In-vitro toxicity of Ukrain on human Ewing sarcoma cell lines

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Abstract

Ukrain™ is advertised as a drug for alternative cancer cures with high activity against progressive Ewing sarcomas (EWS). Since preclinical data of Ukrain™ on EWS are not available so far, we analysed the in vitro toxicity of Ukrain™ on four human EWS cell lines and compared it to the in vitro toxicity of thioTEPA, Chelidonium majus L. alkaloids, doxorubicin, cyclophosphamide, and etoposide. In addition, we studied the toxicity of thioTEPA combined with Chelidonium majus L. alkaloids. Cell viability was determined by the MTT-assay after 48h, 72h, and 96h.

All compounds reduced the growth of EWS cell lines in a time and dose dependent manner. The concentrations which resulted in a growth inhibition of 50% ranged between 6.2 and 31.1 µM for Ukrain™, 1.9 and 26.1 µM for the Chelidonium majus L. extract and 1.7 to 448 µM for thioTEPA. The sensitivity profile of Ukrain™ was comparable to that of the Chelidonium majus L. extract and different from thioTEPA, cyclophosphamide, etoposide, and doxorubicin. Overall doxorubicin was the most toxic drug followed by cyclophosphamide. Ukrain™ and the chelidonium alkaloids were slightly more toxic than etoposide, while thioTEPA showed the lowest toxicity. Co-exposure of thioTEPA with Chelidonium majus L. alkaloids resulted in additive but not in synergistic toxicity.

Though less toxic compared to doxorubicin and cyclophosphamide Ukrain™ was active on Ewing sarcomas in vitro, which might be considered for further preclinical evaluation.

Key words

Ukrain™, ThioTEPA, Chelidonine, Ewing sarcoma

Introduction

The usage of alternative cancer cures is high, and longitudinal data suggest that it is increasing. Ukrain™, which is manufactured by Nowicky Pharma (Vienna, Austria), is a drug used for alternative cancer cures. Ukrain™ is only licensed as a drug in White Russia and not on the European market.

Nowicky Pharma has advertised its product widely on its company webside and produced a number of publications. According to the manufacturer Ukrain™ is a semi-synthetic product generated by thermal adduction between the N,N',N''-Triethylenethiophosphoramide (thioTEPA) and purified alkaloids from Chelidonium majus L. (Greater Celadine) (www.ukrin.com). Ukrain™ was reported to induce apoptosis, to inhibit angiogenesis and metastasis, and to modulate immune function (1-5). In addition, Ukrain™ is claimed to selectively kill cancer cells without affecting normal healthy tissues and to be devoid of mutagenicity, carcinogenicity, and teratogenicity in animals (6-9). In healthy human volunteers no significant toxicity was reported up to a daily dose of 50 mg (5,10). The most frequent side effects observed were local irritation, transient low grade fever, thirst, polyuria, mild nausea and pruritus (11).

According to the manufacturer Ukrain™ is active against colon, breast, bladder, prostate, ovarian, cervix, endometrial, and bronchial carcinomas as well as testicular cancers, melanomas, leukemias, lymphomas, and sarcomas (12-15). So far, two hundred and three cancer patients with advanced disease were reported, who received Ukrain™ over a mean period of 2.5 years. Overall 20% of these patients, for whom no other therapies were available, achieved complete remission (16). Partial remissions were observed in 60% of patients. Especially high rates of complete remission were reported for patients with neuroblastoma (60%) and with Ewing sarcoma (57%) - indicating high activity of Ukrain™ against small, round, blue-cell tumours of childhood.

These reports, however, were severely criticized for considerable methodological shortcomings and lack of rigorous independent replication (17). A number of medical boards like the German society of cancer, the German society of oncology, the German society of complementary oncology of the German alternative practitioner, the study group on „methods of unproven efficacy in oncology" of the Swiss cancer league decidedly refuse the use of Ukrain™ for the cancer treatment.

Despite of clinical trials according to good clinical practice Ukrain™ might add clinical benefit to a group of cancer patients and therefore, needs to be investigated adequately by standard research practice. Since preclinical data on the efficacy of Ukrain™ on Ewing sarcoma have not been published so far, we compared the in vitro toxicity of Ukrain™, thioTEPA, and Chelidonium majus L. alkaloids with the standard anticancer drugs doxorubicin, cyclophosphamide and etoposide on four well characterized human Ewing sarcoma cell lines.

