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Research Article

COMPARATIVE EVALUATION OF MULTIDRUG RESISTANCE AND APOPTOSIS IN DIFFERENT VARIANTS OF HEPATOMA CELLS

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ABSTRACT. Multidrug Resistance is a major obstacle in cancer chemotherapy. The current study was designed to evaluate the multidrug resistance and apoptotic properties of Hepatoma cells. Antiproliferative effect of five anti-cancer drugs: three of bacterial origin namely, puromycin, actinomycin D, doxorubicin and two plant derived drugs viz., colchicine and vinblastine were assessed on heat- resistant variants of dexamethasone–resistant and the dexamethasone- sensitive variants of hepatoma cell lines. These variants showed increased drug resistance to the different anticancer drugs used, i.e., they became moderately multidrug-resistant. The severely heat-treated H56 cells became moderately resistant only to certain drugs. All the experimental variants of Hepatoma cells overexpressed functional P-glycoprotein, a drug-resistant associated marker protein, which attributes to the resistance shown by these cells. The anticancer drugs were capable of inducing apoptosis in both H56 and clone 2 cells and it was found that H56 cells were more prone to undergo apoptosis as compared to clone 2 cells. Furthermore, preliminary immunocytochemical studies revealed that there was a significant difference in expression of p53, a tumor suppressor gene, in H56 and clone 2 cells where H56 cells showed very strong staining for p53 thereby justifying their proneness to apoptosis.

Keywords: *Apoptosis, clone 2 cells, H56 cell lines, hepatoma cells, P-glycoprotein, multidrug resistance.*

INTRODUCTION

Malignant cells have the property of acquiring resistance to a broad spectrum of clinical anticancer drugs, a variety of unrelated cytotoxic drugs and other lipophilic compounds when they are exposed to a chemotherapeutic agent. This phenomenon of a lack of sensitivity in malignant tissue to different anticancer drugs [1] is called multidrug resistance (MDR) [2, 3]. MDR is one of the major challenging aspects of cancer chemotherapy which ultimately results in failure of the treatment [4, 5]. MDR occurs in a short period of time during or after treatment, and can result in cross resistance to numerous mechanically and structurally different chemotherapeutics[2, 6]. Many different mechanisms are ascribed for MDR which include (a) ATP-binding cassette (ABC) transporters that pump out chemotherapeutics [7], (b) mutation of oncogenes that become resistant to various drugs and different treatments [8, 9], (c) an evolving

adaptation of cancer cells to the microenvironment [10, 11], (d) survived cancer stem cells (CSCs) that escape from conventional therapies [12, 13], and (e) activated cell growth factors as well as cell defense systems, and so forth.

The molecular basis of a major form of MDR commonly involves overexpression of P-glycoprotein (P-gp), a plasma transmembrane protein of the ABC transporter family which functions as an ATP-dependent efflux pump for several structurally and functionally untreated cytotoxic drugs [1, 14, 15, 16, 17, 18]. P-gp, discovered 40 years ago, was the first member of the ABC transporter family found in the plasma membrane of cancerous hamster ovary cells [19]. P-gp has ATPase activity [20] and is responsible for the ATP dependent drug efflux activity of drug-resistant neoplastic cells [21, 22]. Several anticancer drugs playing a central role in chemotherapeutic treatment are vulnerable to this P-gp-mediated efflux. P-gp is the first identified and most characterized MDR transporter and has long been recognized as a viable target to overcome MDR in cancer [23].

In oncology, hyperthermia is a strategy used to treat tumors where an external heat source increases tissue temperature and kills malignant cells or impede their further growth [24, 25]. Hyperthermia has been used as an efficient complement to standard cancer treatments like surgery, radiotherapy, chemotherapy, and gene and immunotherapy [26, 27]. Heat may likewise potentiate the cytotoxic effects of certain chemotherapeutic drugs [28, 29, 30, 31].

