Pergamon

Biochemical Pharmacology, Vol. 47, No. 12, pp. 2302–2306, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94) E0099-7

P-glycoprotein expression and function in rat hepatocytes in culture

(*Received* 2 *August* 1993; *accepted* 18 *February* 1994)

Abstract—Expression of P-glycoprotein, which confers multidrug resistance to a broad range of anticancer drugs, was studied in rat hepatocytes in culture. P-glycoprotein was localized in the plasma membrane by immunohistochemical staining and was evaluated by western blotting with C219 as primary antibody and quantification of the coloured spots. The conditions of culture (time in culture, cell density at seeding) had a strong effect on the expression of P-glycoprotein. Expression increases with time in culture. At 10×10^6 cells/75 cm^2 flasks, which is the normal density seeding for hepatocytes in culture, the increase of P-glycoprotein was 17% between 4 and 24 hr in culture, 52% between 24 and 48 hr and 37% between 48 and 96 hr. At low density cell seeding (2×10^6 cells/75 cm^2), the expression of P-glycoprotein was higher than at normal density from the first day in culture (+20%). This difference of expression was maintained until 96 hr of culture and was maximum at 48 hr (+44%). This P-glycoprotein was functional and this overexpression was correlated with a decrease of doxorubicin retention in hepatocytes.

Key words: P-glycoprotein; mdr; rat hepatocytes; culture conditions; anthracyclines; metabolites

P-glycoprotein, a membrane ATP-driven pump able to extrude xenobiotics out of living cells, has been shown to be responsible for a mechanism of cross-resistance against anticancer drugs, which is known as multidrug resistance (mdr*) [1, 2]. It has been established that P-glycoprotein is expressed in normal liver tissue [3], especially in the rat [4] and that this expression is maintained in hepatocyte tissue culture [5]. P-glycoprotein expression increases in preneoplastic and neoplastic nodules induced by carcinogens [6]. This increase has been attributed to the aggression of hepatocytes and is often associated with alterations in drug metabolizing enzymes: decrease of cytochromes P-450, increase of glutathione *S*-transferases [5, 7–9]. Such alterations have also been observed in hepatocytes grown *in vitro* [10–13]. This led us to use rat hepatocytes in culture as a model for the study of the regulation of P-glycoprotein expression in the liver. In a first attempt, we have studied the effect of culture conditions (cell density, growth delay) on P-glycoprotein expression (evaluated by western blotting and immunohistochemistry) and function (evaluated by doxorubicin accumulation).

Materials and Methods

Cell culture. Rat hepatocytes were isolated by collagenase digestion from the livers of male Sprague–Dawley rats weighing 180–200 g [14]. The livers were first washed *in situ* through portal vein by Ca^{2+}-free HEPES buffer (pH 7.65, 37°), then with a 0.025% collagenase solution (Boehringer, Mannheim, F.R.G.) in HEPES buffer. Hepatocytes were seeded at a density of 2×10^6 cells (low density), 4 or 6×10^6 cells (intermediate densities) and 10×10^6 cells (normal density) in 75 cm^2 culture flasks containing 10 mL culture medium. This medium was a mixture of 75% Minimal Eagle Medium and 25% medium 199 (both obtained from Gibco, Uxbridge, U.K.) and was supplemented with 200 μg/mL bovine serum albumin, 10 μg/mL bovine insulin and 10% fetal calf serum. After 4 hr, the cells have attached to the flask and the medium is replaced. Fetal calf serum was absent from the medium which contained 70 μM hydrocortisone hemisuccinate and was changed every day. The cultures were maintained under a 5% CO_2 humidified atmosphere. Hepatocytes were studied after 4, 24, 48 and 96 hr of culture.

