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Sensitive Markers Used to Identify Compounds That Trigger Apoptosis in Cultured Hepatocytes

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Apoptosis may be a major event in chemical-induced injury, and therefore the detection of apoptotic effects when developing new drugs is highly relevant in screening for pharmacotoxicological risk assessment. However, as apoptosis *in vitro* normally degenerates to secondary necrosis, it is possible that it is underestimated, unless sensitive and specific parameters are used. In this present study we have evaluated the usefulness of a set of markers associated with the pivotal steps in the execution phase of apoptosis, in order to detect apoptotic compounds in hepatocytes before significant necrosis takes place. The markers selected include several biochemical parameters (downregulation of the antiapoptotic $bclX_L$ gene, caspase-3 activation, and cytochrome C release from mitochondria), and flow cytometry determinations (analysis of the size of the nuclei, chromatin complexity, and DNA integrity). The effects of several well-known model apoptotic toxicants (galactosamine, tertiary-butyl-hydroperoxide, etoposide, campothecine, and curcumin) were analyzed in hepatocytes. The aim was to identify early markers of apoptosis using known inducers of apoptosis in hepatocytes, as this battery of markers is designed to identify compounds triggering apoptosis in hepatocytes prior to necrosis. Concentrations of the compounds, as low as possible in order to keep 90% of hepatocyte viablility, were selected according to their intracellular lactate dehydrogenase (LDH) leakage, which is well known as an indicator of cell membrane integrity and cell viability. The results demonstrated that (1) the apoptotic effect of 4 out of 5 compounds could be detected in low concentrations of the drugs long before cell necrosis (tertiary-butyl-hydroperoxide-induced apoptosis was only detected at concentrations causing concomitant necrosis) and (2) among the markers evaluated, caspase 3 activation and nucleus and DNA analysis by flow cytometry were used to fulfil the compromise between reliability, sensitivity, and ease of performance, which are critical issues when screening for an apoptotic effect of newly developed drugs.

Key Words: **apoptosis sensitive markers; $bclX_L$; caspase 3; DNA fragmentation; cytochrome C; flow cytometry; DNA analysis; hepatocytes; toxicity.**

For many years it was assumed that chemically induced injury and death occurred primarily by necrosis. Now, however, it is recognized that cell death may also be the result of another mechanism, namely apoptosis, which could be induced by the absence of survival signals or activation of death receptors by different lethal signals (Raffray and Cohen, 1997). A better understanding of the fundamental mechanisms involved in apoptosis has made it possible to define its real significance in many areas of cell biology and more recently in pharmacology and toxicology. In fact, it is now believed that apoptosis could be the major form of chemically induced cell death and that necrosis is much rarer, occurring only in circumstances of gross cell injury (Alison and Sarraf, 1995; Gill and Dive, 2000; Raffray and Cohen, 1997). However, the importance of apoptosis in toxicology has been underestimated because of the difficulty in identifying apoptotic cells in the intact organism, since they undergo striking morphological changes that make them swiftly unrecognizable and are rapidly engulfed by phagocytes (Gill and Dive, 2000; Savill *et al.*, 1993; Raffray and Cohen, 1997). During *in vitro* experimentation, in the absence of phagocytes, a secondary nonspecific degeneration occurs that results in the uptake of vital dyes such as trypan blue that is commonly mistaken for necrosis and is often referred to as secondary necrosis. Therefore, apoptotic cells may be underestimated, particularly *in vitro*, unless specific and sensitive parameters are used. The biochemical stages of apoptosis induced by a toxicant can be described as the imposition of damage by the toxicant, sensing and therefore coupling the damage to the engagement of apoptosis, next follows the execution of the cell that then ends with the disposal of the corpse (Gill and Dive, 2000). In this context, apoptosis provides a mechanism for the disposal of cells damaged by toxicants without perturbing the homeostatic balance of its environment.

There are few reports on chemical-induced apoptosis in parenchymal cells of the liver and kidney, which are the main target organs of cytotoxic drugs. The liver is the most active organ in metabolizing foreign compounds, and although biotransformation catalyzed by cytochrome P450 enzymes generally parallels a detoxification process, this reaction can generate metabolites that are not only more reactive but also more toxic than the parent compound. Therefore the hepatic metab-

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olism of drugs is very frequently the cause of adverse drug reactions in the liver and the reason why the liver is a key target organ for drug toxicity (Bort *et al.,* 1999). Since it is now recognized that apoptosis in the liver plays a central role in the toxicity of many xenobiotics and cytochrome P450-generated metabolites, the detection of the apoptotic potential is of major interest when developing new pharmaceuticals (Feldmann, 1997; Haouzi *et al.,* 2000). The objective of this work is to investigate whether it would be possible to identify compounds that induce the basic mechanisms of apoptosis in hepatocytes, when very mild or nonnecrotic effects are observed, as well as to determine the appropriate apoptotic markers able to predict the apoptotic potential of compounds.

A battery of apoptotic markers for the screening of newly developed drugs would require reliability, sensitivity, simplicity, the accuracy while using a small number of cells, low time consumption, automatization, and reproducibility. Furthermore, one should postpone and store the samples until analysis. With this aim in mind, we have examined the usefulness of the following parameters: analysis of nuclei and DNA by flow cytometry, the expression of the antiapoptotic $bclX_L$ gene, caspase-3 activation, and cytochrome C release from the mitochondria, while also detecting apoptosis of several well-known apoptotic inducers in primary cultured hepatocytes: galactosamine (Itokazu *et al.,* 1999; Tsutsui *et al.,* 1997), campothecine and etoposide, topoisomerase-I and -II inhibtors, respectively (Ferraro *et al.,* 2000; Jarvis *et al.,* 1999), TBOOH (Kanno *et al.,* 2000; Karbowski *et al.,* 1999; Lemasters *et al.,* 1999), and curcumin (Bhaumik *et al.,* 1999; Chen *et al.,* 1999; Ozaki *et al.,* 2000).

