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ROMAN PADUCH[1], MARIA SŁOTWIŃSKA[2],
ANNA STACHURA[2], WOJCIECH RZESKI[1], BARBARA ZDZISIŃSKA[1],
MARTYNA KANDEFER-SZERSZEŃ[1]

[1]Department of Virology and Immunology
[2]Department of Cell Biology
Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin

Comparative analysis of different methodological approaches to the *in vitro* study of tumour cells chemosensitivity

Analiza porównawcza różnych metod badania wrażliwości komórek nowotworowych *in vitro* na chemioterapeutyki

SUMMARY

Drug sensitivity assay was performed using two human tumour cell lines: HeLa and Hep-2 cultivated in two-dimensional monolayer cell cultures and three-dimensional cultures on gelatine sponge Spongostan^R. Two cytostatics with different mechanisms of anti-tumour action were used: cisplatin and etoposide. Chemosensitivity of tumour cells was assessed by counting the number of viable and nonviable cells (cytostatic and cytotoxic activity of drugs), by counting the number of apoptotic cells and by clonogenic assay of viable cells.

We found that the clonogenic assay was more sensitive than the other tests used, especially after long-term (7 days) treatment of tumour cells with cytostatics. A short (24h) treatment with cytostatics gave false results which were not confirmed after prolonged treatment with cytostatics. We suppose that short treatment tests should not be used for examination of the chemosensitivity of tumour cells isolated from patients. Tumour cells growing on Spongostan^R were viable for a longer time than in monolayer cultures and exhibited chemosensitivity comparable to monolayer cell cultures despite of their multilayer growth on gelatine sponge.

STRESZCZENIE

Do badań wrażliwości na cytostatyki użyto dwie ludzkie linie nowotworowe: HeLa i Hep-2, hodowane w formie murawy dwuwymiarowej (płaskiej) oraz w formie przestrzennej, trójwymiarowej, na gąbce żelatynowej Spongostan^R. W badaniach użyto cytostatyki posiadające różny mechanizm działania przeciwnowotworowego, a mianowicie cisplatynę i etopozyd. Wrażliwość komórek

nowotworowych na chemioterapeutyki określano ilością komórek przeżywających i martwych (cytostatyczne i cytotoksyczne właściwości leków), ilością komórek apoptotycznych oraz oceniano klonogenne właściwości komórek przeżywających. Stwierdzono, że ocena klonogennych właściwości komórek jest metodą bardziej czułą w porównaniu z innymi testami, szczególnie po długim (7-dniowym) kontakcie komórek z cytostatykami. Ocena krótkotrwałego (24-godz.) kontaktu komórek nowotworowych z cytostatykami dawała fałszywe wyniki, nie potwierdzone po długotrwałej inkubacji komórek z cytostatykami. Uważamy, że testy polegające na ocenie efektu krótkotrwałej inkubacji komórek z lekami nie powinny być stosowane do oceny wrażliwości na chemioterapeutyki komórek nowotworowych izolowanych od pacjenta. Komórki nowotworowe rosnące na Spongostanie^R były żywe dłużej niż rosnące w hodowlach płaskich i pomimo wielowarstwowego wzrostu na gąbce żelatynowej wykazywały wrażliwość na leki porównywalną z hodowlami płaskimi.

Key words: cisplatin, etoposide, tumour cells, chemosensitivity.

INTRODUCTION

For effective cancer chemotherapy, chemosensitivity testing of anticancer drugs should be performed using fresh surgical specimens obtained from the cancer. Several chemosensitivity *in vivo* and *in vitro* tests have been developed (1, 2, 4, 6, 17, 19, 21, 24). *In vitro* human tumour clonogenic assay (HTCA), thymidine incorporation assay (TIA), succinic dehydrogenase inhibition assay (SDIA) and MTT assay were widely used (3, 7, 11, 13, 16, 18, 22, 23). All of these tests were developed for primary cultures of tumour cells cultivated on flat plastic plates (two-dimensional cultures). The collagen gel matrix method was also reported as a three-dimensional culture method by Freeman and Hoffman (5). They cultured ultra-thin sections of tumour tissues on a gelatine sponge as a histoculture which allowed a favourable growth of tumour cells in long-term cultures. Hoffman et al. (10) and Furukawa (7) established a clinically useful histoculture drug response assay (HDRA), using this culture method. However, the HDRA required higher concentrations of the drugs in comparison to those observed *in vivo*. Multicellular spheroids (MTCS) have been also used as an *in vitro* model system of solid tumours (9, 14). It allows scientists to study gradients of oxygen tension, nutrients and drug concentrations and also the interactions of tumour cells with normal tissues (20). Cells isolated from MTCS are generally more resistant to cytotoxic drugs than the same cells grown as monolayer. Intracellular communication via gap junctions, specific DNA packaging causing increased repair in spheroids, specific microenvironment which may affect the activity of cytotoxic compounds by reducing the proliferation rate of tumour cells, low pH values, oxygen deprivation are the reason of high resistance of tumour cells to cytotoxic drugs. However, three-dimensional histocultures, the sandwich culture and multicellular spheroids are models that should reflect heterogeneity of solid tumours *in vivo* and can led to establish the most appropriate drug test profile (15, 20).