Immunohistochemistry. Immunohistochemistry was performed after fixation of the cells by a mixture of 95% ethanol and 7% acetic acid (v/v) on ice. After incubation for 1 hr with a 10% solution of fetal calf serum in PBS to reduce aspecific label, the cells were incubated for 1 hr at room temperature with C219 antibody (Centocor) diluted at 1/10 in PBS [15]. The label with C219 was revealed by incubation for 1 hr with a peroxidase-labeled rabbit anti-mouse IgG (Diagnostics Pasteur) diluted at 1/100 in PBS.

Western blotting. For western blotting evaluations of P-glycoprotein, the cells were washed two times with PBS, pH 7.4, then recovered by gentle scraping in 40 mM Tris buffer, pH 8 and centrifuged (3 min at 400 *g*). The cells originating from several flasks could be pooled so as to always obtain a pellet containing $10–12 \times 10^6$ cells. After elimination of the supernatant, the cell pellets were kept at −80°. They were resuspended in 250 μL of 5 mM Tris buffer, pH 8, containing 6 mM $MgCl_2$; 1.5 μL of aprotinin solution (containing 32.2 TIU/mL in 0.15 M NaCl and obtained from the Sigma Chemical Co., St Louis, MO, U.S.A.) were added and the cells left in ice for 10 min. The cell pellets were then sonicated three times for 10 sec in ice and cell lysis was checked at the microscope. In each tube were added 250 μL 80 mM Tris, 6 mM $MgCl_2$, pH 8, containing 1.5 μL aprotinin and 50 μL DNAse I (Sigma) diluted in 0.15 M NaCl at a concentration of 204 U/mL. Incubation with DNAse was performed for 30 min at room temperature. Proteins were assayed in cell lysates with Coomassie blue (Bio Rad, Munich, F.R.G.) at 595 nm.

Proteins of whole cell lysates were separated by gel electrophoresis according to Laemmli [16] on a 6.5% polyacrylamide gel. Each well was loaded with 100 μg protein. Protein separation was obtained under a constant voltage of 90 V during 16 hr at 4°, with a Tris 25 mM buffer, pH 8.3, containing 192 mM glycine and 0.1% SDS. Proteins were then transferred on a nitrocellulose membrane under a constant voltage of 150 V during 90 min at 4°, with a 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% methanol [17]. The nitrocellulose membrane was first incubated for 1 hr in a suspension of powdered milk to saturate aspecific binding sites, then for 16 hr at 4° with C219 antibody diluted at 1/100, then after five rinses, with

* Abbreviations: mdr, multidrug resistance; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; PBS, phosphate buffer saline; TIU, Trypsin inhibitor unit; HTC, hepatoma tissue culture.

