Rapid and extensive lethal action of clofibrate on hepatoma cells *in vitro*

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Abstract

Clofibrate, for a long time in use as a hypolipidemic drug, is a well known peroxisomal proliferator (PP) and hepatocarcinogen in rodents. We show here that *in vitro* 1 mM clofibrate induces a rapid and massive death of rat AH-130 hepatoma cells. Cell death was prominent already after 4 h of treatment, with a characteristic 'apoptotic' pattern by conventional microscopy. This was further supported by the pronounced chromatin condensation detectable on 4′,6-diamine-2′-phenylindole dihydrochloride (DAPI) staining, the clearcut internucleosomal DNA fragmentation on agarose-gel electrophoresis (ladder patten), and the accumulation of markedly hypochromic cells observed in flow cytometric DNA histograms. Consistently with the apoptotic features of the process, some parameters commonly used to detect cell death, such as plasma membrane permeabilization to trypan blue or propidium iodide, lack of mitochondrial retention of rhodamine 123, or extracellular release of lactate dehydrogenase, were all virtually negative. However, these same parameters became markedly positive after 24 h of treatment, which was suggestive for the occurrence of 'secondary' necrosis among AH-130 cells. By a combination of flow cytometric parameters, after 4 h on 1 mM clofibrate only 41% of the AH-130 cells could still be categorized as viable (i.e., non-apoptotic and non-necrotic), while 46% of cells appeared apoptotic and 13% necrotic. At 24 h, 67% of cells were necrotic, 20% apoptotic and only 13% non-apoptotic and non-necrotic. Apoptosis was also extensive in AH-130 cells treated with another PP such as nafenopin at 1 mM concentration and in human hepatoma HepG2 cells treated with clofibrate. By contrast, clofibrate did not cause apoptosis on primary rat hepatocyte cultures. These observations indicate that: (i) apart from their well-known cell growth-promoting action, PPs such as clofibrate or nafenopin may exert a substantial cytotoxic action on targets such as the AH-130 or HepG2 hepatoma cells; (ii) this cell death evolves from an initial 'apoptotic' to an eventual 'necrotic' pattern; (iii) detection of cell death requires the adoption of a full panel of tests, adequate to cover the whole evolving death pattern, while such tests may even be substantially misleading whenever applied individually; (iv) the cytotoxicity of clofibrate and similar agents on normal and, particularly, tumoural cells may deserve careful reevaluation.

Keywords: cell death, apoptosis, necrosis, hepatoma cells, peroxisome proliferators, clofibrate, nafenopin

Abbreviations: AGE, agarose-gel electrophoresis; DAPI, 4′,6-diamine-2′-phenylindole dihydrochloride; FL, fluorescence channel; FSC, forward light scatter; HMGCoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PI, propidium iodide; PP, peroxisome proliferator; PPAR, PP-activated receptor; R123, rhodamine 123; SSC, side light scatter; TB, trypan blue

Introduction

The carcinogenic action of PPs is widely documented. Since PPs do not damage DNA directly, these agents are usually categorized as nongenotoxic carcinogens (e.g. Reddie and Lalwai, 1983; Bursch *et al*, 1992; Chen *et al*, 1994). Yet genotoxicity may arise indirectly, due to excess reactive oxygen species and lipid peroxidation products generated in consequence of the PP-induced elevation of peroxisomal β-oxidation enzymes; of special importance, in this connection, is fatty acyl-CoA oxidase, which has hydrogen peroxide as a byproduct (Reddie and Lalwai, 1983; Hwang *et al*, 1993). The induction of these enzymes, as well as of the microsomal cytochrome P450 IVA or of the mitochondrial HMGCoA synthase (Rodriguez *et al*, 1994), is specifically mediated by the PPAR. This is a member of the steroid hormone receptor superfamily that recognizes PP-response elements in the upstream promoter region of the responsive genes and thereby activates their transcription (reviewed in Green and Wahli, 1994). The action as tumour promoters is most often ascribed to enhanced liver cell proliferation (Reddie and Lalwai, 1983; Chen *et al*, 1994, and references therein). PPs may indeed stimulate cell growth *in vivo* (Ohmura *et al*, 1996). In cells of the hepatocytic lineage mitogenesis by amphipathic carboxylates, the main PP class that includes clofibrate, specifically involves their interaction with the liver fatty acid-binding protein (Khan and Sorof, 1994, and references therein). An additional mechanism, not necessarily alternative, stems from the observation that cell death by apoptosis in

preneoplastic or early neoplastic liver lesions is suppressed by a variety of tumour promoters, including PPs (Schulte-Hermann *et al*, 1987; Bursch *et al*, 1992; Columbano *et al*, 1996). Consistently, a PP such as nafenopin (50 μM) has recently been reported to promote the survival of cells of the hepatocytic lineage *in vitro* (Bayly *et al*, 1994; Gill *et al*, 1995).