Recently other three-dimensional method has been developed for testing of tumour cells chemosensitivity in collagen droplet embedded cultures (CD-DST) (12). In this system *in vitro* the sensitivity to various anticancer drugs was similar to the clinical response rate of treated patients. However, this method is technically difficult and time consuming, as the growth or its inhibition of several tumour cells colonies in collagen droplets should be measured.

Gelatine sponges are widely used as scaffolds for cultivation of normal cells in tissue engineering methods and are also useful for cultivation of tumour cells (25). Therefore, we used pig skin gelatine sponge- Spongostan^R for cultivation of tumour cells in three-dimensional cultures. In this study we compared the *in vitro* chemosensitivity to cisplatin and etoposide of two human tumour cells HeLa and Hep-2 cultivated in two-dimensional scaffold-plastic plates and three-dimensional gelatine sponge. To assess tumour cells chemosensitivity we measured antiproliferative and cytotoxic effects of drugs (decrease in viable and increase in nonviable cell number), induction of cell apoptosis and anti-clonogenic activity (decrease in the number of colonies formed by drug-treated tumour cells).

MATERIAL AND METHODS

Reagents

Etoposide (VP-16) and cisplatin (CDDP) were purchased by Sigma St. Louis MO. The compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C. Further dilutions were made in cell culture media immediately before use. Sterile pig skin collagen Spongostan^R, Special was purchased from Ferrosan, Denmark.

Tumour cell cultures

Human cervix carcinoma tumour cell line HeLa (ECACC No 85060701) and Hep-2 human larynx carcinoma (ECACC No 86030501) were grown in RPMI 1640 medium, Eagle's MEM (1 : 1), supplemented with 5% foetal calf serum (FCS), 100 M/ml of penicillin, 100 μg/ml of streptomycin in plastic 75 cm^2 flasks (Costar, Cambridge MA), trypsinized and transferred at a density of 1×10^6 cells/ml (24 h cultures) or 6×10^4 cells/ml (4–7 days cultures) into 6-well plastic plates coated with Spongostan^R (diluted in 0.1% acetic acid and neutralized with 7.6% $NaHCO_3$) and cultivated in an incubator at 37°C. Cells were also transferred into siliconized tubes with pieces of Spongostan^R (0.11 cm^3 wet disc volume in culture medium) and incubated at 37°C in gyratory water bath (100 rpm).

In vitro chemosensitivity test

In 24 h cultures of tumour cells the medium was changed to the new one supplemented with cytostatics and incubated for additional 24 h or 4 and 7 days. In long-term cultures (4 and 7 days) the medium was changed on the 3rd and 5th day of incubation. After that the cells were

trypsinized, the total number of the cells was estimated by counting in haemocytometer, and the number of viable cells was counted using trypan blue exclusion test. The cytostatic effect of drugs was also assessed by clonogenic assay (plating efficiency). Trypsinized cells were washed with the medium supplemented with 10% FCS, diluted in the medium with 0.33% agar to the density of 3×10^2 cells/ml and plated in 6-well plastic plates. Seven days later cell colonies were fixed with ethanol, stained with crystal violet and counted under inverted microscope.

Chromatin condensation and fragmentation or formation of apoptotic bodies in cells treated with cytostatics was also estimated. The cells after treatment with cytostatics were trypsinized, washed with phosphate buffered salt solution (PBS), fixed (acetic acid: methanol 1 : 3 v/v) and centrifuged. The cell pellet was placed on microscope slide, dried in air and stained with hematoxylin-eosin (10% solution of hematoxylin, 0.25% solution of eosin in ethanol). Cells were scored under the microscope for apoptotic morphology.