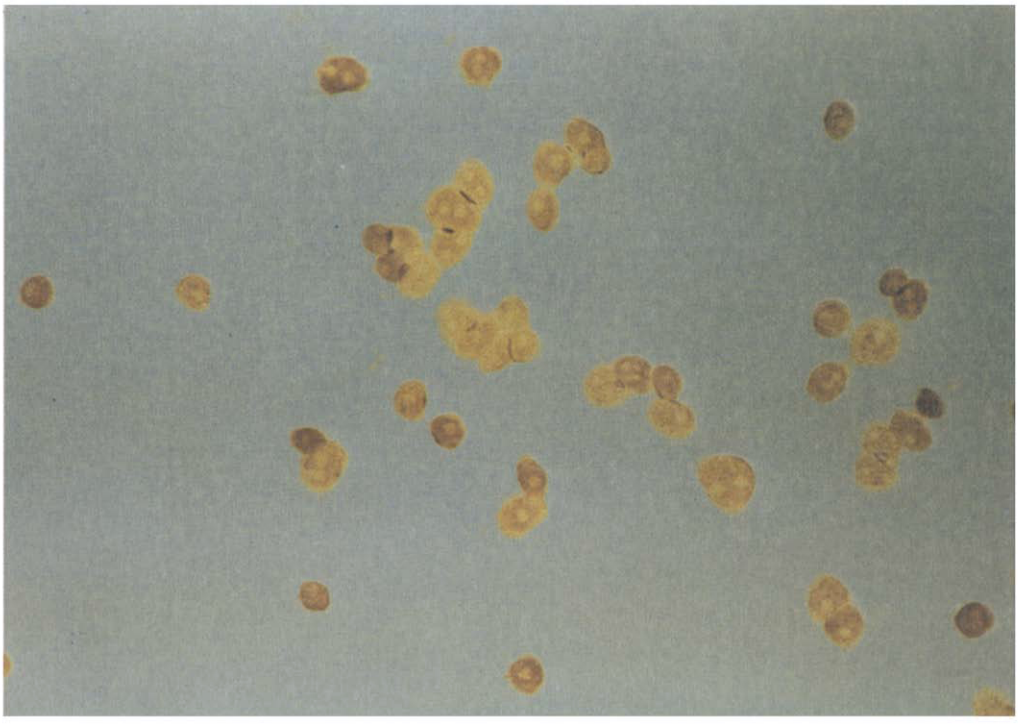

Fig. 1. Immunohistochemical staining of rat hepatocytes with C219 antibody. Hepatocytes have been seeded at the density of 2×10^6 cells and were grown for 4 hr.

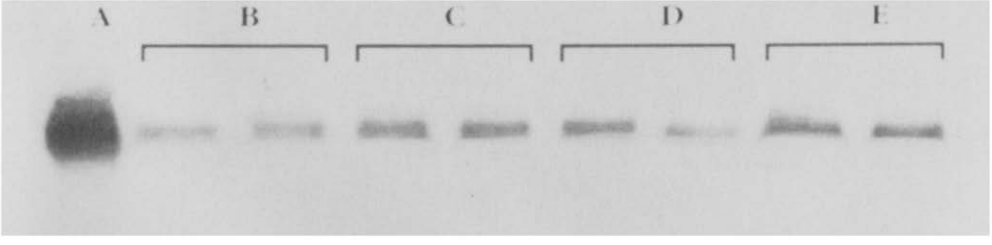

Fig. 2. Immunoblot analysis of Pgp expression in hepatocytes in culture seeded at different densities. (A) resistant HTC cells (control); (B) 10×10^6 hepatocytes/75 cm^2; (C) 6×10^6 hepatocytes/75 cm^2; (D) 4×10^6 hepatocytes/75 cm^2; and (E) 2×10^6 hepatocytes/75 cm^2.

a rabbit anti-mouse IgG antibody labeled with alkaline phosphatase (Diagnostics Pasteur) and diluted at 1/200 in the saturation solution.

Proteins were revealed in a 0.5 mg/mL solution of 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) for 5–15 min. P-glycoprotein appeared on nitrocellulose sheets as a blue spot. Quantification of the spots was performed on a Gemini gel analysis system (Newcastle, U.K.) with a Joyce Loebl software. The individual spots were compared on each nitrocellulose membrane, but no comparison was made between different membranes. A reference P-glycoprotein containing extract from rat HTC cells was run simultaneously. These hepatoma tissue culture cells, rendered resistant to doxorubicin were a gift of Dr J. Robert [18].

Intracellular accumulation of doxorubicin. After 4, 24, 48 or 96 hr in culture with initial seedings of 2 and 10×10^6 cells in 75 cm^2 flasks, doxorubicin (obtained from Farmitalia, France) was added to the culture medium at the concentration of 0.5 μM and maintained for 2 hr. The cell monolayer was then washed, recovered in 1 mL 0.05 M PBS, pH 7, to which was added an adequate amount of daunorubicin (internal standard). After alkalinization with 100 μL of 0.5 M sodium tetraborate, pH 9.8, the cell suspension was extracted with 9 mL of chloroform/methanol 4/1 (v/v) as described by Baurain *et al.* [19]. The organic layer was then evaporated and the dry residue was reconstituted with the mobile phase of the chromatography.