In the course of experiments aimed at altering the lipid composition of tumour cells, we found, unexpectedly, that 1 mM clofibrate induced rapid and massive death of Yoshida AH-130 ascites hepatoma cells *in vitro*. The same result was obtained on human hepatoma HepG2 cells, while only AH-130 cells were killed by another PP, nafenopin, at 1 mM concentration. Clofibrate, discovered some 30 years ago (Thorp and Waring, 1962), has long been in use as a hypolipidemic drug (briefly reviewed in Hahn and Goldberg, 1992). The mechanism of the hypolipidemic action of clofibrate or other fibric acid derivatives, some still of clinical relevance, is not yet clear (Hahn and Goldberg, 1992; Green and Wahli, 1994). In the liver of rodents, clofibrate and other PPs cause conspicuous increases in size and number of peroxisomes, hyperplasia and hepatocellular carcinomas (Reddie and Lalwai, 1983).

In the present paper, we report on the death of rodent and human hepatoma cells by clofibrate and nafenopin, show that it occurs by the apoptotic mode, and briefly discuss the possible relevance of this finding to the action of clofibrate and related agents as tumour promoters. In addition, the present data, further expanding previous observations and concepts (Darzynkiewicz *et al*, 1992; Dive *et al*, 1992), suggest that this clofibrate-induced cell death can be proposed as a useful model to illustrate the relative merit and reliability of a panel of procedures commonly employed in cytotoxicity testing.

Results

Microscopy

The basic morphological changes occurring in AH-130 cells exposed to 1 mM clofibrate are illustrated in Figure 1B and C. Cell shrinkage due to nuclear and cytoplasmic condensation was clearly detectable at 4 h and involved a substantial fraction of the population by 24 h. As observed after DAPI-staining, a striking pattern of chromatin condensation associated with polylobation or fragmentation of nuclei was already prominent by 4 h. A similar pattern was observed when AH-130 cells were incubated in the presence of 1 mM nafenopin (Figure 1D).
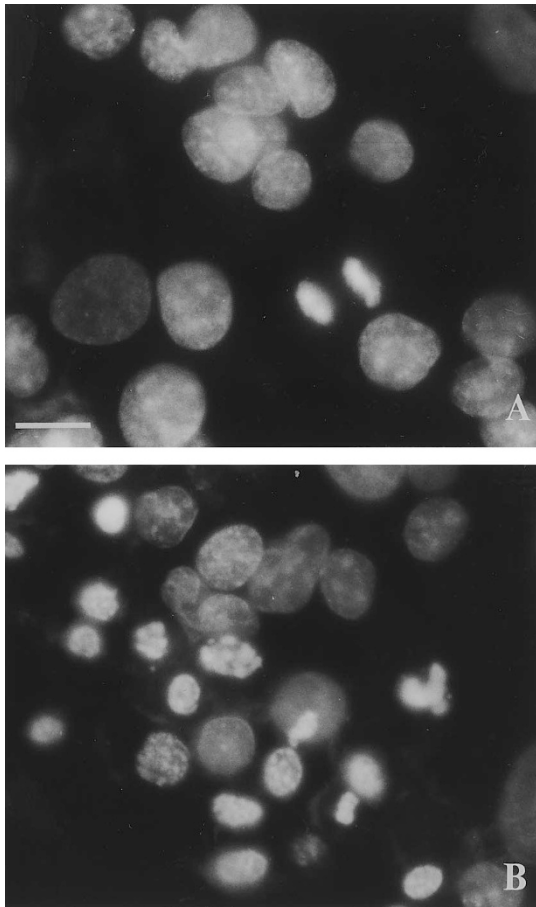
After 4 and 24 h incubation, the percentage of apoptotic cells (apoptotic index, data not shown) amounted to 48 and 95%, respectively, for cells treated with 1 mM clofibrate and to 37 and 94%, respectively, for those on 1 mM nafenopin. Lower concentrations of either drug (0.05 mM) did not cause any appreciable increase of the apoptotic index over the values recorded for controls, incubated with the solvents alone (2 and 8% at 4 and 24 h, respectively).

Moreover, at variance with its previously reported action on rat hepatocytes or FaO hepatoma cells (Bayly *et al*, 1994; Gill *et al*, 1995), 0.05 mM nafenopin did not display