Materials

Reagents

Ukrain™ was provided by Nowicky Pharma (Vienna, Austria). ThioTEPA was purchased from Lederle (Wolfratshausen, Germany) and an ethanol extract from Chelidonium majus L., which contained chelidonine and other alkaloids, was obtained from Pascoe GmbH (Gießen, Germany). Doxorubicin was purchased from Pharmacia (Freiburg, Germany), etoposide from Sigma Aldrich (Deisenhofen, Germany). 4-Hydroxyperoxocyclophosphamide was provided by ASTA medica (Frankfurt, Germany). The Chelidonium majus L. extract was standardized to a chelidonine content of 1.2 mg in 1 g extract. Stock solutions of Ukrain™ and thioTEPA were prepared by dissolution in sterile distilled water. Etoposide was dissolved in dimethylsulfoxide (DMSO) (Sigma Aldrich). Stock solutions were diluted with complete cell culture medium. Controls consisted of complete cell culture medium. The volume of sterile distilled water, DMSO, or ethanol, which was used for the highest drug concentrations did not affect the growth of the four Ewing sarcoma cell lines CADO-ES-1, STA-ET-1, STAET-2.1, and VH-64.

Cell culture

VH-64, STA-ET-1, and STA-ET-2.1 were kindly provided by F. van Valen, Department of Orthopaedics, Muenster, Germany. CADO-ES-1 was purchased from the German Collection of Microorganisms and Cell Culture (DMSZ, Braunschweig, Germany). CADO-ES-1 and VH-64 were derived from lung metastasis of typical Ewing sarcomas. STA-ET-1 and STA-ET-2.1 stem from primary peripheral neuroectodermal tumours.

All cell lines were grown in RPMI 1640 medium (GibcoBRL cell culture, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 200 mM L-glutamine, 10.000 U/ml penicillin G,

10.000 µg/ml streptomycin, 25 µg/ml amphotericin B and 10 % fetal calf serum on collagen coated 7.5 cm^2 tissue culture flasks in a humidified atmosphere of 5 % CO_2 at 37 °C.

Cell viability assay

Chemosensitivity was evaluated by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay (18,19). Cells were grown on collagen-coated 96-well flat-bottom microtiter plates (Becton Dickinson, Heidelberg, Germany). One hundred µl of cell suspension containing 3 x 10^3 CADO-ES-1 or VH-64 cells, 6 x 10^3 STA-ET-1 cells, or 9 x 10^3 STA-ET-2.1 cells were seeded in each well. In order to allow adhesion to the collagen matrix and resumption of exponential growth the cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 72 h before 100 µl of medium containing the respective drugs at different concentrations were added. After 48 h, 72 h, and 96 h 20 µl of MTT reagent (Sigma, Deisenhofen, Germany) was added to each well and the cells were incubated for another 4 h. The MTT reagent was dissolved in phosphate buffered saline (PBS) pH 7.4 (Life Technologies, Karlsruhe, Germany) at a concentration of 5 mg/ml. In viable cells mitochondrial dehydrogenases reduce the yellow soluble MTT to water insoluble blue formazan crystals. An increase in the number of living cells resulted in an increase in total metabolic activity in the sample, which in turn correlated with the amount of purple formazan crystals formed. After 4 h the supernatant was removed and the formazan crystals were dissolved in a solution of sodiumdodecylsulphate (SDS) (20% w/v) solved in dimethylformamide (DMF) and water (50% v/v). The absorbance of the dissolved formazan dye of each well was measured at 550 nm and a reference wavelength of 630 nm using an automated Dynatech MR 7000 microplate reader.

Each drug concentration was tested in four replicates from which mean, standard deviation and coefficient of variation were calculated. Dose-response curves were plotted on a semi-

logarithmic scale with the percentage of viable cells compared to untreated controls versus drug concentrations. The drug concentration capable of 50% growth inhibition relative to untreated controls (= GI_{50}) at the respective time points 48 h, 72 h, and 96 h was calculated with the equation ([% viable cells (>50%)] - 50) / ([% viable cells (>50%)] - [% viable cells (< 50%)]) * (drug concentration above 50% viable cells - drug concentration below 50% viable cells) + (drug concentration below 50% viable cells).