MATERIAL AND METHODS

Culture of hepatocytes. Hepatocytes were isolated from Sprague-Dawley male rats (180–250 g) by reverse perfusion of the liver with collagenase, as previously described (Donato *et al.,* 2001). Hepatocytes were seeded on fibronectin-coated plastic dishes (3.5 $\mu g/cm^2$) at a density of 8×10^4 viable cells/cm^2 and cultured in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% newborn calf serum, 50 mU/ml penicillin, 50 μg/ml streptomycin, 0.2% bovine serum albumin, and 10 nM insulin. One h later, the medium was changed, and after 24 h, cells were shifted to a serum free, hormone supplemented medium (10 nM insulin and 10 nM dexamethasone). Thereafter the medium was changed daily.

Preparation of stock solutions of the compounds. Stock solutions of 25 mM etoposide and 3 mM campothecine were prepared in dimethylsulfoxide, 5 mM curcumin in ethanol, and 80 mM tertiary-butyl hydroperoxide (TBOOH) and 1 M galactosamine in culture medium and diluted with culture medium to obtain the appropriate concentrations. The final concentration of solvents in culture medium was 0.5%, v/v, and the control cells were treated with the same amount of solvent.

Cytotoxicity assay. Hepatocytes were seeded on fibronectin-coated 96-well microtitre plates at a density of 25×10^3 viable cells/well. After treating the compounds, cytotoxicity was assessed by measuring the intracellular lactate dehydrogenase (LDH) leakage, which is widely recognized as a cytotoxic end-point indicator for cell membrane disruption and viability.

The concentrations causing 10 and 50% of LDH leakage (IC_{10} and IC50 respectively) were directly compared to the untreated control cells, then they were mathematically calculated from the concentration-effect curves (Ponsoda *et al.,* 1991).

Flow cytometric analysis. Cell monolayers were kept frozen at –20°C until the time of analysis. Then monolayers were thawed and covered with hypotonic lysis solution (0.1% Na_3 citrate and 0.1% Triton X-100 in distilled water) and kept overnight at 4°C in order to release nuclei. Propidium iodide (PI) (50 μg/mL, final concentration) was added to the nuclei suspension for fluorescent staining of DNA. Nuclei suspensions were incubated with both fluorochromes for 30 min at room temperature in the dark, prior to the analysis in the flow cytometer.

All of the analyses were performed with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Brea, CA) equipped with an air-cooled, argon-ion laser emitting at 488 nm and 15 mW. For each run, 20,000 individual events were collected and stored in list-mode files for off-line mathematical analysis and graphic display using the EPICS XL System II v 3.0 software (Beckman-Coulter, Brea, CA; Fig. 1).

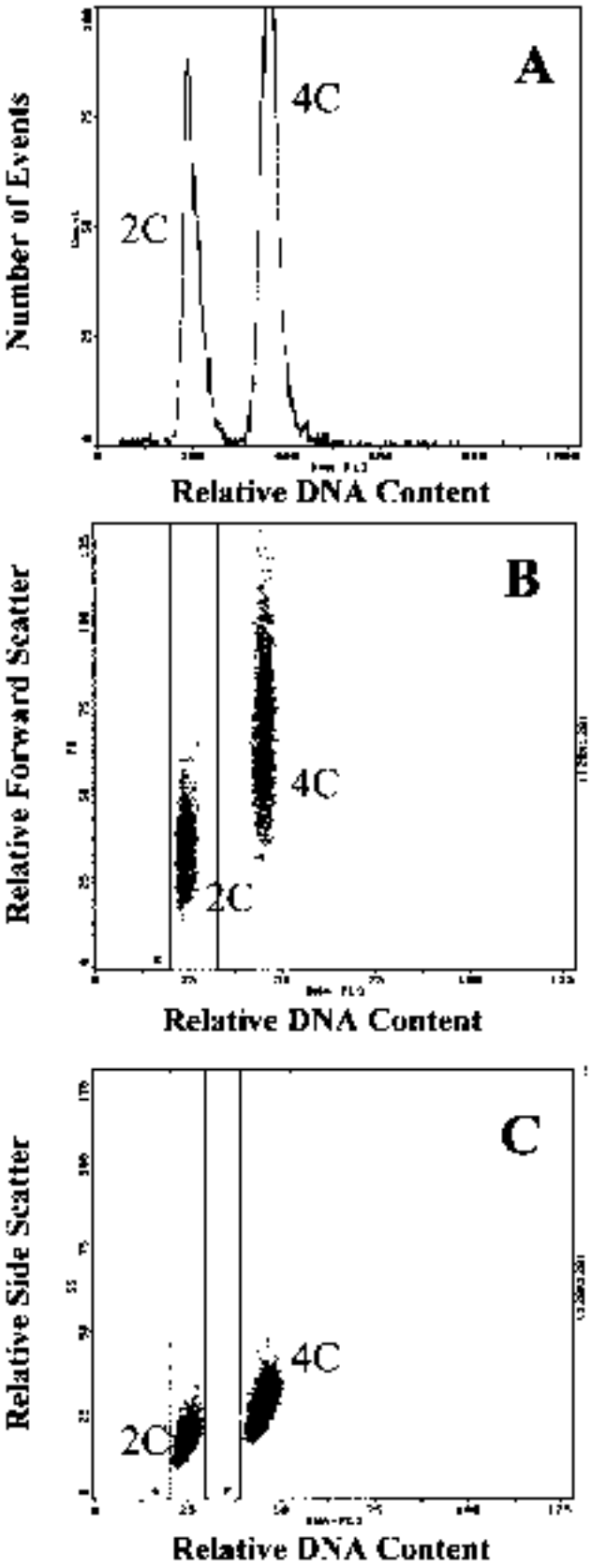
Relative size of nuclei and complexity were estimated from laser light scatter measurements. Laser light scattered in the forward direction (FS) is mostly related to nucleus size (Papa *et al.,* 1987), while laser light scattered sideways and collected at 90° from the laser beam (SS) is indicative of nuclear texture (granularity/complexity; Darzynkiewicz, 1990; Papa *et al.,* 1987). DNA content in individual nuclei (i.e., nuclear ploidy) was estimated from the intensity of PI orange fluorescence (FL3, collected through a 610 DL dichroic filter plus a 575 BP band-pass filter). Aggregates of 2 and more nuclei were hardware-discriminated based upon the ratio of FL3 pulse versus integral signals.