RESULTS AND DISCUSSION

For our *in vitro* model of chemosensitivity assay two different types of human tumour cell lines: HeLa and Hep-2 were chosen and also two anticancer drugs differed in the mechanism of their antitumour activity: cisplatin and etoposide. Cisplatin (cis-diammino-dichloroplatinum) and its derivatives are among the most widely used powerful anticancer drugs. They are used alone or in combination regiments to treat many types of human malignancies, including testicular and ovarian cancers, tumour of the bladder, head and neck and some lung cancers. Cisplatin biological activity is a consequence of covalent adducts formation between the platinum compound and certain bases in DNA (26). Etoposide (4'dimethyl--epipodophyllotoxin-9-(4,6-O ethylidene-beta-D-glukopyranoside, VP-16) is also an important antineoplastic agent used against several tumour types either alone or in combination therapy. The cytotoxicity of VP-16 is generally based on induction of DNA damage by drug interference with breakage-reunion reaction of DNA-topoisomerase II (inhibitor of topoisomerase II) (8).

The chemosensitivity of tumour cells was assessed by the counting of the total number of cells, the number of dead cells, by counting cells undergoing apoptosis and the number of colonies growing from cells treated with cytostatics (clonogenic assay). Moreover, the tumour cells were grown and treated with cytostatics in two--dimensional and three-dimensional culture conditions. The results are presented in the figures. As can be seen from Figure 1A and B, Figure 1E and F, HeLa cells were more sensitive to etoposide than cisplatin, especially when apoptosis of HeLa cells and their clonogenic activity (plating efficiency) was measured. Also Hep-2 cells were more sensitive to etoposide than cisplatin, especially when apoptosis of the cells was examined. Another method used did not show any difference in the chemosensitivity of Hep-2 cells to cisplatin. No significant differences in chemosensitivity of HeLa and Hep-2 cells cultivated in two-dimensional cultures in comparison to that in three-dimensional ones were observed.

For further experiments lower concentrations of cytostatics were chosen. As can be seen from Figure 2 treatment of tumour cells for 4 days caused mainly inhibition of their proliferation and clonogenic activity, while the number of apoptotic cells was low. The most sensitive method was clonogenic assay.

In contrast to 24-hrs test, 4-day test exhibited that HeLa cells were more sensitive to cisplatin than to etoposide, especially when clonogenic activity was measured and more sensitive to cisplatin than Hep-2 cells. No significant influence of the cell culture conditions on chemosensitivity of cells was observed.

When the time of incubation was extended to 7 days (Figure 3), the clonogenic properties of HeLa cells treated with cisplatin and etoposide decreased significantly and the number of apoptotic cells increased, especially in three-dimensional cultures. Hep-2 cells were still more resistant than HeLa cells to cisplatin and etoposide, and both cytostatics only partially inhibited the clonogenic activity of Hep-2 cells.

Summing up, HeLa and Hep-2 cells cultivated on Spongostan^R in three--dimensional culture conditions generally exhibited chemosensitivity comparable to cells cultivated as monolayer in two-dimensional cultures. In contrast to a high resistance of tumour cells cultivated as spheroids (9), cells cultivated on sponge scaffold — Spongostan^R, did not exhibit enhanced resistance to cytostatics. The results of our study strongly suggest that a short (24 hrs) treatment of tumour cells with cytostatics should not be used for examination of chemosensitivity of cells isolated from patients, as the results obtained after a short treatment differed significantly from those obtained after a long one, and the high chemosensitivity of HeLa cells to etoposide after 24 h treatment was not confirmed when the cells were treated with cytostatics for a longer period of time. The most sensitive method of those used in our study was clonogenic assay.

Gelatine sponge was already used by Freeman et al. (5, 10) for cultivation of ultrathin sections of tumour tissues and allowed a favourable growth of the tumour cells in long-term cultures. We confirmed these observations that tumour cells can be grown on Spongostan^R for 7 days without any significant decrease in their viability (data not shown). Moreover, we observed that tumour cells formed multilayer cultures on Spongostan^R with more close cell-cell interactions, which however, did not enhance their resistance to antitumour drugs.

Two technological problems limit the usefulness of our model of the chemosensitivity assay: requirement for a large number of tumour cells and a long duration of the clonogenic assay. In future we intend to develop a miniaturized chemosensitivity assay and to improve and shorten the clonogenic assay.

