Apoptosis-based drug screening and detection of selective toxicity to cancer cells

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The goal of our study was to determine whether an apoptosis assay used after short-term drug exposure could predict selective toxicity to cancer cells. To this end we compared the effect of eight anticancer drugs and 10 toxic compounds without known antitumor activity in cultures of human breast cancer cells and normal diploid fibroblasts by Apoptosis ELISA and growth inhibition assays. There was an overlap in concentration values of drugs and toxins inhibiting proliferation in cancer cells. In contrast, Apoptosis ELISA clearly distinguished between the two groups of compounds. Anticancer drugs induced apoptosis in cancer cells at 0.0015–0.5 μM, while toxins were effective at much higher concentrations of 8.0–50.0 μM. Moreover, six out of the 10 toxins did not induce apoptosis in cancer cells. The normal:cancer cell (N:C) ratio for growth inhibiting concentrations was in a similar range for anticancer drugs and toxins. The N:C ratio for apoptosis inducing concentrations was 33–200 for anticancer drugs and 1.3–3.0 for toxins. Our data indicate that apoptosis assays could be used to detect selective toxicity of anticancer drugs by determining apoptosis induction in cancer cells or through a comparison of apoptosis-inducing concentrations in normal and cancer cells.
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Introduction

Cell-based drug screening assays play an important role in drug discovery, as target-based screening may not be predictive of the drug effect in the whole cell [1]. Since the detection of compounds selectively toxic to cancer cells is the most important goal of drug screening, the cell-based assays, which might predict such selectivity, should be included in the drug discovery process [2]. However, the routinely used short-term growth inhibition assays have limited ability to detect selectivity and only an evaluation of the long-term antiproliferative effects may establish a preferential effect on cancer cells [3].

The goal of our study was to determine whether apoptosis assay performed after short-term drug exposure might predict selective toxicity to cancer cells better than the growth inhibition assays routinely used for drug screening. The feasibility of such an approach was demonstrated by the ability of the apoptosis assay to distinguish between anticancer drugs and toxins without a known antitumor activity in leukemic cell cultures [4]. However, this effect has to be reproduced in cancer cell lines of non-hematological origin as these lines are used in majority of drug screening programs [3,5]. To this end we compared the induction of apoptosis by anticancer drugs and toxic compounds without known antitumor activity in breast cancer cells and normal diploid fibroblasts. The rationale for this approach was based on the assumption that clinical doses of anticancer drugs have some degree of selectivity for the cancer cells. Thus, these tests, which distinguish between anticancer drugs and toxins, could be potentially useful for the selection of anticancer leads by screening of chemical libraries.

The rationale for the inclusion of apoptosis assays in drug screening programs is indicated by the observations of higher sensitivity to drug-induced apoptosis of cancer cells in comparison with normal cells [6–10], although no systematic study of this differential sensitivity to apoptosis and of the relation between sensitivity to apoptosis and sensitivity to proliferation inhibition has been reported. To determine the ability of apoptosis assays to establish selective toxicity to cancer cells we used MDA-MB-468 breast cancer cell line (p53 mutant) [11] and human lung diploid fibroblasts WI-38. Although fibroblasts and epithelial cancer cell lines differ in their origins, the inclusion of WI-38 cells as a normal counterpart is justified by the fact that selectivity indices in long-term proliferation assay were similar whether normal fibroblasts or normal epithelial cells were used [3].

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The Apoptosis ELISA assay used in this study is based on the selective DNA denaturation in condensed chromatin of the apoptotic cells by formamide and reactivity of single-stranded DNA (ssDNA) in apoptotic cells with monoclonal antibodies (mAb) highly specific to ssDNA. These antibodies specifically detect apoptotic cells and do not react with the necrotic cells or non-apoptotic cells with drug-induced DNA damage [12–14]. The specificity of the assay is based on the mAb reaction with condensed chromatin in early and late apoptotic cells. Since chromatin condensation is the most universal and specific apoptotic marker, which indicates irreversible commitment to apoptosis [15], this assay provides a precise and specific assessment of cell death.