The degree of apoptosis was estimated from the percentage of nuclei with a DNA content lower than the diploid (2C) peak in a single-parameter histogram of PI fluorescence distribution. In order to exclude debris from apoptotic nuclei, PI fluorescence measurements were gated on FS versus PI, and only particles with a size similar to diploid nuclei were considered.

The homogeneity of PI staining of isolated nuclei was studied as an estimation of the appearance of changes in chromatin condensation (Mazzini *et al.,* 1983) or DNA strand integrity (Maier and Schawalder, 1986), compatible with those typical of the early steps/stages of apoptosis. For this purpose, the full-peak coefficient of variation (% FPCV: [standard deviation/mean fluorescence intensity] $\times$ 100) of the DNA distribution was calculated by the cytometer software after defining regions of statistical analysis by setting a linear cursor including the diploid (2C) or tetraploid (4C) Gaussian distributions in histograms of PI-fluorescence distribution (Maier and Schawalder, 1986).

Caspase 3 activity. After incubation of hepatocytes in the absence or presence of the compounds for the time indicated, activation of caspase 3 was measured using the fluorogenic substrate Ac-DEVD-AMC as described elsewhere (Donato *et al.,* 2001) with some modifications. In brief, culture medium was centrifuged and floating cells pelleted. These detached cells, along with the cells in the monolayer from the same plate, were lysed in a buffer (10 mM Tris-ClH, 10 mM NaH_2PO_4/$NaHPO_4$, pH 7.5, 130 mM NaCl, 1% Triton X-100, and 10 mM NaPPi) for 1 h on ice. Reaction mixture containing 20 μM Ac-DEVD-AMC and cell lysate in buffer assay (20 mM HEPES pH 7.5, 10% glycerol, and 2 mM DTT) was incubated for 1 h at 37°. The AMC liberated from Ac-DEVD-AMC was measured in a fluorimeter with an ex wavelength at 380 nm and an em at 460 nm.

Cytochrome C release measurement. Hepatocytes were incubated in the absence or presence of the compounds for the time indicated and processed as described (Piqué *et al.,* 2000). Cells were scraped in ice-cold PBS, obtained by centrifugation at 300 $\times$ *g* in the cold and gently lysed for 30 s in 75 μl of an ice-cold buffer containing 250 mmol/l sucrose, 1 mmol/l EDTA, 0.05% digitonin, and 25 mmol/l Tris, pH 6.8, 1mM DTT, and protease inhibitors (1 ug/ml leupeptin and aprotinin, and 0.1 mmol/l PMSF). Lysates were centrifuged for 2 min at 12,000 $\times$ *g* at 4°C and the cytosolic supernatants (50 μg protein each) were subjected to a 15% SDS–PAGE. A 60 ng sample of cytochrome C (from horse heart, C-7752, purchased from Sigma, Madrid, Spain) was included to act as a positive control for size and identity. The gel was transferred to a



PVDF membrane (Millipore), and the blot was probed with a mouse anticytochrome C monoclonal antibody (clone 7H8.2C12; purchased from Pharmingen, San Diego, CA) and developed by using a horseradish peroxidase-coupled antimouse secondary antibody by enhanced chemiluminescence (Amersham).

bclX$_L$ analysis. After incubation of hepatocytes in the absence or presence of the compounds for the time indicated, total RNA was isolated and contaminating genomic DNA was removed by incubation with DNase I Amplification Grade (GIBCO BRL, Paisley, UK). For quantitative RT-PCR analysis, 1 μg of RNA was reverse transcribed as described (Rodríguez-Antona *et al.*, 2000), and diluted cDNA (2 μl) was amplified with a rapid thermal cycler (LightCycler Instrument, Roche Diagnostics) in 15 μl of 1×LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals), 5 mM $MgCl_2$ and 0.3 μM of each primer (see Table 1). After denaturing for 30 s at 95°C, amplification was performed by 40 cycles of 1 s at 94°C, 5 s at 58°C, and 15 s at 72°C. The real-time monitoring of the PCR reaction and precise quantification of the products in the exponential phase of the amplification were done using the SYBR Green I format and a subsequent melting curve analysis was carried out as recommended by the manufacturer.

Statistical analysis. Experiments were performed in at least 3 independent cultures. Statistical analysis was done according to the Student's *t*-test.

RESULTS

Intracellular LDH Leakage as an Indicator of Cytotoxicity of the Compounds

Hepatocytes were incubated with increasing concentrations of the compounds for 4, 8, 14 and 24 h. Intracellular LDH release, as a result of the breakdown of the plasma membrane and the alteration of its permeability, was evaluated as it is a widely recognized cytotoxicity endpoint for the measurement of cell membrane integrity and viability. It allows evaluation of cell death in cultures, as a result of both cell necrosis and secondary necrosis, at the late stage of apoptosis. To avoid the overlapping of necrosis and apoptosis, we used subcytotoxic concentrations of the compounds, using only those which were able to keep hepatocytes viability around 90% (IC_{10}).

All of the compounds evaluated produced a concentration- and time-dependent increase in LDH leakage when added to cultured rat hepatocytes. Accordingly, IC_{10} and IC_{50} values for LDH leakage decreased during the time of treatment (Table 2).

Flow Cytometric Analysis of Nuclear Parameter Markers of Apoptosis

Flow cytometric analysis was performed in cultured hepatocytes treated with the compounds at concentrations that

FIG. 1. Representative results of the flow cytometric simultaneous analysis of biochemical and structural parameters in suspensions of isolated nuclei from rat cultured hepatocytes. (A) DNA content distribution estimated from the fluorescence intensity of propidium iodide. Nuclei are diploid (2C) and tetraploid (4C). (B) Size of diploid and tetraploid nuclei estimated from forward scatter measurements. (C) Complexity of diploid and tetraploid nuclei estimated from side scatter measurements. The scale of corresponding axes in each graph indicates arbitrary units of light scatter or fluorescence intensity. Rectangular boxes in biparametric histograms are the analysis regions set up for the calculation of numerical data.