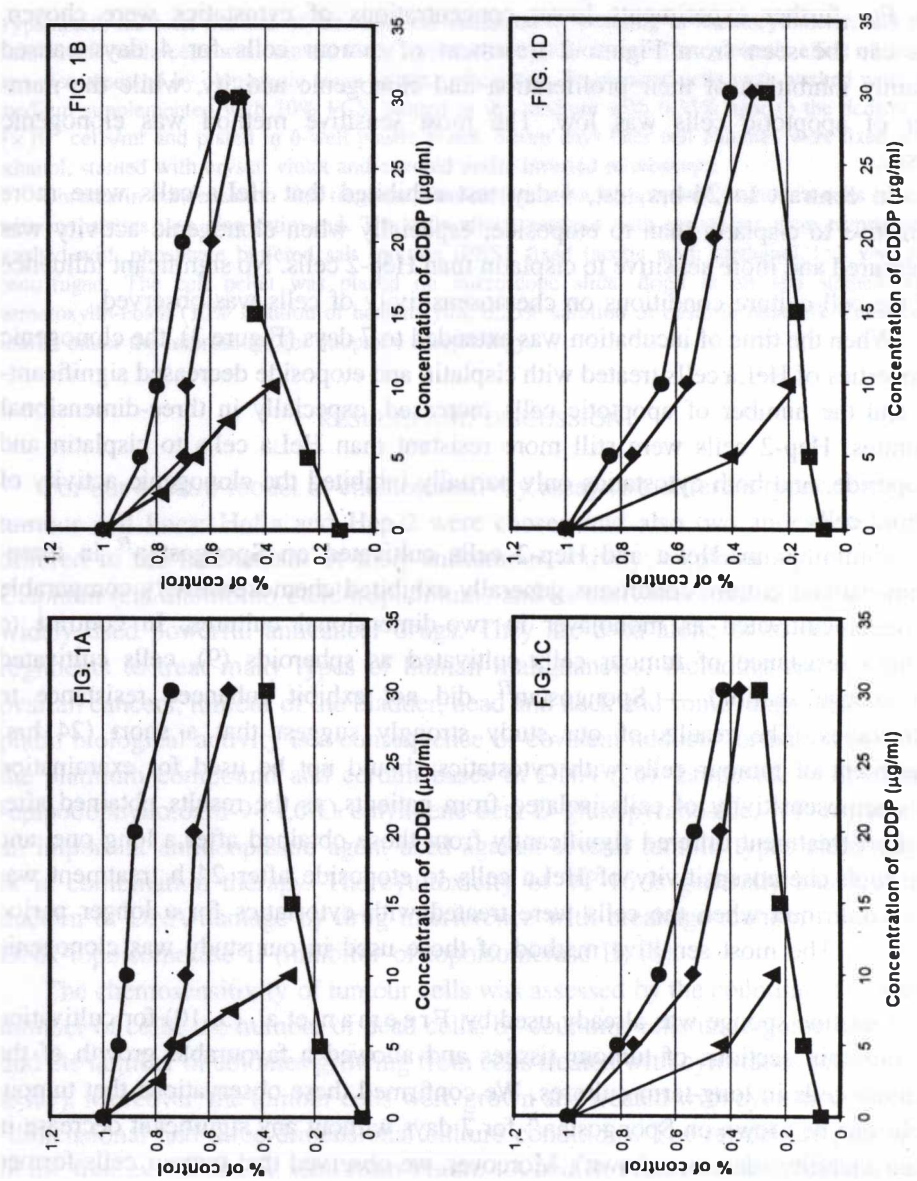
FIG. 1B
FIG. 1D
FIG. 1A
FIG.1C
% of control
Concentration of CDDP (µg/ml)
0
0,2
0,4
0,6
0,8
1
1,2
0
5
10
15
20
25
30
35