any protective effect on AH-130 cells against apoptosis either spontaneous or induced by 1 mM clofibrate.
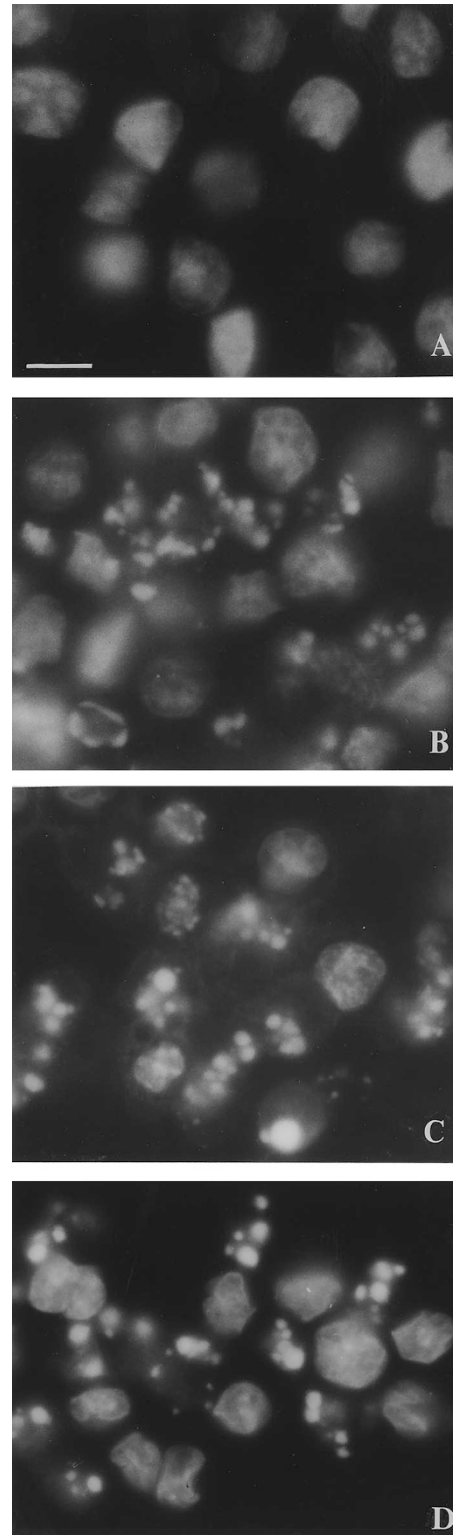

Figure 1 Light microscopic appearance of PP-induced apoptosis in AH-130 cells. Control cells (A) and cells after 4 h (B) and 24 h (C) on 1 mM clofibrate or after 4 h on 1 mM nafenopin (D), stained with DAPI. Bar (A–D): 10 μm.

The cytotoxicity of clofibrate was also assessed on other cell types. No apoptosis was caused by this drug, at concentrations up to 1 mM, in primary cultures of rat hepatocytes (data not shown). By contrast, 1 mM clofibrate was clearly apoptogenic for human hepatoma HepG2 cells (Figure 2B; the percentage of cells with a $<2n$ DNA fluorescence, by flow cytometry, was 1% at time 0, 5% at 2 h, 19% at 4 h, 26% at 8 h). The HepG2 cells, however, were not affected by treatment with 1 mM nafenopin.


Figure 2 Light microscopic appearance of clofibrate-induced apoptosis in HepG2 cells. Control cells (A) and cells after 4 h on 1 mM clofibrate (B), stained with DAPI. Bar (A–B): 10 μM.
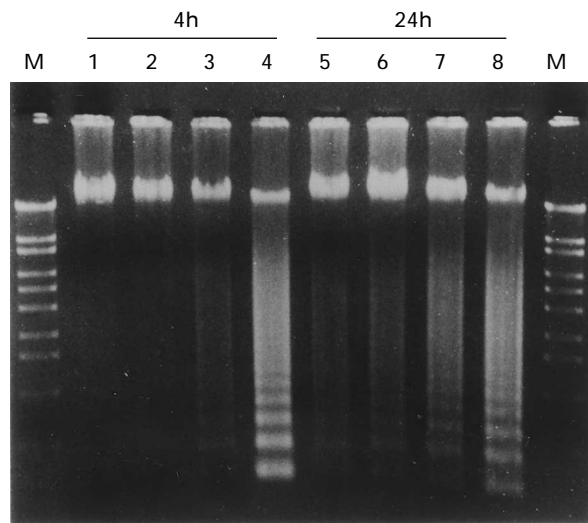

Figure 3 Agarose gel electrophoresis of DNA. DNA from AH-130 cells incubated for 4 h or 24 h, as indicated, with no clofibrate (controls: lanes 1 and 5) or with 0.25 mM (lanes 2 and 6), 1.0 mM (lanes 3 and 7), and 2 mM (lanes 4 and 8) clofibrate. Molecular weight markers (M): StyI digest of ϕ.
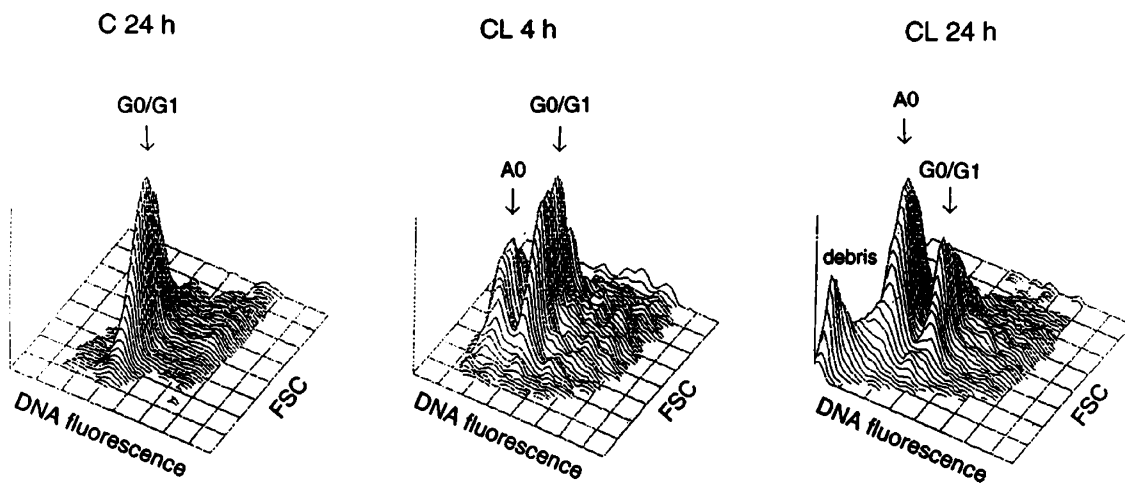
Figure 4 Forward light scatter (FSC) *vs* DNA fluorescence. Representative contour plots of ethanol-fixed AH-130 cells: control cells at 24 h (left panel) and cells exposed to 1 mM clofibrate for 4 h (center) and 24 h (right). A0 region denotes cells with a normal size (FSC) and a subdiploid DNA fluorescence, well distinct from debris.