Results

Ukrain™ as well as the other tested drugs inhibited the growth of all four Ewing sarcoma cell lines in a time and dose dependent manner. After 72 h and 96 h Ukrain™ significantly inhibited the growth of all cell lines treated with concentrations between of 0.05-50 µM. Table 1 summarizes the GI_{50}-concentrations determined for Ukrain™, Chelidonium majus L. extract, thioTEPA, etoposide, doxorubicin and 4-hydroxyperoxocyclophosphamide after 48 h, 72 h, and 96 h.

Overall doxorubicin was the most effective drug on the four Ewing sarcoma cell lines followed by 4-hydroxyperoxocyclophosphamide. With a mean GI_{50} (calculated from all cell lines and time points; $GI_{50\text{-mean}}$) of 0.2 µM doxorubicin was about 30 times more potent than 4-hydroxyperoxocyclophosphamide with a mean GI_{50} of 6.5 µM. The mean GI_{50} concentrations for Ukrain™ and the Chelidonium majus L. extract were 11.9 µM and 12.3 µM, which were about 60 times higher than the mean GI_{50} concentration of doxorubicin. Etoposide ($GI_{50\text{-mean}}$: 14.9 µM) was about 75 times and thioTEPA ($GI_{50\text{-mean}}$: 89.2 µM) more than 450 times less potent than doxorubicin.

STA-ET-1 and VH-64 were the cell lines most sensitive to Ukrain™ and Chelidonium majus L. extract, while both CADO-ES-1 and STA-ET-2.1 showed less sensitivity to Ukrain™ and the Chelidonium majus L. extract. ThioTEPA was most active in STA-ET-1 followed by CADO-ES-1 and VH-64. STA-ET-2.1 was the cell line most resistant to thioTEPA. Like thioTEPA etoposide and 4-hyroxyperoxocyclophosphamide showed the highest toxicity on STA-ET-1 followed by CADO-ES-1 and VH-64. STA-ET-2.1 was the cell line most resistant to etoposide and 4-hydroxyperoxocyclophophamide. Doxorubicin was most toxic on STA-ET-1, while it showed the least toxicity on CADO-ES-1. VH-64 and STA-ET-2.1 were of intermediate sensitivity.

Since our formula for the calculation of GI_{50} differed from the formulas used by the NCI, we also calculated growth inhibition of 50% ($GI_{50\text{-}NCI}$), total growth inhibition (TGI_{NCI}), and reduction of cell viability by 50% ($LC_{50\text{-}NCI}$) according to the NCI formulas (http://dtp.nci.nih.gov/branches/btb/ivclsp.html). For the Ewing sarcoma cell lines we determined $GI_{50\text{-}NCI}$ concentrations between 4.32 and 11.8 µM (mean: 7.57 µM) after Ukrain™ exposure for 48h. Concentrations of TGI_{NCI} were 11.5 – 34.2 µM (mean 27.0 µM) and $LC_{50\text{-}NCI}$ concentrations ranged from 33.9 to >50 µM (mean: 40.5 µM). This is about two times above the mean $GI_{50\text{-}NCI}$ (mean: 3.2 µM), TGI_{NCI} (mean: 15.8 µM), and $LC_{50\text{-}NCI}$ (mean: 67.6 µM) concentrations determined by the NCI on the 60 human tumour cell lines.

We further tested, whether thioTEPA combined with Chelidonium majus L extracts, without thermal adduction resulted in synergistic toxicity compared to Chelidonium majus L. alkaloids alone. The cell lines were incubated with either 5 µM or 50 µM thioTEPA and with increasing concentrations of Chelidonium majus L. extract. On all cell lines and at each time point the combinations of thioTEPA and Chelidonium majus L. extract were more toxic than thioTEPA or the Chelidonium majus L. extract alone (Figure 1). However, the addition of thioTEPA to the Chelidonium majus L. extract without the process of thermal adduction did not synergistically increase the toxicity of the chelidonium majus L extract (Figure 2).