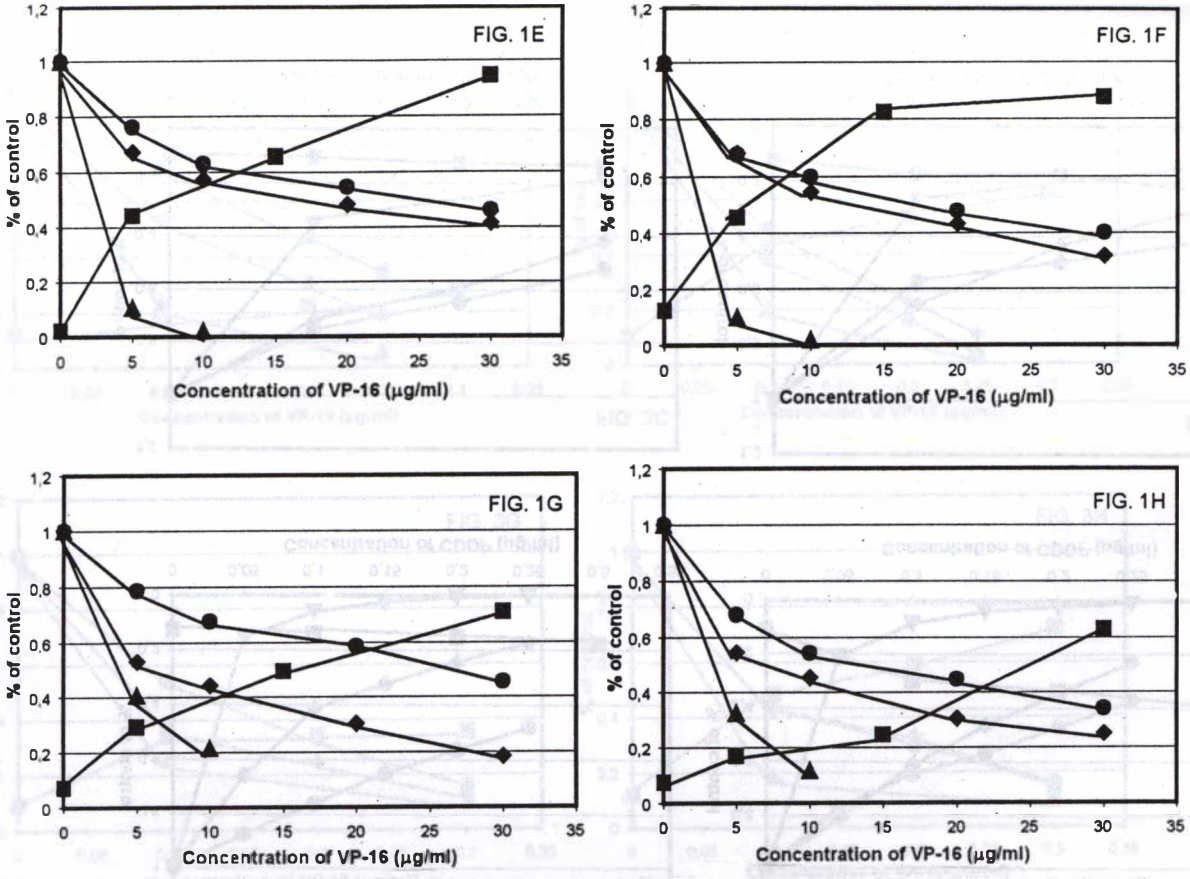

Fig. 1. The effect of 24h-treatment with cisplatin (CDDP) of HeLa cells cultured in two-dimensional (2D) model (**A**), in three-dimensional (3D) model (**B**), Hep-2 cells cultured in two-dimensional (2D) model (**C**) and three-dimensional (3D) model (**D**)
The effect of 24h treatment with etoposide (VP-16) of HeLa cells cultured in two-dimensional (2D) model (**E**), in three-dimensional (3D) model (**F**), Hep-2 cells cultured in two-dimensional (2D) model (**G**) and in three-dimensional (3D) model (**H**); • — cell number, ♦ — cell viability, ▲ — clonogenic activity (plating efficiency), ■ — apoptosis

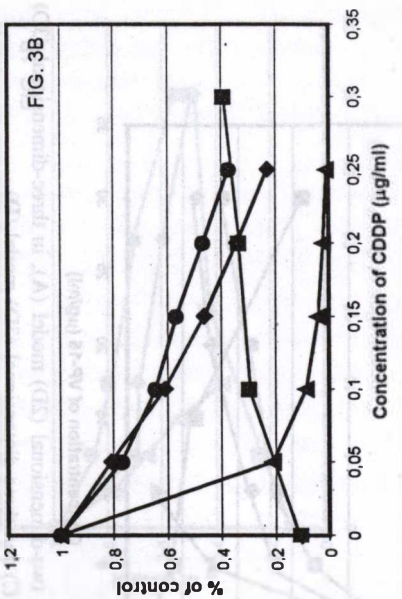
FIG. 3B
% of control
Concentration of CDDP (µg/ml)

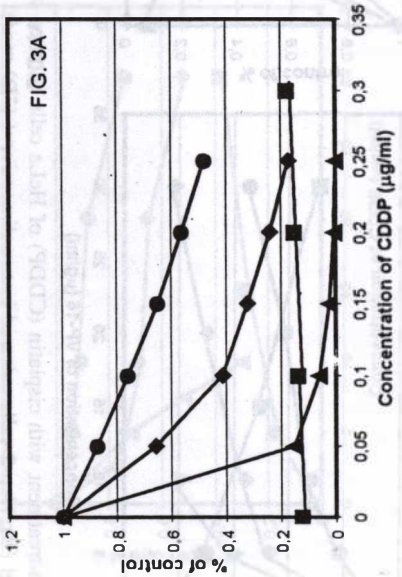
FIG. 3A
% of control
Concentration of CDDP (µg/ml)