DNA electrophoresis

Cleavage of nuclear DNA into multiples of 180–200 bp, detectable as 'DNA laddering' by AGE, is usually considered a hallmark of apoptosis (reviewed in Peitsch *et al*, 1994). Analysis of the DNA extracted from clofibrate-treated AH-130 cells showed (Figure 3) a clearcut ladder pattern at both 4 and 24 h, the extent of DNA fragmentation increasing with exposure time and clofibrate concentration. Although in some systems such internucleosomal chromatin cleavage is not detectable, its occurrence confirms the apoptotic nature of the process.
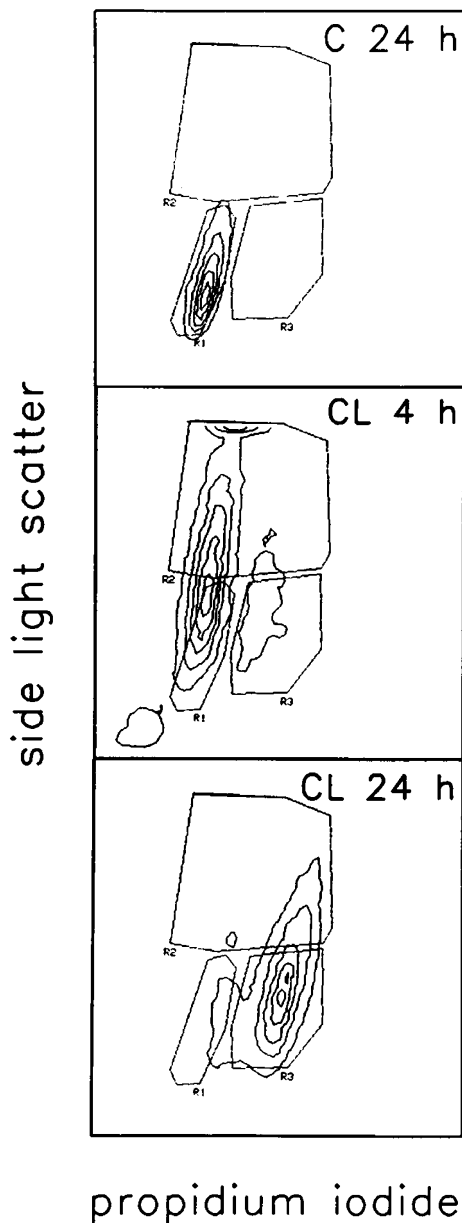
Figure 5 Side light scatter (SSC) *vs* PI exclusion. Representative contour plots of unfixed AH-130 cells: control cells at 24 h (top panel) and cells exposed to 1 mM clofibrate for 4 h (center panel) and 24 h (bottom panel), as indicated. Areas indicated as R1, R2, R3 correspond to viable, early and late apoptotic cells, respectively, as defined in the legend to Figure 6.
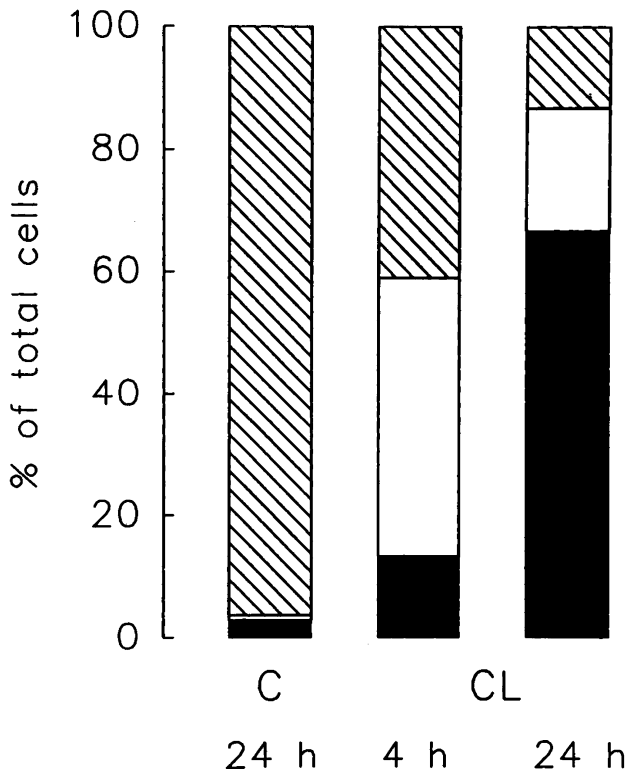
Flow cytometry