Materials and methods

Materials

Tissue culture medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Drugs, chemicals and peroxidase-conjugated anti-mouse IgM were obtained from Sigma (St Louis, MO). The mixture of primary anti-ssDNA mAb F7-26 with peroxidase-conjugated anti-mouse IgM for one-step Apoptosis ELISA immunostaining was supplied by Apostain (Miami, FL). The generation and binding specificity of anti-ssDNA mAb has been described previously [12].

Cell culture and drug treatment

The MDA-MB-468 breast cancer cell line was maintained as an adherent culture in IMEM (Richter) medium supplemented with 5% FBS at 37°C in a humidified incubator in 5% CO_2 atmosphere. Normal human fibroblasts (WI-38) obtained from ATCC (Rockville, MD) were grown in DMEM medium supplemented with 10% FBS. For experiments, 10^4 cells in 200 µl of complete medium were seeded per well in 96-well flat-bottomed culture-treated Costar plates. Plates were incubated at 37°C in a humidified incubator in 5% CO_2 atmosphere for 24 h before treatments. Culture medium was then replaced with 200 µl of complete medium containing drugs and chemicals in final concentrations. Each drug dilution was added to six wells. Control wells received 200 µl of complete medium. After drug addition, incubation was continued 48 h for breast cancer cells and 48–72 h for fibroblasts. The effects of treatments were analyzed with the Apoptosis ELISA and growth inhibition assays.

Apoptosis ELISA

Detection of the apoptotic cells by ELISA with antibody to ssDNA was performed as described earlier [4,14]. Plates incubated with drugs were centrifuged, culture medium was removed by vacuum using immunowasher (Nunc, Naperville, IL) and replaced with 200 µl of 80% methanol in PBS. After 30 min fixation at room temperature, plates were centrifuged, fixative was removed by vacuum and plates were dried in an oven at 56°C for 20 min. Wells with dried cells were filled with 50 µl of formamide and plates were incubated for 30 min in an oven preheated to 56°C to denature DNA of the apoptotic cells. Plates were then cooled for 5 min at 4°C. Single-stranded DNA was stained using the following steps: (i) blocking of non-specific binding sites with 200 µl of 3% non-fat dry milk for 1 h at 37°C, (ii) staining with anti-ssDNA mAb F7-26 mixed with peroxidase-conjugated anti-mouse IgM for 30 min [14], (iii) washing with PBS, and (iv) incubation with peroxidase substrate and measurement of mean absorbance of six wells at 405 nm in a plate reader (MRX Revelation plate reader; Dynatech, Chantilly, VA). The data is presented as a mean of three to four independent dose–response experiments ± SEM. Mean values of ELISA absorbance for each drug concentration, calculated from repeated experiments were used to create a single dose–response curve for each compound, with drug concentrations inducing apoptosis being determined from these curves.

Growth inhibition assays

The sulforhodamine B (SRB) growth inhibition assay was performed as described by Monks *et al.* [16] with slight modifications. Plates were centrifuged, the medium was replaced with 50 µl of cold 10% trichloroacetic acid and plates were incubated at 4°C for 30 min. Fixative was aspirated, and plates were washed 3 times with distilled water and dried at room temperature. Following drying, cells were stained with 50 µl of 0.4% SRB in 1% acetic acid for 20 min. SRB was removed, plates were washed with 1% acetic acid, the bound SRB was solubilized with 100 µl of 10 mM unbuffered Tris-based solution and the absorbance was measured at 550 nm in a plate reader.

The tetrazolium-based MTT assay, which detects reduction of 3-(4,5-dimethylthiazolyl)-2,5-diphenyformazan bromide by mitochondrial dehydrogenase of viable cells to a blue formazan product, was performed as described by Carmichael *et al.* [17]. Following incubation with drugs, 50 µl of 2 mg/ml MTT was added to each well, plates were incubated at 37°C for 4 h and the medium was replaced with 150 µl of DMSO. The absorbance in control and drug-treated wells was measured in a plate reader at 550 nm. Mean values of SRB or MTT absorbance for each concentration from repeated experiments were used to create a single dose–response curve for each compound and the concentrations inhibiting growth by 50% (IC_{50}) were calculated from these curves.