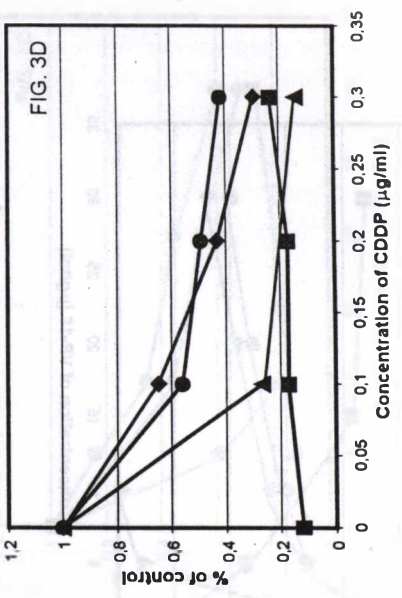
FIG. 3D
% of control
Concentration of CDDP (µg/ml)

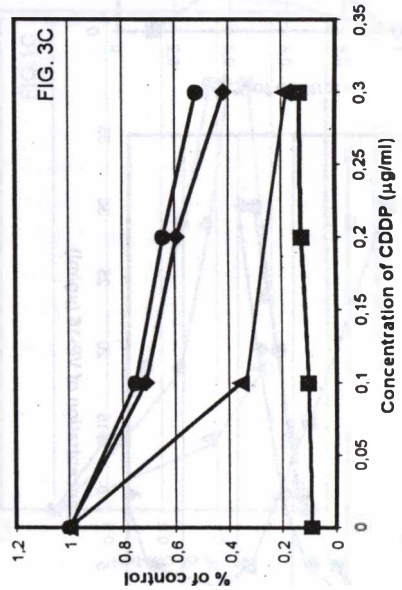
FIG. 3C
% of control
Concentration of CDDP (µg/ml)