The death process elicited by clofibrate in AH-130 cells was analyzed in detail flow cytometrically (cf. Darzynkiewicz *et al*, 1992; Dive *et al*, 1992). At 4 h, the onset of apoptosis was associated with an increased SSC of cells, in agreement with previous observations (Swat *et al*, 1991; Barbiero *et al*, 1995), though basal SSC levels had recovered by 24 h (not shown). Figure 4 illustrates the distribution of DNA fluorescence *vs* FSC, a parameter for cell size. In control cells, the pattern after 24 h *in vitro* was similar to that observed at time 0 or after a 4 h incubation (not shown). In cells exposed to 1 mM clofibrate, flow cytometry detected a substantial accumulation of cells characterized by a <2n DNA fluorescence (in the A0 region, according to Telford *et al*, 1991). Already prominent after a 4 h treatment, when it accounted for $41 \pm 7\%$ of cells, this subpopulation became even more conspicuous by 24 h ($63 \pm 8\%$), when also the FSC was decreased. A further peak, well distinct from the A0 region, was evident at 24 h (Figure 4, right): characterized by very low FSC and faint PI staining, it represented debris from necrotic cells (cf. Darzynkiewicz *et al*, 1992; Dive *et al*, 1992).

Figure 5 illustrates how the distribution of AH-130 cells treated with 1 mM clofibrate evolved with respect to two other parameters evaluated on non-fixed cells, namely, SSC, which reflects 'cell density', and PI exclusion, as a measure of plasma membrane integrity. SSC was substantially increased at 4 h, as often observed in apoptosing cells (Swat *et al*, 1991; Barbiero *et al*, 1995), though by 24 h it had largely receded to control levels. Figure 5 further shows that PI, while excluded by virtually all of the control cells and by most of those treated with 1 mM clofibrate for 4 h, was taken up by the vast majority of cells after 24 h on clofibrate; this finding argues for the occurrence of 'secondary necrosis' among AH-130 cells (Wyllie *et al*, 1980; Desjardins and MacManus, 1995).

As a further viability test, R123 mitochondrial retention in AH-130 cells was evaluated by flow cytometry (not shown). This parameter closely correlated with PI-exclusion in both control and experimental cells. On 1 mM clofibrate, cells that did not retain R123 were >14% at 4 h, but amounted to >65% by 24 h. In other models of apoptosis (Barbiero *et al*, 1995, and reviewed in Kroemer *et al*, 1995) the integrity of mitochondria is compromised earlier than that of the plasma membrane or of lysosomes (by acridine orange retention).

The overall information obtained by flow cytometry is summarized in Figure 6. As detailed in the legend, combining different flow cytometric parameters (DNA fluorescence, FSC, SSC, PI exclusion, R123 retention) permitted to discriminate among viable (neither necrotic nor apoptotic), early apoptotic, and late apoptotic AH-130 cell subpopulations. Control cells were virtually all viable at 4 h, and still >90% viable by 24 h. On 1 mM clofibrate, not more than 41% of the population could be considered viable by 4 h, and only 13% by 24 h, which figures are pretty close to those obtained microscopically by counting the proportion of apoptotic cells (see above). It is clear, though, that at 4 h most dying cells were still at an early

Figure 6 Frequency of apoptotic cells by flow cytometry. AH-130 cells incubated with no clofibrate (C) or with 1 mM clofibrate (CL) for 4 h and 24 h. Based on combined flow-cytometric parameters, as evaluated on fixed or unfixed cells, cells were attributed to three classes: (1) *viable* (dashed bars): that is non-apoptotic and non-necrotic: with normal SSC, excluding PI, retaining R123, and outside the A0 region; (2) *early apoptotic* (open bars): with increased SSC, excluding PI, retaining R123, and included in the A0 region; (3) *late apoptotic* (full bars): with normal SSC, not excluding PI, not retaining R123, and included in the A0 region ('secondary necrosis', Wyllie *et al*, 1980). Data expressed as percentages of total cells (means of three experiments). Differences between means were statistically significant ($p < 0.05$) on ANOVA followed by Newman-Keuls test.

stage of the apoptotic process, and only a few (13%) at a late stage, whereas by 24 h two-thirds of the cells were at a late apoptotic stage.

Conventional cell integrity parameters

The effect of clofibrate on AH-130 cell integrity was further evaluated by two routine procedures, namely TB exclusion and LDH release (Tables 1 and 2). In controls or in cells incubated with 0.05 or 0.25 mM clofibrate at both 4 and 24 h, only a negligible fraction of cells failed to exclude the dye. By contrast, on 1 mM clofibrate the proportion of cells stained by TB at 4 h increased moderately, while about 70% of the cells became permeable to TB by 24 h. Moreover, a significant decrease of cell number was observed only after 24 h treatment with 1 mM clofibrate (Table 3). These figures are consistent with those obtained flow-cytometrically with the PI-exclusion test (see Figures 4 and 5).