Results

Breast cancer cells

The effects of eight anticancer drugs and 10 toxic compounds without known antitumor activity were evaluated in cultures of human MDA-MB-468 breast cancer cells by Apoptosis ELISA and growth inhibition

assays after 2-day exposure. Multiple concentrations of drugs and toxins were used and effective doses were calculated from the dose–response curves. The typical results for anticancer drug cisplatin are presented in Figures 1 and 2, and the data for all compounds are summarized in Table 1. Apoptosis induction was characterized by the concentration increasing Apoptosis ELISA absorbance values more than 2-fold—from 0.4 (control) to 1.0. The antiproliferative activity was measured by the SRB and MTT assays IC_{50}.

The data presented in Figures 1 and 2 illustrate significant differences in the concentrations of cisplatin inducing apoptosis and inhibiting proliferation as determined by the SRB assay. Extensive apoptosis was induced by exposure to 0.03 μM cisplatin, while a decrease of SRB absorbance was detected only after treatment with concentrations higher than 0.375 μM. Similarly, for all the other drugs tested, apoptosis-inducing concentrations were significantly lower than the concentrations inhibiting growth by 50% (Table 1). The growth inhibiting concentrations measured by SRB and MTT assays were in a similar range, demonstrating that for anticancer drugs both growth inhibition assays have lower sensitivity than the Apoptosis ELISA.

Since the major goal of *in vitro* drug screening is to identify the agents with probable antitumor activity *in vivo*, we compared induction of apoptosis by toxic chemicals without known antitumor activity with the effects of anticancer drugs. We were interested to determine whether Apoptosis ELISA could be helpful in discriminating between these two types of compounds. Discrimination between anticancer drugs known to have at least limited selectivity for tumor cells and non-selective toxins demonstrates the potential usefulness of the assay for the prediction of selectivity.

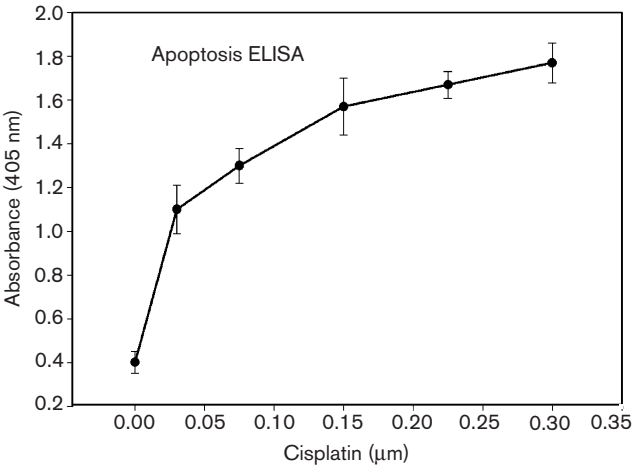
The toxic chemicals used in this study were selected from the list of reference compounds used for the

Table 1 Concentrations of anticancer drugs and toxins inducing apoptosis and inhibiting growth in culture of MDA-MB-468 breast cancer cells

Compound	Apoptosis ELISA (μM)	SRB assay IC_{50} (μM)	MTT assay IC_{50} (μM)
Anticancer drugs			
adriamycin	0.01	0.1	0.05
cisplatin	0.03	1.4	1.38
taxol	0.0015	0.01	0.01
6-thioguanine	0.5	2.0	1.5
vincristine	0.05	0.15	0.25
mitomycin C	0.1	3.5	2.5
etoposide	0.25	10.0	10.5
staurosporine	0.002	0.035	0.025
Toxins			
thioridazine	8.0	7.0	7.0
chlorpromazine	10.0	6.8	8.8
amitryptiline	30.0	30.0	35.0
quanidine sulfate	50.0	75.0	62.5
cyclohexemide	negative	1.8	1.8
benzothonium	negative	3.5	3.0
mercuric chloride	negative	7.5	7.5
cadmium chloride	negative	15.0	15.0
N-dimethylformamide	negative	200.0	175.0
2,4-dinitrophenol	negative	350.0	400.0