Discussion

The National Cancer Institute screened the in vitro toxicity of Ukrain™ on 60 human tumour cell lines as part of its Developmental Therapeutic Program (NSC 631570; http://dtp.nci.nih.gov). Since Ewing sarcomas were not among the tested tumour types, we compared the toxicity of Ukrain™ to standard anticancer drugs in four human Ewing sarcoma cell lines in vitro.

In our experimental setting Ukrain™ reduced the growth and viability of Ewing sarcoma cell lines in a dose and time dependent manner. The effects of Ukrain™ were superior to that of thioTEPA and comparable to that of etoposide, which has been proven effective in the treatment of Ewing sarcomas in vivo. However, Ukrain™ was inferior to doxorubicin and the activated form of cyclophosphamide, which are one of the most active drugs in the treatment of Ewing sarcomas (20-22).

The Ewing sarcoma cell lines tested were in mean about two times less sensitive to Ukrain™ than the 60 human cell lines tested by the NCI Developmental Therapeutic Program. This difference might be explained by the different methods used for staining of viable cells. The NCI uses the SRB-protein-assay to determine cell viability and the SRB-protein-assay has been shown to result in lower IC_{50} values than the MTT-assay (23). In addition the cell lines used might have influenced the test results as well.

The resistance profile of Ukrain™ on the four Ewing sarcoma cell lines was comparable to that of Chelidonium majus L. alkaloids and not to thioTEPA. Similar observations were reported for HeLa, Hs27, Graham 293 and Vero cells. The effects of Ukrain™ on these cell lines were comparable to those of chelidonine but not to those of thioTEPA (24,25).

Panzer et al. found Chelidonium majus L. alkaloids at least in a part of the commercial preparation (26). Regarding the sensitivity profile of Ukrain™ and Chelidonium majus L. extract on the four Ewing sarcoma cell line Chelidonium majus L. alkaloids might well have

contributed to the toxicity observed on the four Ewing sarcoma cell lines. The combination of thioTEPA with Chelidonium majus L. extract without thermal adduction increases the toxicity because of the toxicity of each compound, however, this combination at least without thermal adduction did not result in synergistic toxicity on the four Ewing sarcoma cell lines.

Oral preparations of Chelidonium majus L. extracts are used for spasmodic diseases of the billary tract and spasmodic gastro-intestinal diseases. Intravenous applications of Chelidonium majus L. are not available on the European market. The oral preparations of Chelidonium majus L. were repeatedly warned of because of severe side effects like i.e. hepatitis, cholestasis, necrosis of the liver parenchyma and lethal liver failure of several different drugs made from chelidonium-plant (27-29). Thus, regarding the toxicities of oral Chelidonium majus L. preparations the use of intravenously applied Chelidonium majus L. extracts should be considered with care in general.

In the preclinical screen Ukrain™ showed activity against Ewing sarcoma cell lines. It was less effective than the anticancer drugs with the highest therapeutic activity in the treatment of Ewing sarcomas, but it showed a different sensitivity profile on the Ewing sarcoma cell lines compared to the standard anticancer drugs, cyclophosphamide, etoposide, and doxorubicin used for the treatment of Ewing sarcomas.

It would be irresponsible to advocate the use Ukrain™ without additional preclinical information and sufficient clinical data on its activity, tolerability, and safety, which have to be obtained with state of the art clinical trials methodology according to good clinical practice. On the other hand, the in vitro data should encourage the manufacturer to further explore the efficacy of Ukrain™ to provide both clinician and patient evidence based data for therapeutic decision making.

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Table 1: GI_{50} concentrations of Ukrain™, thioTEPA, chelidonium majus L. extract, and standard anticancer drugs on four Ewing sarcoma cell lines.