TABLE 1
PCR Oligonucletide Primers

Gene	Accession	5′nt Forward primer	5′nt Reverse primer
$bclX_L$	X82537	155 GAG ACC CCC AGT GCC ATC AAT	464 CCC CGC CAA AGG AGA AGA AG
β-Actin	V01217	1670 GCC AAC CGT GAA AAG ATG AC	2592 GAA GGA AGG CTG GAA GAG AG

would not cause significant LDH leakage. Figure 2 shows the effect of the different treatments on the appearance of apoptotic nuclei with subdiploid DNA content. As shown in Figure 2A, following 4 h of incubation none of the treatments induced any significant increase in apoptotic nuclei above the small percentage observed in control cultures. However, at 8 h most of the drugs tested induced apoptosis that was judged according to the increased percentage of subdiploid nuclei. Only TBOOH at the subcytotoxic doses tested did not cause any significant enhancement of apoptotic events as compared with the corresponding control cultures. At 8 h incubation the effects of galactosamine, etoposide, campothecine, and curcumin were significant, as shown in Figure 2B, while at 24 h, the percentage of apoptotic nuclei was much higher in all cases as compared with both the corresponding 24-h control cultures and the 8-h treated cells (Fig. 2A).

Loss of nuclear DNA integrity during apoptosis is preceded and accompanied by extensive modifications of chromatin conformation (Darzynkiewicz *et al.*, 1997) that in turn alter nuclear morphology. For this reason, we studied the effect of the different treatments on nuclear morphological parameters at 8 h (especially size and complexity of nuclei, i.e., the first time point at which apoptosis consistently appears) by flow cytometry (Fig. 1). In order to obtain homogeneous data, these measurements were restricted to the population of diploid (2C) nuclei, by means of analyzing the regions defined on biparametric dotplots of DNA content versus forward scatter or DNA content versus side scatter. As seen in Figure 3A, camptothecin, curcumin, TBOOH and, especially, etoposide increased nuclear size in the 2C population, while galactosamine did this only when tested at the highest dose. In the case of curcumin, the increase is dose-dependent. Regarding the alterations in nuclear complexity, all treatments, except for galactosamine and TBOOH, increased the intensity of side scatter in a dose-dependent fashion, which indicates an increase in the granular properties of the nuclei (Fig. 3B).

The changes in nuclear morphology described above are evident in the population of nuclei belonging to cells that do not exhibit the classic signs of overt apoptosis, such as the loss of DNA which follows DNA strand breaks. In order to ascertain whether the homogeneity of PI staining of nuclei with a normal DNA content could be an early indicator of commitment to apoptosis, the coefficient of variation of the PI fluorescence distribution was calculated at the base of both the 2C and 4C gaussian peaks (full-peak coefficient of variation [FPCV]). As can be seen (Table 3), treatment with the different apoptogenic drugs caused a slight but consistent increase in FPCV of PI distribution in both ploidy groups.

Caspase 3 Activation by Xenobiotics

Time-course analysis of caspase 3 activation was examined in cultures treated with a concentration of the compounds close to the maximal nontoxic concentration (IC_{10}, Table 2). The kinetics of caspase 3 activation were measured during the first 14 h of treatment. A time-dependent increase in caspase 3 activity was observed for up to 8 h, thereafter the activity significantly decreased to the basal levels found in untreated control cells (Fig. 4). Cultured hepatocytes were then exposed to increasing subcytotoxic concentrations of the compounds over an 8-h period, and caspase 3 activity was measured at the end of the treatment. Campothecine, etoposide, and curcumin showed a significant concentration-dependent activation of caspase 3 (Figs. 5A–5C), while a moderate effect was pro-

TABLE 2
Cytotoxicity of Compounds Assessed by Intracellular LDH Leakage

	IC_{10}				IC_{50}			
Compound	4 h	8 h	14 h	24 h	4 h	8 h	14 h	24 h
Camptothecin	11 μM	9.6 μM	5.3 μM	3.4 μM	35 μM	29 μM	17 μM	13 μM
Galactosamine	42 mM	33 mM	24 mM	19 mM	130 mM	103 mM	59 mM	61 mM
Etoposide	467 μM	457 μM	262 μM	176 μM	1100 μM	905 μM	543 μM	326 μM
Curcumin	63 μM	52 μM	26 μM	14 μM	156 μM	135 μM	47 μM	28 μM
TBOOH	120 μM	80 μM	60 μM	30 μM	280 μM	230 μM	130 μM	100 μM