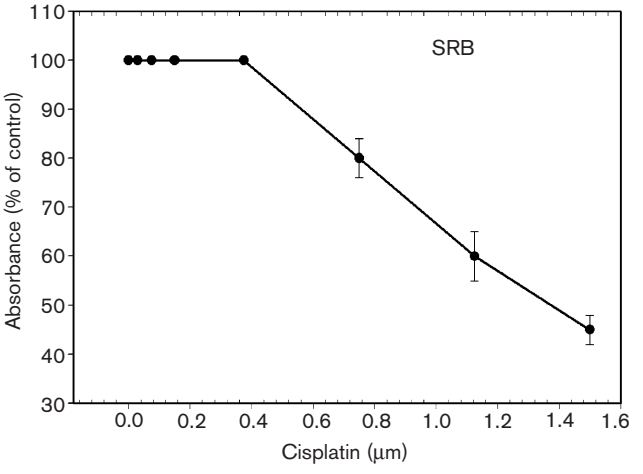
Apoptosis induction was characterized by concentrations increasing the Apoptosis ELISA absorbance more than 2-fold—from 0.4 (control) to 1.0. These concentrations were calculated from a single dose–response curve determined from mean values of ELISA absorbance from four independent experiments. Mean values of SRB or MTT absorbance for each drug concentration from four experiments were used to create a single dose–response curve with the IC_{50} concentrations being calculated from these curves.

Fig. 1



Induction of apoptosis by cisplatin in MDA-MB-468 breast cancer cells. Cultures grown in 96-well plates were treated with cisplatin for 48 h, fixed and analyzed by Apoptosis ELISA as described in Materials and methods.

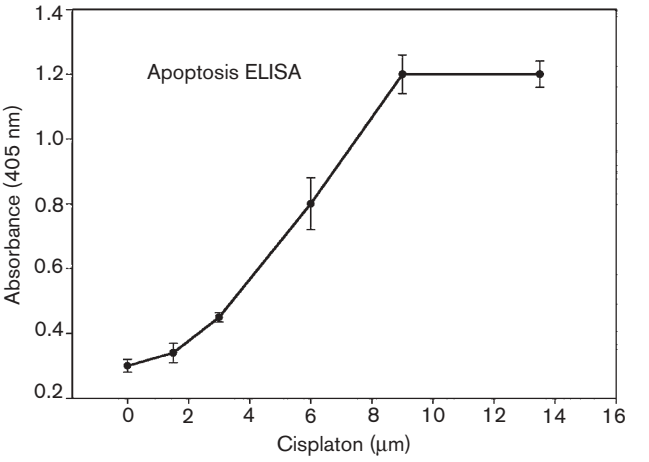
Fig. 2



Effect of cisplatin on growth of MDA-MB-468 breast cancer cells. Cultures grown in 96-well plates were treated with cisplatin for 48 h and analyzed by SRB growth inhibition assay as described in Materials and methods.

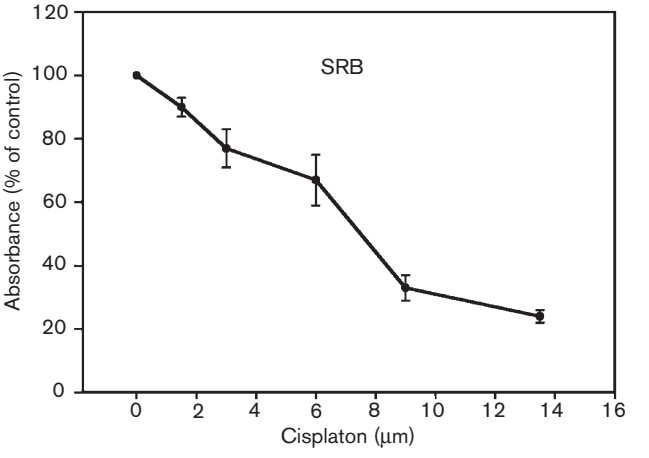
development of *in vitro* toxicity screens [18,19]. Although some of these compounds are used clinically for the non-oncological diseases, they are toxic and do not have known antitumor activity. First, these compounds were tested by the SRB assay and then compounds which inhibited cell proliferation were evaluated by the Apoptosis ELISA (Table 1). There was an overlap in concentration values of drugs and toxins inhibiting proliferation: all anticancer drugs and five out of 10 toxins were active at concentrations of 10 μM and lower. Thus, SRB or MTT IC_{50} alone could not distinguish between the two groups of compounds. In contrast, there was significant difference in concentrations inducing apoptosis between the two groups of compounds without any overlap: 0.0015–0.5 μM for anticancer drugs and 8.0–50.0 μM for toxins. Moreover, six out of 10 toxins tested did not induce apoptosis at any of the concentrations tested (0.5–2.0 IC_{50}) (Table 1). The important difference between the two types of compounds was the fact that the concentrations inducing apoptosis were significantly lower than those inhibiting proliferation for anticancer drugs, while toxins induced apoptosis and inhibited proliferation at similar concentrations.

