after 2 h exposure (B) in the indicated cell lines. In (B) the effect of 3 μg/ml of SDZ PSC 833 is also shown. See Materials and methods.

accumulation was found highest for RPMI 8226/S, K562 and ACHN whereas for the U937 GTB and vcr10 cells the proportion of hydrolyzed calcein confined intracellularly was somewhat lower (Figure 4A and B). Both the MDR cell lines showed very low accumulation of the dye. When 3 μg/ml PSC was added the cellular accumulation of calcein increased in the Pgp expressing cell lines, approaching the levels observed in the parental cell lines. PSC had no effect on accumulation in non-MDR cell lines (Figure 4B).

Calcein efflux

In Figure 5 the efflux of calcein from cells preloaded with 2.5 μg/ml calcein/AM for 2 h is shown. There

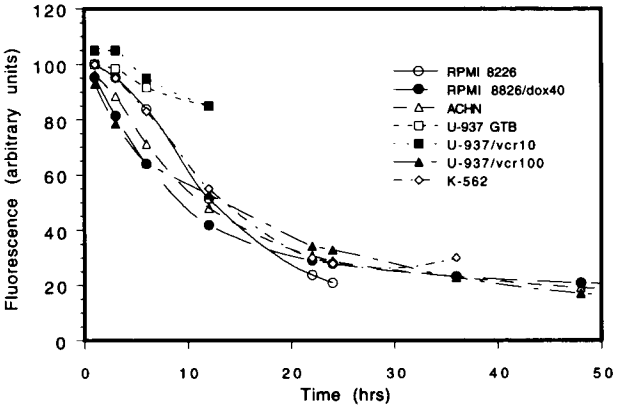


Figure 5. Time-dependent efflux of calcein/AM from the indicated cell lines after 2 h incubation at 2.5 μg/ml. The graph shows one of two experiments.

was a very slow rate of calcein efflux from U-937 GTB and vcr10 cells over the first 12 h compared with the other cell lines. At the next timepoint investigated (22 h), membrane integrity was lost with clear morphological evidence of cell death precluding further study of dye efflux. The remaining cell lines showed efflux curves with a rapid first phase up to 12 h, which for ACHN and the MDR cell lines was followed by a slower phase (12–72 h). These cell lines did not show evidence of significant cell death at later timepoints. The second slower efflux phase could also be discerned for RPMI 8226/S and K562 although significant cell death was observed before 72 h. When the exposure for the first 12 h was calculated as area under the fluorescence intensity–time curve (AUC) normalized for cell number, RPMI 8226/S, K562 and ACHN showed the highest AUC, with U-937 GTB and vcr10 showing intermediate levels, and the MDR cell lines showing the lowest level of exposure (Table 2).

Discussion

In the present paper we demonstrate a significant cytotoxic activity, not shared by other closely related fluorescein derivatives, of calcein/AM alone at relatively low concentrations. The effect was clearly dependent on the cytoplasmic hydrolysis of the AM ester to free calcein since the latter compound had no effect. Indeed, the MDR lines which showed the lowest degree of hydrolysis and accumulation were also less sensitive to the cytotoxic effect of calcein. This observation together with the fact that sensitivity could be restored by the addition of PSC is consistent with calcein/AM being a substrate for Pgp.[7,8] However, there was no absolute relation-

ship between cellular calcein accumulation and cytotoxicity since ACHN, which showed a high degree of calcein accumulation, was resistant to the cytotoxic effects of calcein. This was the case also when total exposure during the first 12 h was calculated revealing higher exposure in ACHN compared with calcein sensitive U-937 GTB cells. This differential pattern may indicate that other factors, like target expression and/or sensitivity, may play a role in determining calcein/AM sensitivity.

The cytotoxic effects of calcein in the sensitive cell lines also showed no apparent schedule dependency since similar concentration–response curves were obtained irrespective of the exposure times from 30 min to 72 h. This may be a potential pharmacokinetic advantage if calcein/AM is to be evaluated *in vivo*, allowing for quick distribution and cellular retention from a single bolus administration. The delivery by esterase-dependent trapping may also provide some protection for the normal hematological precursor cells which have a reported low non-specific esterase activity.[19] There may also be some selectivity for tumor cells from solid tumors since, at least on a per cell basis, many solid tumor samples show a higher esterase activity compared to their hematological counterparts.[20] Calcein/AM also show density-dependent effects which may explain why the use of the agent as a cytoplastic viability dye has been feasible without apparent cytotoxicity.[21] The recommended density of cells/ml loading media in these applications is very much higher (about 100 times) than used in the present paper.[21]

What is then the primary cellular target for calcein/AM? The major part of the intracellularly accumulated dye is probably distributed in the cytoplasm.[1] However, a non-cytoplasmic localization for at least a fraction of the dye cannot be excluded and this issue requires techniques like confocal imaging to be completely resolved. Indeed, for cell lines tested the efflux of calcein is apparently biphasic. The persistant fluorescence may be due to covalent association with intracellular macromolecules with long half-life, as suggested by others.[2] U-937 GTB and vcr10 cells are an exception from this efflux pattern by showing unusually high initial retention. However, it cannot be excluded that the initial rapid phase was missed by the present sampling scheme. Indeed, in the U-937 GTB and vcr10 cells, only approximately 50% of the hydolyzed calcein was retained after washing, which should be compared with 80–90% for the other cell lines. Irrespective of this, the stable cytoplasmic dye retention in the U-937 GTB cell line during the first 12 h makes this cell line especially interesting in the search for specific cytoplasmic molecules with high affinity for calcein/AM.

One potential target for calcein/AM may be cytoplasmic tubulin. However, the Vcr resistant U-937/vcr 10 for which tubulin alterations has been implicated as a possible resistance mechanism[14] showed similar degree of calcein/AM sensitivity, uptake and efflux characteristics as the parental cell line. Moreover, preliminary results using primary cultures of tumor cells from patients show no high degree of cross-resistance with tubulin active drugs, including vinca alkaloids and paclitaxel.

The mechanism for calcein/AM induced cell death is also not clear. However, the fact that the loss of membrane integrity is delayed to 12–48 h in the various calcein/AM sensitive cell lines indicates the potential involvement of programmed cell death (apoptosis).[22] Morphological and biochemical evidence on this point is clearly needed.

The reason for the difference between calcein/AM and the other structurally similar fluorescein derivatives tested with respect to cytotoxic activity is also not clear. All compounds possess planar aromatic domains which are the characteristic feature of Pgp blocking agents.[23] However, only the calcein molecule contains a basic nitrogen atom and extended carbon side chains which are additional structural requirements associated with Pgp-blocking efficacy.[23] The structure–activity relationships for calcein/AM and related compounds for cytotoxicity remains to be determined.

Conclusion

Calcein/AM show cytotoxic activity at relatively low concentrations in several human tumor cell lines. The cellular target appears to be located in the cytoplasm to which the drug is delivered by esterase-dependent intracellular trapping. These observations warrant further preclinical investigation of the antitumor activity of calcein/AM and related compounds on *in vitro* and *in vivo* tumor models. Evaluation of calcein/AM antitumor efficacy on primary cultures of tumor cells from patients is currently underway.

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