HPLC was performed on a Lichrocart RP18 cartridge (Merck) combined with a Lichrocart Lichrosorb RP18 microcolumn (Merck). The solvent was a mixture of acetonitrile and 0.05 M ammonium formate buffer, pH 4.0 [20] at the following proportions: 5 min isocratic with 20% acetonitrile, 5 min gradient from 20 to 40% acetonitrile, 5 min isocratic with 40% acetonitrile. Detection of anthracyclines and eventual metabolites was achieved with a spectrofluorometer Jasco FP210 with excitation and emission wavelengths respectively set at 467 and 550 nm.

Results

By immunohistochemistry, P-glycoprotein was found

Table 1. Relative amounts of P-glycoprotein in hepatocytes seeded at low density (2×10^6 cells/flask) compared to cells seeded at normal density (10×10^6 cells/flask)

	Hours in culture			
	4	24	48	96
% Increase in low density cells in comparison with normal density cells	+20.2 ± 0.5	+33.7 ± 8.5	+44.3 ± 11.7	+34.4 ± 6.6

Values are means ± SEM of two to seven experiments, in duplicate.

Table 2. Relative amounts of P-glycoprotein in hepatocytes seeded at 2×10^6, 4×10^6, 6×10^6 cells per flask compared to cells seeded at normal density (10×10^6 cells/flask) after 48 hr in culture

	2×10^6	4×10^6	6×10^6
% Increase in cells at different densities in contrast with normal density cells (10×10^6)	+44.3 ± 11.7	+31 ± 5.8	+19.8 ± 5.5

Values are means ± SEM of six experiments, in duplicate.

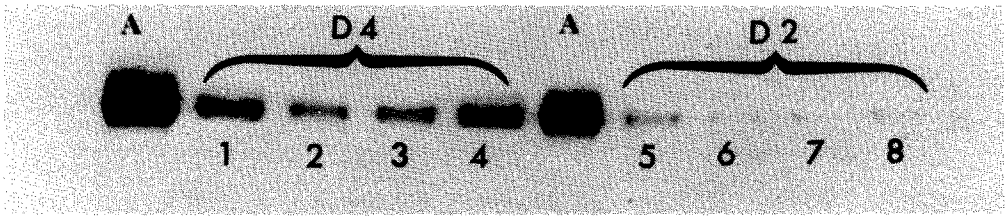

Fig. 3. Immunoblot analysis of Pgp expression in hepatocytes in cultures as a function of time in culture. (A) resistant HTC cells (control); (D4) day 4; (D2) day 2; (1-4-5-8) 2×10^6 hepatocytes/75 cm^2; and (2-3-6-7) 10×10^6 hepatocytes/75 cm^2.

localized at the level of the plasma membrane of rat hepatocytes (Fig. 1). The immune label was especially important at the junctions of hepatocytes when they begin to form biliary canalicular-like structures.

By western blotting, it was possible to quantify the amount of P-glycoprotein. There was only one protein band reacting with the C219 antibody, at a molecular weight of 150–170 kDa, similar to the protein labeled by this antibody in doxorubicin-resistant HTC cells (Figs 2 and 3). Table 1 presents the variation of P-glycoprotein amounts in whole cell lysates as a function of cell density at seeding. It clearly appeared that the level of P-glycoprotein was inversely correlated with cell density, whatever was the duration of the culture, from 4 to 96 hr. The data on four different cell densities at 48 hr in culture confirm the inverse correlation between P-glycoprotein expression and cell density (Table 2).

Table 3 presents the evolution of P-glycoprotein levels during hepatocyte culture. It appears that the level of P-glycoprotein progressively increased from 4 to 96 hr post-seeding, both at low and normal cell densities.