Fig. 3



Induction of apoptosis by cisplatin in normal diploid fibroblasts WI-38. Cultures grown in 96-well plates were treated with cisplatin for 48 h, fixed and analyzed by Apoptosis ELISA as described in Materials and methods.

Fig. 4



Effect of cisplatin on growth of normal diploid fibroblasts WI-38. Cultures grown in 96-well plates were treated with cisplatin for 48 h and analyzed by SRB growth inhibition assay as described in Materials and methods.

Normal fibroblasts

Cultures of normal diploid fibroblasts were treated for 2 or 3 days with multiple concentrations of eight anticancer drugs and four toxins, which induced apoptosis in cancer cells. The 2-day exposure was performed to compare the effect on normal and cancer cells after a similar duration of treatment. The 3-day exposure of fibroblasts was used to compare the effect on normal and cancer cells after a similar generation time. The doubling time was 26.7 h for breast cancer cells and 40 h for fibroblasts. Thus, 2 days for cancer cells and 3 days for fibroblasts were equal in terms of cell doubling time. Effective doses of drugs and toxins were calculated from dose–response curves. Apoptosis induction was characterized by concentrations increasing Apoptosis ELISA absorbance more than 2-fold—from 0.3 (control) to 0.8. The antiproliferative activity was measured by the SRB assay IC_{50} after 2- and 3-day exposure. The typical results for cisplatin are presented in Figures 3 and 4, and the data for all compounds are summarized in Table 2.

All eight anticancer drugs inhibited cell proliferation in WI-38 fibroblasts (Table 2). Apoptosis was induced by five drugs after 2-day and by six drugs after 3-day treatment. Etoposide and 6-thioguanine did not induce apoptosis in normal fibroblasts even at concentrations 2-fold higher than SRB IC_{50}, while vincristine induced apoptosis only after 3-day exposure. Toxic compounds, which induced apoptosis in breast cancer cells, also induced apoptosis in normal fibroblasts (Tables 1 and 2). The concentrations of anticancer drugs inducing apoptosis in fibroblasts were similar to or higher than IC_{50} measured by the SRB assay. This is in striking contrast with the fact that apoptosis induction in cancer cells occurred at lower drug concentrations than the inhibition of proliferation.

Selective toxicity

Analysis of the data presented in the previous sections was performed to determine whether the apoptosis assay could be more useful for the evaluation of selective

Table 2 Concentrations of anticancer drugs and toxins inducing apoptosis and inhibiting growth in cultures of normal diploid fibroblasts WI-38 after 2 or 3 days exposure to drugs

Compound	Apoptosis ELISA (μM)		SRB assay IC_{50} (μM)	
	2 days	3 days	2 days	3 days
Anticancer drugs				
adriamycin	0.5	0.38	0.25	0.25
cisplatin	6.0	6.0	7.5	6.0
taxol	0.05	0.05	0.05	0.05
thioguanine	negative	negative	20.0	20.0
vincristine	negative	2.0	1.0	1.0
mitomycin C	20.0	12.5	10.0	5.0
etoposide	negative	negative	10.0	5.0
staurosporine	0.2	0.2	0.025	0.01
Toxins				
thioridazine	10.0	10.0	9.0	5.0
chlorpromazine	20.0	20.0	20.0	10.0
amitryptiline	40.0	40.0	50.0	20.0
quanidine sulfate	150.0	75.0	150.0	50.0

Apoptosis induction was characterized by concentrations increasing the Apoptosis ELISA absorbance more than 2-fold—from 0.3 (control) to 0.8. These concentrations were calculated from a single dose–response curve determined from mean values of ELISA absorbance from three independent experiments after 2 or 3 days exposure to drugs. Mean values of SRB absorbance for each drug concentration from three experiments were used to create a single dose–response curve with the IC_{50} concentrations being calculated from these curves.