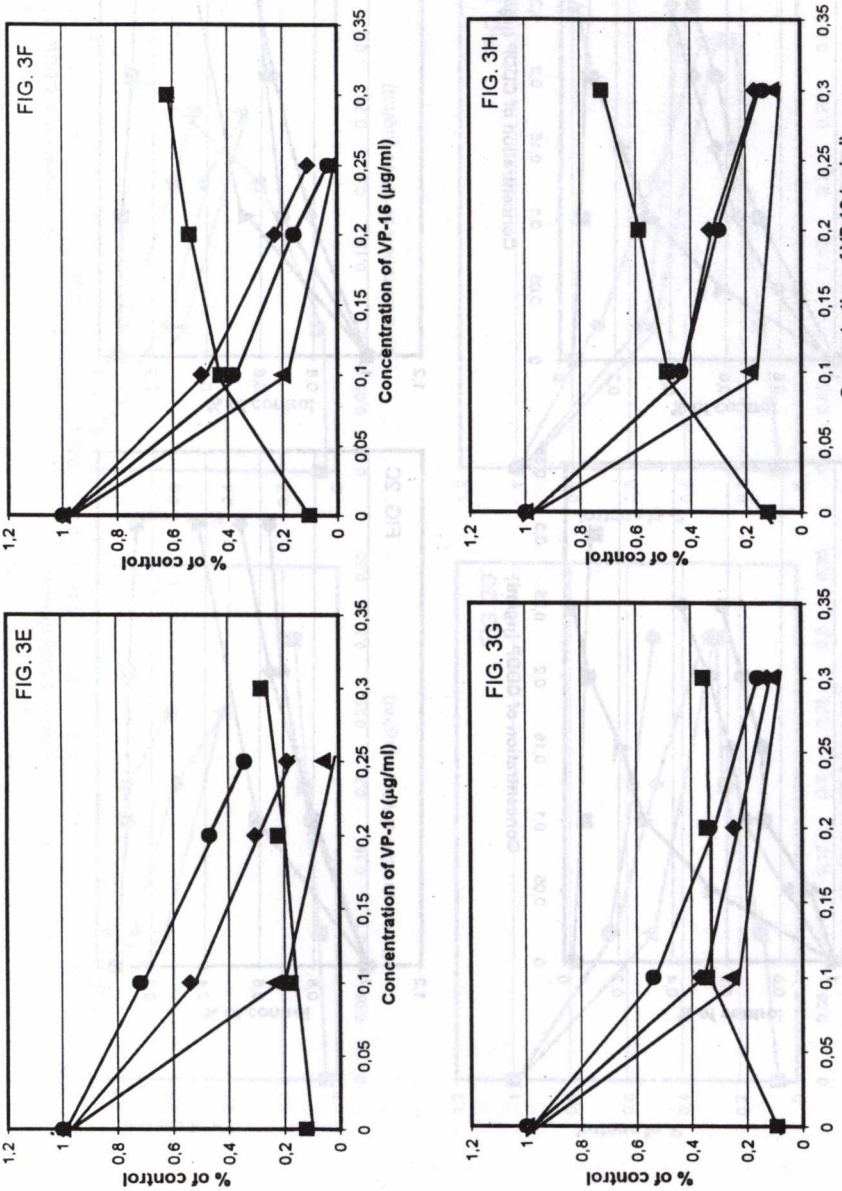

Fig. 2. The effect of 4-day treatment with cisplatin (CDDP) of HeLa cells cultured in two-dimensional (2D) model (**A**), in three-dimensional (3D) model (**B**), Hep-2 cells cultured in two-dimensional (2D) model (**C**) and three-dimensional (3D) model (**D**)
The effect of 4-day treatment with etoposide (VP-16) of HeLa cells cultured in two-dimensional (2D) model (**E**), in three-dimensional (3D) model (**F**), Hep-2 cells cultured in two-dimensional (2D) model (**G**) and in three-dimensional (3D) model (**H**)

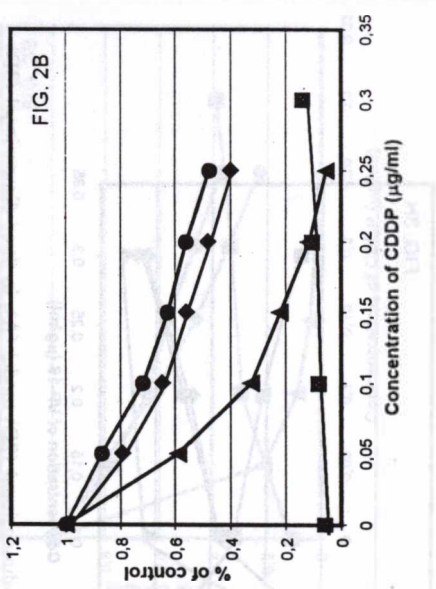
FIG. 2B
% of control
Concentration of CDDP (µg/ml)
0
0,2
0,4
0,6
0,8
1
1,2
0,05
0,1
0,15
0,2
0,25
0,3
0,35

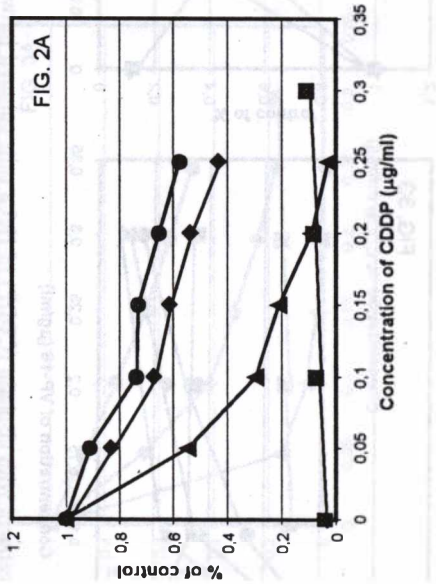
FIG. 2A
% of control
Concentration of CDDP (µg/ml)
0
0,2
0,4
0,6
0,8
1
1,2
0,05
0,1
0,15
0,2
0,25
0,3
0,35

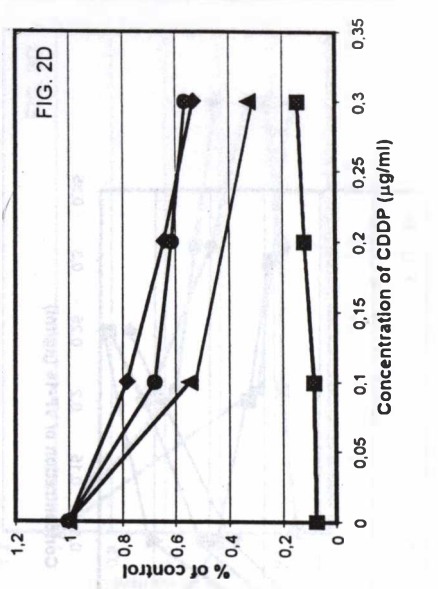
FIG. 2D
% of control
Concentration of CDDP (µg/ml)
0
0,2
0,4
0,6
0,8
1
1,2
0,05
0,1
0,15
0,2
0,25
0,3
0,35