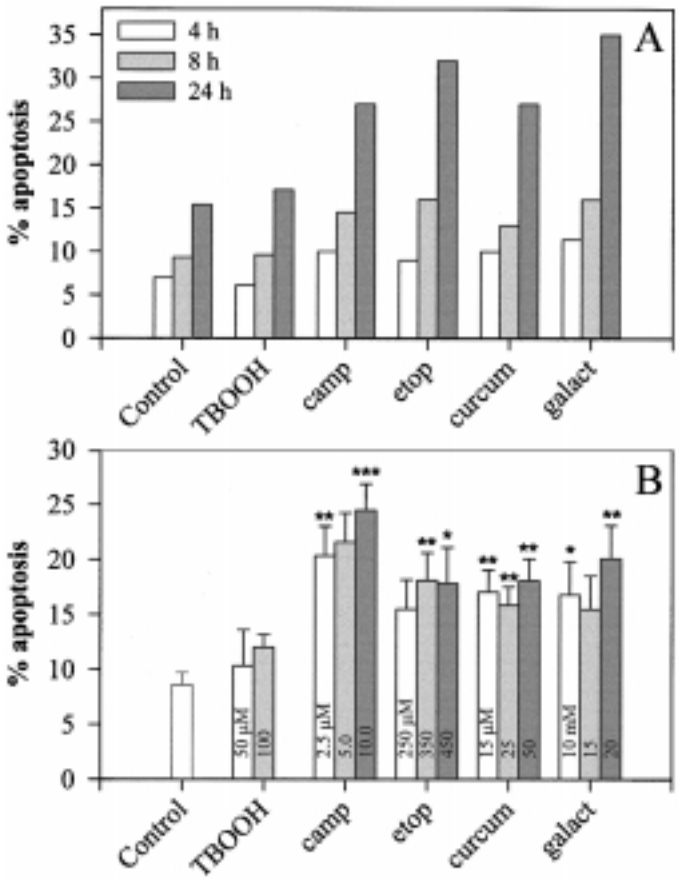


FIG. 2. Effect of the xenobiotics on the appearance of apoptotic nuclei with subdiploid DNA content evaluated by flow cytometry. The degree of apoptosis was estimated from the percentage of nuclei with DNA content lower than the 2C peak as a result of the loss of nuclear DNA integrity in a single-parameter histogram of PI fluorescence distribution. (A) Evaluation of the time-dependent apoptotic effect on hepatocytes. Cultures were exposed to a concentration of the compounds close to the maximal nontoxic concentration (10 μM campothecine, 450 μM etoposide, 20 mM galactosamine, 50 μM curcumin, and 100 μM TBOOH). Data corresponding to a representative experiment are shown. (B) Dose-dependent effect evaluated after an 8-h incubation with increasing concentrations of the drugs. Data represent the mean ± SD of 3 different experiments, (*$p < 0.01$; **$p < 0.005$; ***$p < 0.001$).

duced by galactosamine (2.6 ± 0.3 fold increase versus control; Fig. 5D) and no effect was produced by TBOOH (Fig. 5E).

Cytochrome C Release Induced by Xenobiotics

In the mitochondrial pathway of the apoptosis cytochrome C is released from the intermembrane space of the mitochondria to the cytoplasm. Therefore, cytochrome C was analyzed in the cytoplasmic fractions of hepatcytes, which had been treated with the compounds for 14 h, at concentrations without causing any observable LDH leakage.

Curcumin, in particular, was able to induce cytochrome C release, as was demonstrated by the bands of protein identified by western-blotting analysis after processing the cytoplasmic fractions by SDS-PAGE (Fig. 6).

$bclX_L$ Downregulation by Xenobiotics

mRNA levels of $bclX_L$ were analyzed in cultured hepatocytes treated with different compounds at concentrations without causing any observable LDH leakage. Hepatocytes were exposed for an 8-h period and $bclX_L$ mRNA was measured at the end of the treatment by real-time quantitative RT-PCR (Fig. 7).

Campothecine caused a significant decrease (ca. 50%) in $bclX_L$ mRNA in all of the doses that were assayed (2.5, 5, and 10 μM). D-galactosamine was also very effective (decrease of 60%) when assayed at the highest concentration (30 mM). Etoposide and curcumin caused a more moderate effect, and no changes in $bclX_L$ mRNA levels were observed by TBOOH treatment.

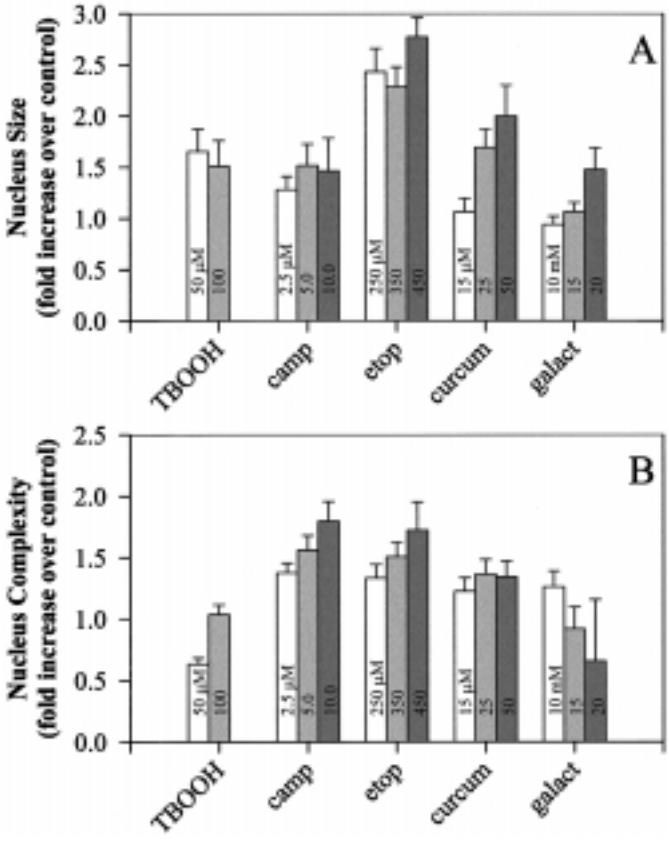


FIG. 3. Effect of xenobiotics on nuclear morphological parameters by flow cytometry: nucleus size and complexity. Loss of DNA integrity and modifications of chromatin conformation during apoptosis are accompanied by alterations in nuclear morphology. Dose-dependent changes in nucleus size (A) and complexity (B) were analyzed in the population of diploid (2C) nuclei, by means of analysis regions defined on biparametric dotplots of DNA content versus forward scatter or DNA content versus side scatter. Data are expressed as the mean ± SD of 3 different experiments.

TABLE 3
Analysis of the the Full Peak Coefficient of Variation of the PI Fluorescence Distribution (FPCV)

	FPCV (%)	
Dose group	2C Population	4C Population
Control	7.50	5.09
TBOOH		
50 μM	7.49	5.48
100 μM	8.70	5.89
200 μM	8.66	6.55
Campothecine		
2.5 μM	8.66	6.06
5 μM	9.33	6.04
10 μM	9.31	6.40
Etoposide		
250 μM	8.54	5.56
350 μM	8.99	5.81
450 μM	9.27	6.27
Curcumine		
15 μM	8.51	5.58
25 μM	8.75	6.19
50 μM	9.36	7.86
Galactosamine		
10 mM	9.00	5.80
15 mM	8.36	5.80
20 mM	8.75	5.90

Note. The FPCV was calculated at the base of both 2C and 4C gaussian peaks in order to assess the homogeneity of PI staining of nuclei with a normal DNA content. Data correspond to a representative experiment.