Although basically similar, the LDH release data (Table 2) indicate a degree of damage slightly higher than that measured by TB-exclusion testing. At first sight, this may appear surprising for a small molecule such as TB (or PI)

should cross a 'leaky' plasma membrane much more easily than LDH. However, the results of simple counts showed that in 24 h 1 mM clofibrate caused a 14% decrease in the total number of cells. Since this change presumably reflected, in part at least, complete disintegration of a significant proportion of cells, the corresponding contribution of LDH to the medium probably accounted for the excess cell death measured by LDH release *vs* the values detected by TB-exclusion testing.

TB exclusion and LDH release were also evaluated on cells exposed to nafenopin (Tables 1 and 2). The results with 0.05 mM nafenopin at 4 and 24 h (data not shown) and with 1 mM nafenopin at 4 h closely reproduced those obtained with clofibrate at corresponding concentrations and times. By contrast, leakiness to TB and LDH release were both markedly lower after 24 h on 1 mM nafenopin than on clofibrate and, consistently, the number of nafenopin-treated cells did not decrease significantly (data not shown).

Discussion

The present observations show that clofibrate and nafenopin were rapidly and extensively lethal for rat ascites hepatoma AH-130 cells *in vitro*. Also the HepG2 cells, a human hepatoma line, were rapidly killed by clofibrate, though less extensively than the AH-130 cells, but nafenopin had no lethal action on them. Other rat hepatoma cells (JM2 and Morris 7777, unpublished observations) or rat hepatocytes in primary culture were not affected by clofibrate. This induced cell death, which occurred by apoptosis, was particularly investigated on clofibrate-treated AH-130 cells.

Clofibrate was apoptogenic at 1 mM, which apparently is a quite high concentration; however, similar or even higher concentrations have been used in biochemical studies on tissue cultures (Chawla and Lazar, 1994; Perrot *et al*, 1991), and 250 mg/kg b.w. is a usual dosage for clofibrate in rat chemical hepatocarcinogenesis protocols (cf. Reddy and Lalwai, 1983).

The death process in AH-130 cells

Apoptosis is the mode by which the AH-130 cells spontaneously die in the animal at rates that increase as the tumour shifts from the exponential to the plateau growth phase (Tessitore *et al*, 1993). In AH-130 cells treated with 1 mM clofibrate, apoptosis was already prominent after 4 h and became massive by 24 h. Cell death was easily detected by light microscopy, on smears stained with haematoxylin and eosin or DAPI, as well as by flow cytometry, from the appearance of a subpopulation of cells with a < 2 n DNA fluorescence. Such hypodiploid peak or A0 region in the cytogram correlated with the occurrence of a clearcut internucleosomal DNA fragmentation, as detected by AGE (see Nicoletti *et al*, 1991; Telford *et al*, 1991). At 4 h, the onset of apoptosis was associated with an increased SSC of cells, in agreement with previous observations (Swat *et al*, 1991; Barbiero *et al*, 1995), though basal SSC levels had recovered by 24 h, most cells had shrunk to a smaller size, as

Table 1 Effect of clofibrate and nafenopin on trypan blue exclusion in AH-130 cells.

	Incubation time	
	4 h	24 h
Controls	0.4 ± 0.1^a	2.1 ± 0.1^a
Clofibrate		
0.25 mM	2.0 ± 0.1^a	2.4 ± 0.2^a
1.00 mM	9.4 ± 0.6^b	68.1 ± 9.5^c
Nafenopin		
1.00 mM	3.0*	16.4*

Data expressed as percentages of cells not excluding trypan blue (means $\pm$ SD, n=6). Differences between groups labelled with different letters are statistically significant (p<0.05) on ANOVA followed by Newman-Keuls test. *Data refer to two experiments.

Table 2 Effect of clofibrate and nafenopin on lactate dehydrogenase release from AH-130 cells

	Incubation time	
	4 h	24 h
Controls	3.0 ± 0.2^a	10.3 ± 0.9^b
Clofibrate		
0.25 mM	7.4 ± 0.3^{ab}	9.8 ± 1.0^b
1.00 mM	9.7 ± 0.5^b	77.8 ± 9.4^c
Nafenopin		
1.00 mM	5.3*	28.9*

Lactate dehydrogenase (LDH) activity released to the medium expressed as percentage of the total activity in cell suspensions. Total LDH activity at time 0 corresponded to 783 ± 108 nmoles of NADH consumed/min per 10^6 cells. Presentation and statistical analysis of data as in Table 1.