Table 3 Selective toxicity characterized by the ratio of concentrations inducing apoptosis or inhibiting proliferation in normal (N) to cancer (C) cells

	Apoptosis ELISA (N:C ratio)		SRB assay (N:C ratio)	
	A	B	A	B
Anticancer drugs				
adriamycin	50.0	38.0	2.5	2.5
cisplatin	200.0	200.0	5.4	4.3
taxol	33.0	33.0	5.0	5.0
thioguanine	>100.0	>100.0	10.0	10.0
vincristine	>100.0	40.0	6.7	6.7
mitomycin C	200.0	125.0	2.9	2.9
etoposide	>80.0	>80.0	1.0	0.5
staurosporine	100.0	100.0	0.7	0.3
Toxins				
thioridazine	1.3	1.25	1.3	0.7
chlorpromazine	2.0	2.0	3.0	1.5
amitryptiline	1.3	1.3	1.6	0.7
quanidine sulfate	3.0	1.25	2.0	0.7

A=Ratio of concentrations affecting normal and cancer cells after a similar duration of exposure (2 days).
B=Ratio of concentrations affecting normal and cancer cells after exposure for a similar cell doubling time: 3 days for normal cells and 2 days for cancer cells.
For drugs not inducing apoptosis in normal cells the N:C ratios were calculated using maximal concentrations tested ($2 \times IC_{50}$).

toxicity to cancer cells than growth inhibition assays. The criteria for the assay application in drug screening are its ability to discriminate between anticancer drugs and toxic compounds and to detect selective effects on cancer cells in comparison with normal cells. To characterize the selectivity, we calculated the ratio between concentrations affecting normal and cancer cells from the data presented in Tables 1 and 2. The comparison was made for the concentrations affecting normal and cancer cells after treatment for a similar duration (A) or after exposure for the same population doubling time (B) (Table 3).

The normal: cancer cells (N:C) ratios of growth inhibiting concentrations IC_{50} determined by the SRB assay for eight anticancer drugs were in the range 0.3–10.0, with four out of eight anticancer drugs having a ratio below 3. The N:C ratios of SRB assay IC_{50} for four toxic compounds were 3.0 or below (range 0.7–3.0). Thus, there was an overlap in selectivity indices (N:C ratio) for anticancer drugs and toxins, indicating that the SRB assay could not detect selective toxicity for cancer cells.

In contrast to the SRB assay, Apoptosis ELISA clearly detected a selective effect of anticancer drugs on breast cancer cells in comparison with diploid fibroblasts. The N:C ratios for apoptosis inducing concentrations were in the range 33–200 for anticancer drugs. For drugs not inducing apoptosis in fibroblasts (Table 2) the ratios were calculated using the highest concentration tested, which did not increase the Apoptosis ELISA absorbance. For four toxins inducing apoptosis in normal and cancer cells, the N:C ratio was 1.25–3.0 after treatment for similar duration and 0.7–1.5 after treatment for similar population doubling time, which is at least 11- to 22-fold lower than the lowest index of selectivity for anticancer drugs.

Normal fibroblasts were less sensitive to anticancer drugs than cancer cells, as demonstrated by the higher values of IC_{50} measured by the SRB assay (Tables 1 and 2). However, the higher sensitivity of cancer cells to apoptosis induction by anticancer drugs in comparison with normal fibroblasts was not a simple reflection of higher sensitivity to drugs. Indeed, for all anticancer drugs the N:C ratios for apoptosis-inducing concentrations were significantly higher than the N:C ratios for growth-inhibiting concentrations (Table 3). In contrast, there was no such difference between N:C ratios for toxins.