DISCUSSION

It is generally believed that mild forms of injury induce apoptosis, while more severe forms of insult result in necrosis. Moreover, in the liver, as in other tissues, many compounds can even cause apoptosis and necrosis simultaneously, and often the intensity of the initial insult decides the prevalence of either apoptosis or necrosis (Alison and Sarraf, 1995; Feldmann, 1997). Therefore, it is of great pharmacotoxicological relevance to assess whether a new drug under development causes significant apoptosis to hepatocytes.

The 2 most well studied pathways of apotosis include the surface death receptor pathway (i.e., Fas) that recruits the effector caspase 8 and mediates transduction of the death signal in cells (Maeda, 2000) and the mitochondria-initiated pathway. Mitochondria are deeply involved in the regulation of cell death, undergoing membrane permeabilization, which commits hepatocytes to apoptosis (Kroemer *et al.*, 1997; Pessayre *et al.*, 1999; Zamzami *et al.*, 1998). Furthermore, several mitochondrial proteins such as cytochrome C and certain procaspases are released into cytoplasm (Kroemer *et al.*, 1997). This process precedes nuclear apoptosis and is inhibited by the presence of the antiapoptotic proteins of the bcl-2 family (Yang *et al.*, 1997; Zamzami *et al.*, 1998). The only member of the bcl-2 family present in hepatocytes is $bclX_L$ (Feldmann, 1997; Zamzami *et al.*, 1998), which may bind directly to cytochrome C in the cytosol to quench its negative effects. Proapoptotic members of bcl-2 family (bax, bak, and bid) translocate to the mitochondrial membrane inducing cytochrome C release, which is not always coincident with loss of mitochondrial membrane potential (Piqué *et al.*, 2000). Cytochrome C forms a complex with cytoplasmic factors allowing activation of caspase-9, which in turn cleaves and activates other caspases, such as caspase-3. This constitutes the execution phase of apoptosis, and it appears to be the point of no return of apoptosis (Yang *et al.*, 1997). In this context, the decreased expression of $bclX_L$ and the release of cytochrome C are more specific biomarkers for the mitochondrial pathway of apoptosis (Gill and Dive, 2000; Zamzami *et al.*, 1998). The caspases are the most important effector molecules that play a central role in the execution of apoptosis (Cain, 2000). Progression of the caspase activation cascade ends with the activation of caspase 3 that occurs in early apoptosis, long before DNA fragmentation appears. Once caspase 3 has been activated, there is no way back to normal viability; the program for cell death is irreversibly activated. Therefore, it is considered a very specific and sensitive apoptotic marker irrespective of how cell death is initiated (Cain, 2000).

Nevertheless, although 2 main pathways of apotosis have been described, the diverse molecules (chemicals and death receptor ligands) may differentially alter these apoptotic mechanisms. Several examples can be found in the literature. For example in human leukemia cells, induction of cytochrome C release in absence of caspase 3 activation by etoposide has been reported (Hirpara *et al.*, 2000) as having been a Fas-induced apoptosis by multiple pathways (Rouquet *et al.*, 1996). Apoptotic cell death through both caspase-dependent and

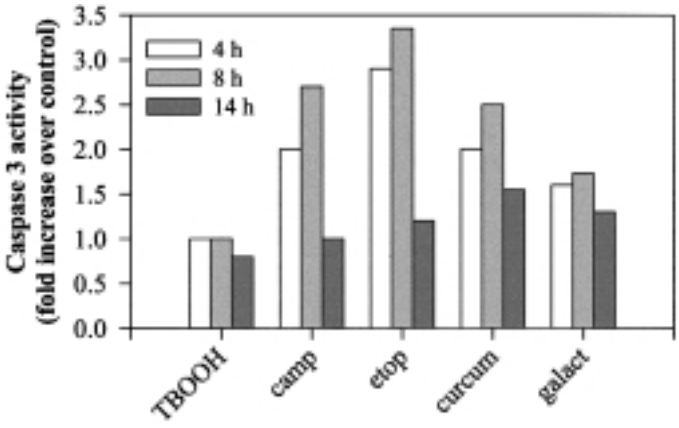


FIG. 4. Time-course analysis of caspase 3 activation. The kinetics of caspase 3 activation was examined in cultures treated with a concentration of the compounds close to the maximal nontoxic concentration for up to 14 h. Cultures were exposed to a concentration of the compounds close to the maximal nontoxic concentration (5 μM campothecine, 450 μM etoposide, 20 mM galactosamine, 50 μM curcumin, and 100 μM TBOOH). Data correspond to a representative experiment.