Drug	Cell line	GI_{50} [µM]		
		48h	72h	96h
Ukrain™	CADO-ES-1	29.8	12.7	9.26
	VH-64	7.57	7.19	6.97
	STA-ET-1	8.69	7.28	6.19
	STA-ET-2.1	31.1	8.38	7.21
Chelidonium majus L. extract	CADO-ES-1	24.6	18.9	12.1
	VH-64	10.7	5.33	2.24
	STA-ET-1	8.02	2.19	1.88
	STA-ET-2.1	24.4	26.1	11.4
ThioTEPA	CADO-ES-1	141	4.09	2.49
	VH-64	235	76.1	15.5
	STA-ET-1	19.7	4.27	1.65
	STA-ET-2.1	448	101	21.9
Etoposide	CADO-ES-1	22.4	9.66	5.76
	VH-64	36.4	8.37	0.46
	STA-ET-1	1.44	0.55	0.25
	STA-ET-2.1	71.9	20.2	1.52
Doxorubicin	CADO-ES-1	0.71	0.45	0.48
	VH-64	0.18	0.05	0.04
	STA-ET-1	0.06	0.03	0.04
	STA-ET-2.1	0.21	0.05	0.07
4-Hydroxyperoxocyclophosphamide	CADO-ES-1	15.3	2.49	0.99
	VH-64	15.5	7.79	2.56
	STA-ET-1	2.67	0.99	0.69
	STA-ET-2.1	15.1	10.6	3.65

Legends:

Figure 1:

Cell viability of CADO-ES-1, STA-ET-2.1, and VH-64 exposed to varying concentrations of chelidonium majus L. extract with or without 5 µM of thioTEPA for 48h. The amount of viable cells after 48h was compared to the amount of viable cells determined at the start of the experiment (0h).

Figure 2:

CADO-ES-1, STA-ET-2.1, and VH-64 exposed to varying concentrations of chelidonium majus L. extract with or without 5 µM or 50 µM of thioTEPA for 48h. After 48h the amount of viable cells exposed to chelidonium majus L. extract was compared to the cell viability of either untreated cells for the incubations with chelidonium majus L. extract alone or cells exposed to 5 µM or 50 µM thioTEPA over 48h, respectively, for the co-incubation experiments.

Figure 1:

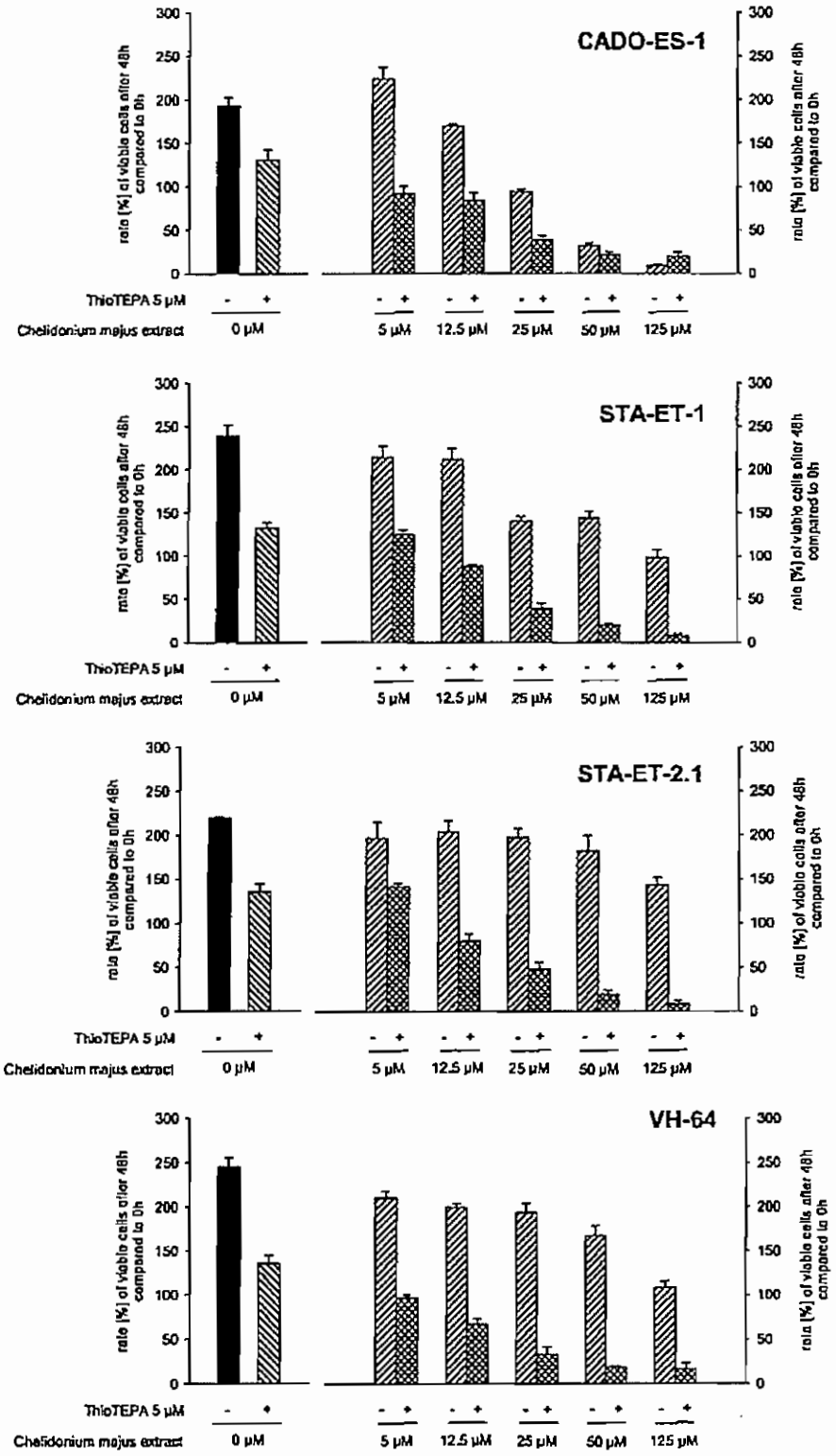
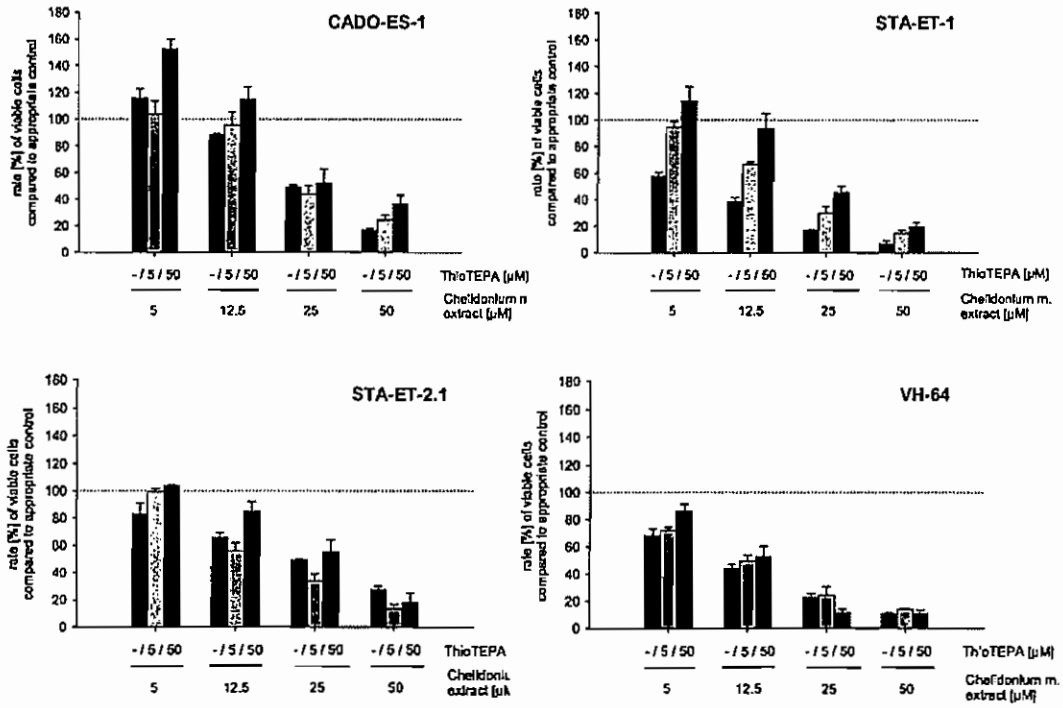

CADO-ES-1
STA-ET-1
STA-ET-2.1
VH-64
rate [%] of viable cells after 48h compared to 0h
ThioTEPA 5 µM
Chelidonium majus extract
0 µM
5 µM
12.5 µM
25 µM
50 µM
125 µM

Figure 2:


CADO-ES-1
STA-ET-1
STA-ET-2.1
VH-64
rate [%] of viable cells compared to appropriate control
-/5/50
ThioTEPA [µM]
Chelidonium m. extract [µM]
5
12.5
25
50