Intracellular doxorubicin accumulation progressively decreased as a function of the duration of the culture. At normal density, doxorubicin accumulation measured at 96 hr was only 15% of the value measured at 4 hr post-seeding (Table 4). Furthermore, there was, 4 hr after seeding, a marked reduced accumulation of doxorubicin at normal cell density compared to low cell density. This reduction was no longer appreciable for the other culture times.

Discussion

We have observed in this study that P-glycoprotein was localized mainly at the level of biliary canalicular-like structures developed by hepatocytes in culture, similarly to the observations of Fardel *et al.* [5]. This is consistent with *in situ* observations. In the whole liver, P-glycoprotein has been shown to be exclusively present on the biliary side of the hepatocytes and on the apical surface of small biliary ducts. [3].

We have shown a significant increase of P-glycoprotein levels in rat hepatocytes in culture as a function of culture duration. This increase is paralleled by a decrease in doxorubicin accumulation, which is in favor of a functional role for P-glycoprotein in hepatocytes in culture. This result is in agreement with the observations of Fardel *et al.* [5], who observed by Northern blots on the same experimental model an increase of *mdr*1 gene expression as a function of culture time, from 4 to 96 hr after seeding.

Chin *et al.* [21] have shown that environmental stress

Table 3. Relative increase of P-glycoprotein in hepatocytes recovered after 24, 48 or 96 hr in culture

	% Increase between		
	4 and 24 hr	24 and 48 hr	48 and 96 hr
Low density cells	+20.8 ± 10.0	+35.7 ± 1.0	+31.3 ± 6.2
Normal density cells	+17.3 ± 0.8	+51.7 ± 11.8	+36.8 ± 13.2

Values are means ± SEM of three experiments in duplicate.

Table 4. Doxorubicin accumulation in hepatocytes recovered after 4, 24, 48 or 96 hr in culture

	Intracellular doxorubicin (pmol/10^6 hepatocytes)			
	4 hr	24 hr	48 hr	96 hr
Low density cells	266 ± 23	134 ± 13	65 ± 7	24 ± 6
Normal density cells	144 ± 7	114 ± 17	79 ± 10	23 ± 6

Values are means ± SEM of two to four experiments made in duplicate.

such as heat shock, sodium arsenite or calcium chloride could induce a 7–8-fold increase in mdr1 gene expression in adenocarcinoma HTB46 cells in culture. It can therefore be hypothesized that P-glycoprotein overexpression in rat hepatocytes is a response to the stress of tissue dissociation and cell isolation, which are known to induce a rapid cell de-differentiation [22, 23]. Increasing the culture time is followed by the expression of fetal-type proteins to the detriment of adult-type isoforms [24]. However, P-glycoprotein is not especially high in fetal hepatocytes [6]. Using agents known to maintain a differentiated state of hepatocytes in culture, such as dimethylsulfoxide or nicotinamide, Fardel *et al.* [25] were able to delay the overexpression of P-glycoprotein without abolishing it. However, they obtained the same result with agents stimulating cell proliferation, such as pyruvate or epidermal growth factor. It is therefore difficult to attribute the progressive increase of P-glycoprotein levels during hepatocyte *in vitro* culture to the loss of differentiation or to the absence of cell proliferation. In a tumor model originating from human colon carcinoma cells, which expressed P-glycoprotein, differentiating agents are able to increase P-glycoprotein expression up to 25-fold [26]. However, this increase is accompanied by an inhibition of P-glycoprotein phosphorylation, which impairs its function selectively toward certain drugs: then, a given amount of P-glycoprotein cannot predict for drug efflux. In the hepatocyte model in contrast, the variations of P-glycoprotein levels with time are correlated with its function as doxorubicin transporter. We have shown that P-glycoprotein expression was enhanced when cells were cultivated at low density than at normal density. Pelletier *et al.* [27] and Dimanche-Boitrel *et al.* [28] have described another type of drug resistance, which is maximum at cell confluence in culture, which they have called confluence-dependent resistance. This was shown on tumor cell in culture originating from colon cancers, especially the HT29 line which does not express P-glycoprotein. Confluence-dependent resistance is accompanied by a decrease in drug accumulation (anthracyclines, vincristine, etoposide), but it may also concern other molecules such as ions, nucleotides and oses [29]. It has been attributed to a decrease of passive influx of drugs and metabolites into confluent cells due to changes in plasma membrane fluidity. This may not be related to our observation, which is most probably in relation to changes in P-glycoprotein expression. The variation of P-glycoprotein in function of duration of culture and cell density at seeding may be the source of discrepancy in toxicity and metabolism studies *in vitro* of drugs effluxed from the cells by P-gp.