Discussion

In the present study we have shown that anticancer drugs induce apoptosis in cancer cells at much lower micromolar concentrations than toxic compounds without antitumor activity. We also observed that N:C cell ratios (selectivity index) of apoptosis-inducing concentrations were significantly higher for anticancer drugs than for toxic compounds. Thus, according to our data the following parameters might indicate possible anticancer leads during drug screening: induction of apoptosis in cancer cells at a concentration below 1 μM or at least a 30-fold lower apoptosis-inducing concentration in cancer than in normal cells.

Anticancer drugs used in the study included drugs with various mechanisms of action: alkylating agents, topoisomerase II inhibitors, antimetabolites, microtubule inhibitors and the protein kinase inhibitor staurosporine. Although staurosporine is not used clinically, its derivative 7-hydroxystaurosporine demonstrates clinical antitumor activity [20]. The selectivity *in vitro* was demonstrated here for genotoxic drugs or microtubule inhibitors, and it remains to be seen if this type of screening will detect selectivity of compounds with other mechanism of actions, such as signal transduction inhibitors. Only apoptosis-based screening of chemical libraries and drugs discovered by target-based screening will address this concern.

The ability of Apoptosis ELISA to detect selective toxicity to cancer cells, and to discriminate between anticancer drugs and toxic compounds, indicates it potential usefulness in drug screening. In contrast to the apoptosis assay, measurement of growth inhibition by the SRB and MTT assays did not detect selective toxicity of the anticancer drugs and did not distinguish between two types of compounds. The important role of selectivity assessment in the drug discovery process was demonstrated by Vassilev *et al.* [3]. Selective toxicity was detected using a three-step screening procedure with evaluation of growth inhibition after 7-day exposure to drugs in a panel of normal and cancer cells. A selective effect on cancer cells was observed after treatment with IC_{90}, but not with IC_{50} concentrations, probably because IC_{90} better reflected the ability of drugs to induce apoptosis [3]. Indeed, in our study selective toxicity was detected by direct measurement of apoptosis using a one-step procedure after a short-term exposure to drugs.

For the detection of anticancer leads during screening of chemical libraries it is important that a rapid measurement of apoptosis-inducing concentrations in cancer cells clearly distinguished between anticancer drugs and toxic compounds without known antitumor activity. The potential usefulness of apoptosis-based screening was underscored here by the inability of routinely used growth inhibition assays to detect selective toxicity to cancer cells.

The detection of selective toxicity by the apoptosis assay probably reflects higher sensitivity to apoptosis in tumor cell lines in comparison to non-transformed or normal cells [6–10]. Importantly, high sensitivity of malignant cells to apoptosis also occurs *in vivo*, as apoptotic indices are higher in tumor than in normal tissue and increase with tumor progression [21,22]. The high sensitivity of tumor cells to apoptosis probably reflects loss of cell cycle checkpoint controls, which limits the ability to repair drug-induced damage and leads to rapid cell death by apoptosis [3,7,23]. Importantly, the toxins tested here were much less effective inducers of apoptosis than anticancer drugs.

The application of apoptosis induction estimates in routine drug screening requires the use of sensitive and specific apoptosis assays, which could be performed in microtiter plates. Probably, two apoptosis assays could be useful for drug screening. The anti-ssDNA mAb assay described in this study is sensitive and specific, but it is not a homogeneous one-step (mix and measure) assay and probably could be most useful in a secondary screening to detect selective toxicity. Caspase assays are specific and homogeneous, although their sensitivity is limited by the fact that caspase activation does not demonstrate irreversible apoptosis and drugs, which induce apoptosis by caspase-independent pathways, will not be detected by these assays [24,25].

In conclusion, we observed that concentrations inducing apoptosis in cancer cells were much lower for clinically useful anticancer drugs than for toxic compounds without antitumor activity. The N:C cell ratio of apoptosis-inducing concentrations was significantly higher for anticancer drugs than for toxic compounds. These data demonstrate that the apoptosis assay could be used to detect selective toxicity to cancer cells during drug screening.

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