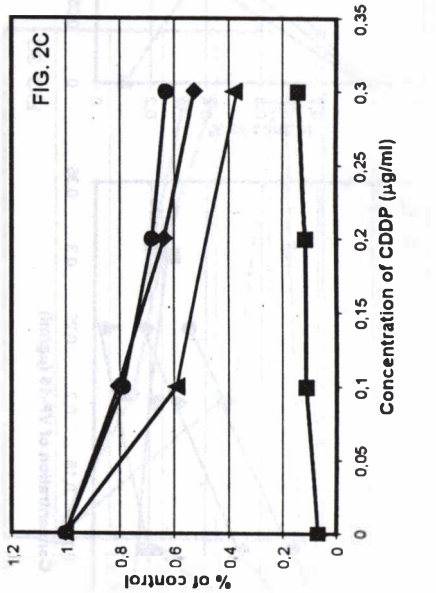
FIG. 2C
% of control
Concentration of CDDP (µg/ml)
0
0,2
0,4
0,6
0,8
1
1,2
0,05
0,1
0,15
0,2
0,25
0,3
0,35

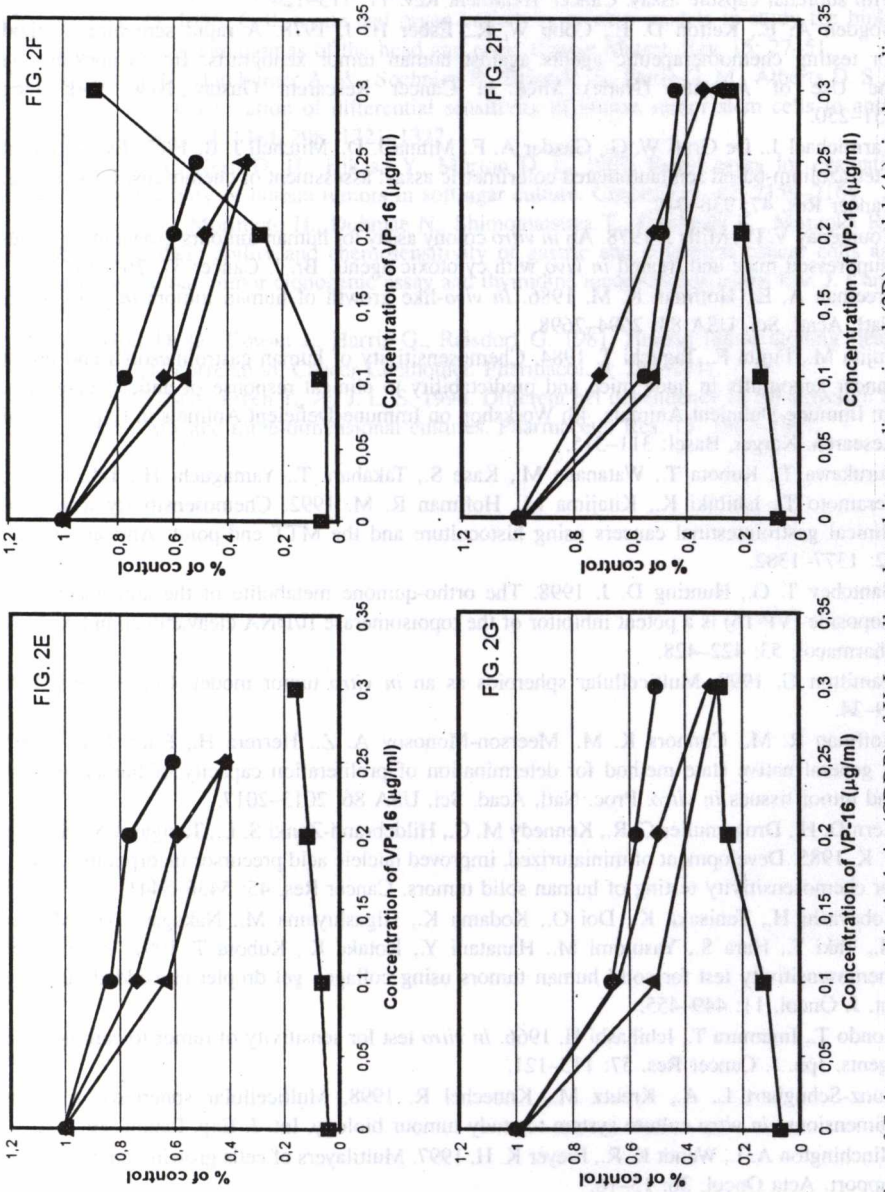

Fig.3 The effect of 7-day treatment with cisplatin (CDDP) of HeLa cells cultured in two-dimensional (2D) model (**A**), in three-dimensional (3D) model (**B**), Hep-2 cells cultured in two-dimensional (2D) model (**C**) and three-dimensional (3D) model (**D**)
The effect of 7-day treatment with etoposide (VP-16) of HeLa cells cultured in two-dimensional (2D) model (**E**), in three-dimensional (3D) model (**F**), Hep-2 cells cultured in two-dimensional (2D) model (**G**) and in three-dimensional (3D) model (**H**)

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