Acknowledgements—The authors wish to thank Dr Jacques Robert, Fondation Bergonié, Bordeaux, for critically reading the manuscript and Mme Nicole Hourmant for her technical assistance. This work was supported by grant No. 6324 from ARC.

Service de Pharmacologie CHU, 29609 Brest Cedex France

MARIE-ANNICK LE BOT,* HELENE SWIRSKY-SIMON, DOMINIQUE KERNALEGUEN, CHRISTIAN RICHE

REFERENCES

1. Bradley G, Juranka PF, Ling V, Mechanism of multidrug resistance. *Biochim Biophys Acta* **948**: 87–128, 1988.
2. Pastan I, Gottesman MM, Multiple-drug resistance in human cancer. *N Engl J Med* **316**: 1388–1393, 1987.
3. Thiebaut P, Tsuruo T, Hamada H, Gottesman HH, Pastan I, Willingham MC, Cellular localization of the multidrug-resistance gene product P glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* **84**: 7735–7738, 1987.
4. Kamimoto Y, Gatmaitan Z, Hsu J, Arias IM, The function of Gp 170, the multidrug resistance gene product in rat liver canalicular membrane vesicles. *J Biol Chem* **264**: 11693–11698, 1989.
5. Fardel O, Ratanasavanh D, Loyer P, Ketterer B, Guil-

* Corresponding author: Dr M.-A. Le Bot, Service de Pharmacologie, CHU, 29609 Brest Cedex, France. Tel.(33) 98 22 33 10; FAX (33) 98 31 64 74.