Table 3 Effect of clofibrate on AH-130 cell number

Clofibrate (mM)	Incubation time	
	4 h	24 h
0	470 ± 73^{ab}	525 ± 78^b
0.25	469 ± 69^{ab}	544 ± 62^b
1.00	412 ± 52^a	452 ± 38^a

Values expressed as 10^4 cells/ml suspension; [a] 400 was the seeding density and thus is the time 0 figure for all groups. Presentation and statistical analysis of data as in Table 1.

also denoted by the decreased FSC, and had generally progressed to more advanced stages of the apoptotic process. Notably, after 4 h on 1 mM clofibrate virtually all cells appeared 'viable' by conventional tests for cell necrosis that measure either the leakiness of plasma membranes to dyes such as trypan blue (by light microscopy) and propidium iodide (by flow-cytometry) or the extracellular release of cytoplasmic constituents such as LDH. Therefore, these tests almost totally failed to diagnose an already conspicuous cell death process, which, by contrast, was clearly detected using appropriate procedures that have been recently developed to monitor and quantify apoptosis. By 24 h, cell death was also demonstrated with conventional tests for cell necrosis. Flow cytometric techniques were particularly apt to monitor the death process, for they rapidly discriminated between apoptotis and necrosis or between early and late stages of

apoptosis in the whole cell population (Darzynkiewicz *et al*, 1992; Dive *et al*, 1992). However, once dead cells had fully disintegrated, death could only be measured by a test such as the LDH release assay. Of interest, the development of such secondary changes was far less pronounced when apoptosis was elicited by 1 mM nafenopin, as consistently shown by all the parameters measured (apoptotic index, TB exclusion, LDH release). The reason for such a difference remains obscure, however. By all evidence, clofibrate or nafenopin caused death in AH-130 cells mainly by the apoptotic mode, while only subsequently the apoptosed cells, particularly those exposed to clofibrate, underwent changes that can be conventionally categorized as 'secondary necrosis' (Wyllie *et al*, 1980; Desjardins and MacManus, 1995).

Mechanisms of the lethal action of clofibrate and nafenopin

An analysis of the mechanism(s) underlying the apoptogenic action of clofibrate and nafenopin on the rat AH-130 cells and the reason why the human HepG2 cells were only susceptible to clofibrate is beyond the scope of the present report, and can only be conjectural thus far.

Among the pleiotropic effects of clofibrate, shared with nafenopin only in part, many are potentially relevant to induction of apoptosis. Suppression of DNA and RNA synthesis (Parekh and Chitins, 1991) or inhibition of HMGCoA reductase (Castillo *et al*, 1990; Haughom and Spydevold, 1992) might well play a role, since apoptosis may be caused by inhibitors of macromolecular syntheses (e.g. Hickman, 1992; Amenta *et al*, 1993; Sherwood and Schimke, 1994; Barbiero *et al*, 1995) or by the HMGCoA reductase inhibitor lovastatin (e.g. Bansal *et al*, 1991; Pérez-Sala and Molinedo, 1994; Han and Wyche, 1996). Also, neither the action of clofibrate as enzyme inducer (see Introduction) nor the suggestion that PP toxicity may result from PPAR interferring with retinoid action (Green and Wahli, 1994) can be disregarded. The status of the PPAR expression in the different cells herewith tested is currently under investigation and we ignore whether it is related to the present differential effects. However, the rapidity of the lethal effect of clofibrate on AH-130 or HepG2 cells might be difficult to reconcile with the possibility of an involvement of the PPAR. On the other hand, clofibrate and other PPs are known to exert antiproliferative and differentiation-promoting effects on cells such as 3T3-L1 preadipocytes (Chawla and Lazar, 1994) and, in normal rat liver, clofibrate can stimulate the production of TGF-β (Rumsby *et al*, 1994), which is a known inhibitor of growth and inducer of apoptosis on rodent hepatocytes or rodent and human hepatoma cells (Lin and Chou, 1992; Bursch *et al*, 1993; Fukuda *et al*, 1993).

Nafenopin at 0.05 mM concentration has been recently shown to suppress apoptosis, whether spontaneous or elicited by agents such as TGF-β, and to promote cell survival of primary hepatocyte cultures and cells of the FaO rat hepatoma line (Bayly *et al*, 1994; Gill *et al*, 1995). On the AH-130 cells, not only we did not observe any protection of 0.05 mM nafenopin against spontaneous or clofibrate-induced apoptosis, but, rather, 1 mM nafenopin

was as apoptogenic as clofibrate. Whether the reason for such divergent effects pertains to the cell type is not known as yet and deserves further investigations.

Possible significance of the lethal action of clofibrate

Two apparently contradictory effects have been reported for PPs as tumor promoters. Short-term treatment consistently causes a decrease in the number and size of glutathione S-transferase P1-1 or γ-glutamyltranspeptidase-positive pre-neoplastic lesions, whereas longer treatment results in more and larger tumors in livers with the same preneoplastic lesions (Chen *et al*, 1994, and references therein). The phenotypic shifts observed in preneoplastic hepatic lesions in response to short-term treatment with PP may reflect inhibition of certain subsets of cell populations and promotion of others (Chen *et al*, 1994). Whether changes in rates of cell proliferation or of cell deletion or of both processes are involved is not entirely clear. In the course of chemical carcinogenesis, preneoplastic liver lesions are known to undergo extensive remodelling and cell turnover (Columbano *et al*, 1984; Rotstein *et al*, 1986; Bursch *et al*, 1992; Canuto *et al*, 1993; Chen *et al*, 1994; Piga *et al*, 1995), implying that they contain a significant proportion of cells whose survival is compromised. Under such circumstances, additional stimulation with a PP could further upset the finely-tuned balance of extra- and intracellular signals, to such an extent as to precipitate cell death (Wyllie, 1992; Passilly *et al*, 1996).