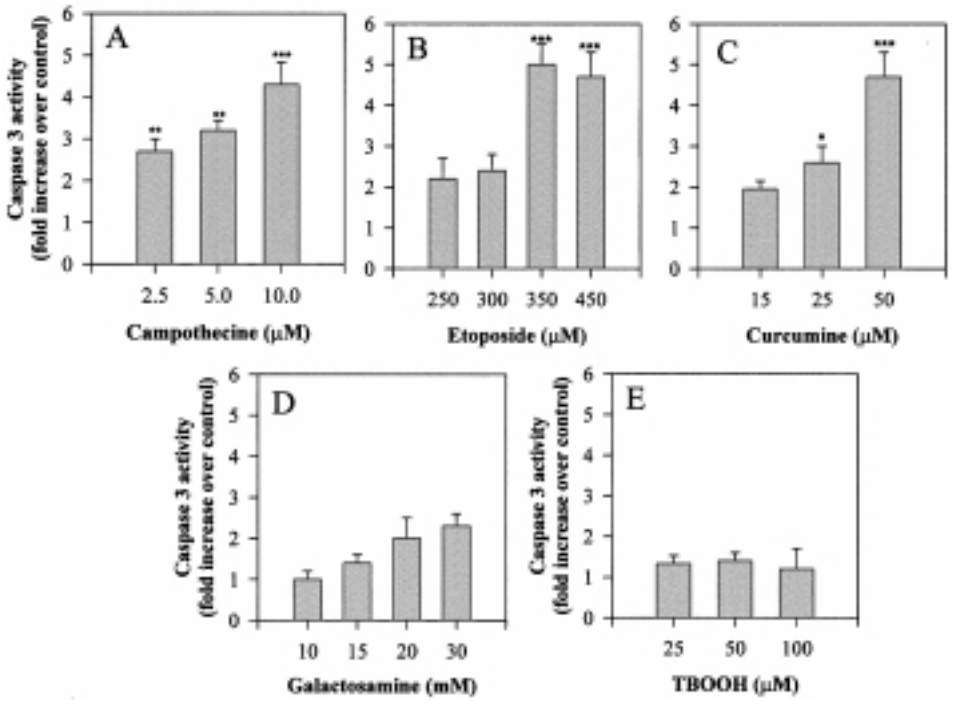


FIG. 5. Dose-dependent effect of compounds on caspase 3 activation. Cultured hepatocytes were exposed to increasing subcytotoxic concentrations of the compounds, for an 8-h period, and caspase 3 activity was measured at the end of the treatment. Effects of campothecine (A), etoposide (B), curcumine (C), galactosamine (D), and TBOOH (E). Data are expressed as fold increase the control values (17.60 ± 7.05 mU/min × mg cellular protein), and represent the mean ± SD of 3 different experiments, (*p <0.01; **p <0.005; ***p < 0.001).

caspase-independent pathways in primary culture of hepatocytes has also been described (Jones *et al.*, 2000). More recently aspirin has been shown to trigger cytochrome C release preceeding caspase activation and loss of mitochondrial membrane potential (Piqué *et al.*, 2000). Therefore, several apoptotic markers associated with the pivotal steps of the execution phase should be evaluated to accurately identify compounds able to induce apoptosis after mild injury. Our selection of apoptotic markers was aimed at identifying apoptotic compounds irrespective of the pathway of how cell death was initiated and also trying to find a compromise between specificity, sensitivity, and ease of detection. Some markers involve the use of intact cells (flow cytometric analysis of nuclei) and others the evaluation of biochemical parameters in subcellular fractions (cytochrome C release, bclX_L gene expression, and caspase 3 activation).

Several assays have been described involving the use of DNA intercaling dyes in order to estimate the number of apoptotic cells in culture (propidium iodide, acridine orange, Hoechscht 33258), but flow cytometry allows a much more accurate automatized analysis. Other assays can be applied to living cultured cells: (1) the use of annexin V allows the detection of phosphatidyl serine, which is restricted to the inner surface of the plasma membrane, but it is externalized during

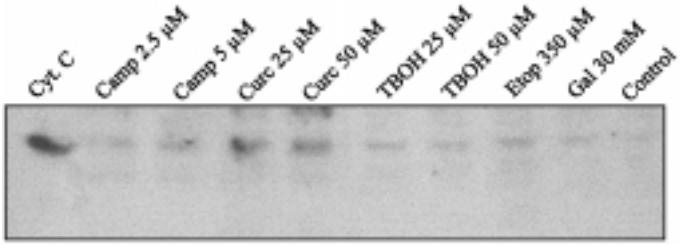


FIG. 6. Effect of compounds on cytochrome C (Cyt. C) release from the mitochondria to the cytoplasm. Cytochrome C was analyzed in hepatcytes after 14-h treatment with concentrations of the compounds not causing any observable LDH leakage, by western-blotting and SDS-PAGE of the cytoplasmic fractions. Mouse anticytochrome C monoclonal antibody ecognized a single band in cytosolic lysates of treated hepatocytes that comigrated with cytochrome C. Data correspond to a representative experiment.

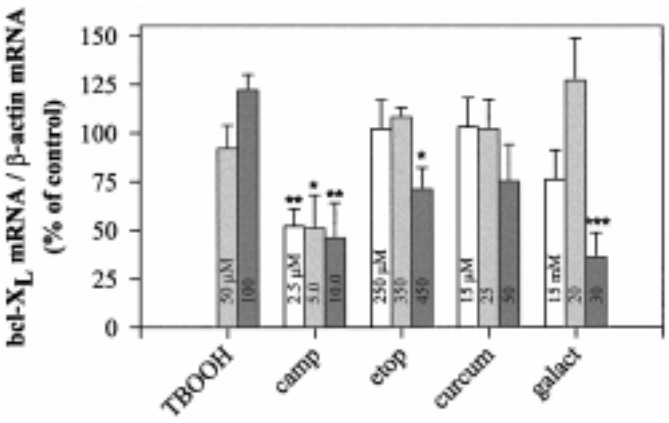


FIG. 7. Dose-dependent effect of compounds on bcl-X_L expression. mRNA levels of bcl-X_L were analyzed by real-time quantitative RT-PCR in cultured hepatcytes exposed for 8 h to concentrations of the compounds without causing any observable LDH leakage. Data are expressed as percentage of the control values (100 ± 14) and represent the mean ± SD of 4 different experiments, (*p < 0.05; **p < 0.01; ***p < 0.005).

apoptosis triggering phagocytosis *in vivo* (Ernst *et al.,* 1998; Fadok *et al.,* 1992); and (2) the poly (adenosine diphosphate-ribose) polymerase (PARP) is cleaved by the activated caspase-3 leading to apoptosis (Cain, 2000). Methods used to evaluate DNA fragmentation and localization of DNA strand breaks, as a result of the activation of endonucleaes, in tissue sections and in cultured cells have also been developed. Among them are the DNA laddering and the TUNEL assay, which are widely used methods (Cain, 2000). TUNEL assay is considered a standard assay for apoptosis, both in flow and image cytometry. This assay is used to detect the DNA strand breaks that are a hallmark of the late stages of apoptosis, leading to DNA fragmentation and loss of low-molecular, internucleosomal DNA fragments that may be revealed by DNA gel electrophoresis (the "DNA ladder" assay). However, these assays provide information that overlaps with other apoptotic markers used here. These assays need a huge number of cells and are time-consuming; therefore, they may not be fully fit for throughput screening purposes.

Apoptotic effects as a consequence of a mild injury were analyzed after incubating hepatocytes with concentrations of the model drugs without causing observable intracellular LDH leakage, therefore indicating the integrity of the outer cell membrane (Table 2).