louzo A, Overexpression of the multidrug resistance gene product in adult rat hepatocytes during primary culture. *Eur J Biochem* **205**: 847–852, 1992.
6. Thorgeirsson SS, Huber BE, Sonel S, Fojo A, Pastan I, Gottesman MM, Expression of the multidrug resistance gene in hepatocarcinogenesis and regenerating rat liver. *Science* **236**: 1120–1122, 1987.
7. Gottesman MM, Multidrug-resistance during chemical carcinogenesis: a mechanism revealed?. *J Natl Cancer Inst* **80**: 1352–1353, 1988.
8. Volm M, Zerban H, Mattern J, Efferth TH, Overexpression of P-glycoprotein in rat hepatocellular carcinomas induced with *N*-nitrosomorpholine. *Carcinogenesis* **11**: 169–172, 1990.
9. Volm M, Efferth T, Mattern J, Pommerenke EW, Resistance mechanisms in murine tumors with acquired multidrug resistance. *Drug Res* **42**: 1163–1168, 1992.
10. Paine AJ, Villa P, Ligands maintain cytochrome P 450 in liver cell culture affecting its synthesis and degradation. *Biochem Biophys Res Commun* **97**: 744–750, 1980.
11. Paine AJ, Lesley JH, Allen CM, Long term maintenance and induction of cytochrome P-450 in rat liver cell culture. *Biochem Pharmacol* **31**: 1175–1178, 1982.
12. Croci T, Williams GM, Activities of several phase I and phase II xenobiotics biotransformations enzymes in cultured hepatocytes from male and female rats. *Biochem Pharmacol* **34**: 3029–3035, 1985.
13. Vandenberghe Y, Ratanasavanh D, Glaise D, Guillouzo A, Influence of medium composition and culture conditions on glutathione S-transferase activity in adult rat hepatocytes during culture. *In Vitro Cell Dev Biol* **24**: 281–288, 1988.
14. Guguen C, Guillouzo A, Boisnard M, Le Cam A, Bourel M, Etude ultrastructurale de monocouches d'hépatocytes de rat adulte cultivés en présence d'hémisuccinate d'hydrocortisone. *Biol Gastroenterol* **8**: 223–231, 1975.
15. Kartner N, Evernden-Porell D, Bradley G, Ling V, Monoclonal antibodies detecting P glycoprotein in multidrug resistance cell line. *Nature* **316**: 820–823, 1985.
16. Laemmli UK, Change of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
17. Towbin H, Staehelin T, Gordon J, Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci* **76**: 4350–4354, 1979.
18. Benchekroun MN, Pourquier P, Schott B, Robert J, Doxorubicin-induced lipid peroxydation and glutathione-peroxidase activity in tumor cell lines selected for resistance to doxorubicin. *Eur J Biochem* **211**: 141–146, 1993.
19. Baurain R, Deprez-De Campaneere D, Trouet A, Determination of daunorubicin, doxorubicin and their fluorescent metabolites by high-pressure chromatography: plasma levels in DBA2 mice. *Cancer Chemother Pharmacol* **2**: 11–14, 1979.
20. Israel M, Pegg WJ, Wilkinson PM, Garnick MB, Liquid chromatography analysis of adriamycin and metabolites in biological fluids. *J Liquid Chromatogr* **1**: 795–809, 1978.
21. Chin KV, Tanaka S, Darlington G, Pastan I, Gottesman MM, Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cell. *J Biol Chem* **265**: 221–226, 1990.
22. Guguen-Guillouzo C, Tichonicky L, Szajnert MF, Shapira F, Kruh J, Changes of some chromatin and cytoplasmic enzymes in primary culture of adult rat hepatocytes. *Biol Cell* **31**: 225–234, 1978.
23. Pariza MW, Yager JD Jr, Goldfard S, Gurr JA, Yanagi S, Grossman SH, Berber JE, Borber TA, Petter VR, Biochemical autoradiographic and electron microscopic studies of adult rat liver parenchymal cells in primary culture. In: *Gene Expression and Carcinogenesis in Culture Liver* (Eds. Gershenson LE and Thomson EB), pp. 137–167. Academic Press, New York, 1975.
24. Guguen Guillouzo C, Gregori C, Schapira F, Modification of pyruvate kinase isoenzymes in prolonged cultures of adult rat hepatocytes. *Biochimie* **9**: 1065–1071, 1975.
25. Fardel O, Loyer P, Morel F, Ratanasavanh D, Guillouzo A, Modulation of multidrug resistance gene expression in rat hepatocytes maintained under various culture conditions. *Biochem Pharmacol* **44**: 2259–2262, 1992.
26. Bates SE, Currier SJ, Alvarez M, Fojo AT, Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate. *Biochemistry* **31**: 6366–6372, 1992.
27. Pelletier H, Millot JM, Chauffert B, Marfait M, Genne P, Martin F, Mechanisms of resistance of confluent human and rat colon cancer cells to anthracyclines: alteration of drug passive diffusion. *Cancer Res* **50**: 6626–6631, 1990.
28. Dimanche-Boitrel MT, Pelletier H, Genne P, Petit JM, Le Grimmellec C, Canal Pierre, Ardiet C, Bastian G, Chauffert B, Confluence-dependent resistance in human colon cancer cells: role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells. *Int J Cancer* **50**: 677–682, 1992.
29. Epifanova OI, Differential sensitivity of proliferating and resting cells to anti-tumor drugs and its probable causes. In: *Regulation and Control of Cell Proliferation* (Eds. Lapis K and Jeney A), pp. 434–457. Akademia Kiado, Budapest, 1984.