Irrespective of the precise underlying mechanism(s), the observation that clofibrate exerts strong lethal action on cells of both rodent and human origin such as the AH-130 and the HepG2 suggests that the cytotoxicity of clofibrate and related agents on normal and, particularly, tumoural cells may deserve careful reevaluation.

Materials and methods

Cells and treatments

Male Long-Evans rats, weighing 120 – 150 g and maintained on a semi-synthetic diet (Piccioni, Brescia, Italy) and water *ad libitum* were used for serial weekly transplantation of the Yoshida AH-130 ascites hepatoma ($20 – 30 \times 10^6$ tumour cells inoculated i.p. in 1 ml). Under sterile conditions, 6 day-old ascites tumours were collected with a syringe and cells were separated from the ascitic fluid by low-speed centrifugation, washed in RPMI 1640 medium, resuspended (4×10^6/ ml) in the same medium supplemented with 10% newborn calf serum, 1% (w/v) bovine serum albumin (fraction V, fatty acid free), and transferred to Petri dishes.

Rat hepatocytes were isolated according to standard procedures (Seglen, 1973) and cultured as indicated by Bayly *et al*. (1994). HepG2 cells, derived from a human hepatoblastoma, were grown in DMEM supplemented with 10% foetal calf serum in standard conditions.

Clofibrate, dissolved in dimethylsulphoxide, or nafenopin, dissolved in N-N-dimethylformamide, were added (1 : 100 in volume) to culture media to the final concentrations specified. Controls were treated with solvents alone. HepG2 cells were incubated for 2 – 4 – 8 h and the other cells for 4 or 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air, then harvested and analyzed.

DNA distribution

DNA staining was performed as described elsewhere (Barbiero *et al*, 1995), with some modifications (Bianciotto *et al*, 1995). Briefly, cells were washed in PBS, fixed in ice-cold 70% ethanol for at least 30 min, incubated at room temperature in PBS containing DNase-free RNase (Type II-A) and PI at final concentrations of 0.4 and 0.18 mg/ml, respectively, then analyzed with a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA, USA) equipped with a 488 nm argon laser and two filters, respectively transmitting at 585 nm (FL2) and above 620 nm (FL3). FSC and SSC were measured simultaneously. Data were recorded on a Hewlett Packard computer (HP 9000, model 300), using CellFit software (Becton & Dickinson).

Cell integrity

A panel of independent parameters was used.

Plasma membrane integrity was checked both microscopically and flow cytometrically. TB exclusion was evaluated on cells suspended in the presence of the dye (0.8 mg/ml); 400 cells were counted for each sample and results expressed as percentages of TB-positive cells. For the PI-exclusion test, cells unfixed and suspended in PBS (10^6 cells/ ml) were incubated with PI (10 μg/ml) and analyzed by flow cytometry as detailed elsewhere (Barbiero *et al*, 1995).

Mitochondrial transmembrane potential was evaluated by a R123-retention test. Unfixed AH-130 cells suspended in PBS (10^6 cells/ml) were incubated for 30 min 37°C with R123 (10 μg/ml) and analyzed by flow cytometry as described elsewhere (Barbiero *et al*, 1995). In some experiments cells were both preincubated with R123 and subsequently stained with PI, as specified above, to compare PI exclusion and R123 retention.

LDH activity was assayed (Kornberg, 1955) on total cell suspensions, after sonication, and on clear supernatants after centrifugation at $600 \times g$ for 10 min. The activity determined in the supernatant was expressed as percentage of total activity in the cell suspension.

DNA fragmentation

The occurrence of internucleosomal DNA fragmentation was evaluated by the AGE method as described by Kaufmann (1989). Cells were washed twice with ice-cold phosphate-buffered saline, suspended at 2×10^6 cells/ml in a lysis buffer (2 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulphate, 500 mM Tris-HCl, pH 9.0), incubated with RNAse (20 μg/ml) for 1 h at 37°C and then with proteinase K (100 μg/ml) for 1 h at 37°C, and finally extracted with phenol/chloroform. Aliquots of 10 μg DNA were electrophoresed on a 1.0% agarose gel and viewed under UV illumination after staining with ethidium bromide (0.5 μg/ml).

Light microscopy

Cells were fixed in methanol, stained with the DNA-specific fluorochrome DAPI and examined in an epiilluminated fluorescence microscope (Dialux, Leitz, Germany). The apoptotic index (percentage of apoptotic cells) was determined on at least 1000 cells per sample.

Statistical analysis

Data are expressed as means $\pm$ SD. Significance of differences was assessed by ANOVA, followed by the Newman-Keuls test.

Chemicals

Nafenopin (2-methyl-2[1,2,3,4-(tetrahydro-1-naphtyl)-phenoxyl]prionic acid) was a kind gift from Ciba-Geigy (Basel, Switzerland); DAPI was purchased from Boehringer Mannheim (Germany); clofibrate (2-(p-chlorophenoxy)-2-methyl-propionic acid ethyl ester), DNase-free RNase (Type II-A), PI, R123, and other reagents from Sigma (St. Louis, MO, USA).

Acknowledgements

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