High doses of D-galactosamine have been reported to induce apoptosis in hepatocytes both *in vitro* and *in vivo* before causing necrosis (Stachlewitz *et al.,* 1999; Tsutsui *et al.,* 1997). Increased DNA fragmentation, but almost no association with caspase 3 activation, has also been reported (Itokazu *et al.,* 1999). These previous findings are consistent with the results obtained in our study.

Curcumin, the active ingredient of the rhizome of Curcuma longa, has antiproliferative and antitumoral effects on several cell types both *in vivo* and *in vitro* (Bhaumik *et al.,* 1999; Chen *et al.,* 1999; Ozaki *et al.,* 2000), and it is a potent inhibitor of transcriptional factors involved in cell survival (Ozaki *et al.,* 2000). Previous reports have shown its ability to induce apoptosis in osteoclasts and AK-5 tumor cell line by producing reactive oxygen species, loss of mitochondrial membrane potential, and DNA fragmentation (Bhaumik *et al*., 1999; Ozaki *et al*., 2000). We also analyzed the apoptotic effect of topoisomerase-I and -II inhibitors, campothecine, and etoposide respectively, both of which are known to produce apoptosis in both primary cells and immortalized cell lines (Ferraro *et al.,* 2000; Jarvis *et al.,* 1999). Our results show that long before LDH leakage all the apoptotic markers evaluated became altered, which means that both curcumine and topoisomerase inhibitors are potent apoptotic inducers in hepatocytes.

Many toxins induce apoptosis by producing active oxidants (Buttke and Sandstrom, 1994; Kurose *et al.,* 1997). This is the case of hydrogen peroxides, such as TBOOH, which have been described as triggering apoptosis in cultured hepatocytes (Kanno *et al.,* 2000; Karbowski *et al.,* 1999; Rauen *et al.,* 1999). It has been reported that in hepatocytes oxidative stress induced by TBOOH, NAD(P)H oxidation has caused an increase in mitochondrial Ca^{2+}, and it has been shown that mitochondrial generation of reactive oxygen species precede and contribute to the mitochondria onset of the MTP. Progression to necrosis or apoptotis depends on the effect the MTP has on cellular ATP levels. If ATP levels fall sharply, necrotic killing ensues, but if the levels are partially maintained, apoptosis follows the MTP (Lemasters *et al.,* 1999). Exposure of hepatocytes to subcytotoxic concentrations of TBOOH produced a very moderate alteration of the apoptotic markers evaluated here, and only, when the necrotic process was observed together with cytotoxic concentrations, the apoptotic parameters were altered concomitantly (data not shown).

Among the methods using intact cells, flow cytometry is probably the most efficient (Vermes *et al.,* 2000). Loss of DNA integrity following endonuclease activation leads to a population of subdiploid cells or nuclei observable when stained with a DNA-specific fluorochrome (Darzynkiewicz *et al.,* 1997). In addition, we have evaluated the changes in nuclear morphology (size and complexity, Fig. 1) in the population of nuclei belonging to cells that do not exhibit the classic signs of overt apoptosis. Our results suggest that the coefficient of variation of the PI fluorescence distribution (FPCV) as an indication of the homogeneity of PI staining of nuclei with a normal DNA content can be used as an early indicator of commitment to apoptosis. Cytometric analysis showed that most of the drugs tested increased the percentage of subdiploid DNA content, which is a well established marker of the late phase of apoptosis. Moreover, the alterations observed in nuclear size and complexity in the population of nuclei belonging to cells that do not exhibit the classic signs of overt apoptosis, were consistent with the biochemical markers. Although there are no reports describing specifically the effect of cytotoxic drugs on nuclear size, early reports using flow cytometric analysis showed that phenobarbital, an inhibitor of apoptosis, decreased this size (Maier and Schawalder, 1986). Regarding the alterations in nuclear complexity, all the compounds except galactosamine increased the intensity of side scatter in a dose-dependent fashion, which indicates an increase in the granular properties of the nuclei. These changes are compatible with an increased degree of chromatin condensation, a consistent change in most models of apoptotic cell death, which is considered the earliest morphological alteration in apoptosis-committed cells (Sun *et al.,* 1994)

Treatment with the different apoptogenic drugs also caused a slight but consistent increase in FPCV of PI distribution in both the 2C and 4C ploidy classes. Previous flow cytometric analysis showed that DNA stainability is affected by chromatin conformation (Mazzini *et al.,* 1983). The alterations in chromatin conformation at this early stage of culture injury may affect the accessibility of fluorochromes to DNA, thus changing the fluorescence properties of stained nuclei. Heterogeneity of DNA staining in isolated nuclei might also arise from the presence of individual nuclei with decreased DNA content due

to DNA strand breaks and subsequent loss of low molecular weight of DNA fragments during the incubation of isolated nuclei in an aqueous buffer (Maier and Schawalder, 1986). Therefore, our results are consistent with the presence of a percentage of nuclei with early signs of apoptosis caused by xenobiotics, which contributes to the variability of DNA staining of cells belonging to the same ploidy class. Thus, the mechanism of the increased heterogeneity can be attributed to changes in chromatin conformation, DNA strand breaks, or to both. Our results suggest that the combination of morphological and DNA-dependent flow cytometric parameters that are relatively simple to determine may be of interest in identifying apoptotic drugs.

Our results demonstrate that apoptosis can be detected in hepatocytes long before cell necrosis by low concentrations of the drugs. Four out of 5 well-known apoptotic compounds could be predicted at subcytotoxic concentrations with this battery of markers. Moreover, our results show that for a reliable screening of apoptosis in hepatocytes several key markers should be used simultaneously. The selection of apoptotic markers should be made on the basis of (1) ability to identify apoptotic compounds irrespective of the pathway of how cell death was initiated; (2) ability to find a compromise between specificity, sensitivity, ease of performance, and reproducibility; (3) needing only a small amount of cells, (4) low cost methods, and (5) use of automatized analysis whenever possible. In this regard, our results suggest that among the markers that have been evaluated, it is the combined use of caspase 3 activation, and nuclei and DNA analysis by flow cytometry that fulfill the requirements for screening the apoptotic effect of new drugs on hepatocytes.

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