Sometimes, cells that have been pre-exposed to thermal stress i.e., lethal temperatures of 43-45°C for short periods of time of about 10 to 30 minutes, can acquire a transient resistance to the killing effect of a subsequent heat stress. This induced transient resistance, distinct from the stable genetic heat resistance, is called thermotolerance [29], [32, 33, 34]. This thermotolerance triggered by heat preconditioning whereby cells become resistant to a subsequent lethal insult, is an adaptive survival response [35, 36]. Development of thermotolerance in tumor cells interferes with the efficacy of hyperthermic treatment, restricts the direct anti-tumour effects of thermal therapy [37] and results in an enhanced resistance to some anticancer drugs [29].

The circumvention of resistance to chemotherapy has become one of the primary and essential objectives of modern approaches to deal with cancer therapy. Several chemotherapeutic drugs kill drug-sensitive cells by apoptosis [38]. Physiological or apoptotic cell death is an active, genetically programmed self-destructive or suicidal process that consists of a cascade of well-regulated events with the involvement and participation of specific genes [39]. The three main stages of programmed cell death are signalization, control and execution [40]. Disturbance of any these stages may result in the failure of the apoptotic program.

In some cases, cancer cells make more death suppressing proteins than normal cells. In other cases, cancer cells deactivate the gene p53, a tumor suppressor gene, which promotes cell death. The p53 gene called as the guardian of the genome helps organism to cope with DNA damage by either stalling cell division or inducing cell death. When mutations accumulate in a cell's DNA they can activate or deactivate specific genes, causing the cell to behave in disruptive manner. Mutations in p53 gene are the most common genetic defect in human cancers, occurring in more than half of all cases [38]. If normal p53 is present any damage may be repaired before progression through the cell cycle and so these cells may appear resistant.

Resistance to apoptosis has emerged as an important category of treatment resistance [41]. The possibility of manipulation of apoptotic cell death in order to intervene

therapeutically in cancer, where failure or suppression of apoptosis is likely to contribute both to the initial development of cancer and to the appearance of tumor cells resistant to chemotherapy, is the central focus of research in recent times. The different cell types vary profoundly in their susceptibility to apoptosis induction and it is currently unclear what determines the distinct cellular thresholds for apoptosis induction. Mild thermotolerance developed at 40°C is generally associated with the accumulation of heat shock proteins (HSPs) and resulted in an apoptosis-resistant phenotype [35], [42, 43, 44, 45, 46]. HSPs play a significant yet complex role in the regulation of apoptosis. The activation of the mitochondrial pathway of apoptosis by moderate hyperthermia (42 to 43 °C) was attenuated in thermotolerant cells [47]. Stable, heat- resistant rat hepatoma variants have been isolated by using repeated heating cycles at 45 °C [48] and these heat-resistant variants showed increased drug resistance to several anticancer medications; they became moderately multidrug-resistant and expressed a slightly elevated level of P-gp [49]. Thus, the present work aims to examine whether heat-treatment will result in increased drug resistance in H56, another hepatoma cell clone. Furthermore, induction of apoptosis induced by different drugs in H56 cells and a preliminary analysis of p53 expression was also studied.

MATERIALS AND METHODS

Cell lines, Media and Culture conditions

Table 1. *Details of cell lines used and their properties*

Cell Lines	Properties
Faza 967	A well-differentiated descendent of H4IIEC3 Reuber rat hepatoma cells [50, 51, 52].
Clone 2	Glucocorticoid (dexamethasone)-resistant, dedifferentiated cells, isolated from the glucocorticoid sensitive Faza 967 clone by using increasing concentrations of dexamethasone [53].
H56	Dedifferentiated, dexamethasone-sensitive, heat-sensitive rat hepatoma cells isolated from H4IIEC3.
S-H56-26; S-H56-106; S-H56-125	Dedifferentiated, dexamethasone-resistant, heat sensitive rat hepatoma cells.
S-H56-26(10X80); S-H56-106 (10x80); S-H56-125 (10x70)	Dedifferentiated, dexamethasone-resistant, heat-resistant rat hepatoma cells.

Heat- resistant variants were isolated by 10 repeated cycles of heat exposure (45 °C for 70, 80 minutes). After each exposure, the surviving cells were regrown at 37 °C [48]. The cells were grown in F12 medium supplemented with 5% fetal calf serum, in a humidified atmosphere of 8% CO_2 in air [53].

Assays:

1. *Cell viability test:* The dose-response curves of the hepatoma cell lines equal number of cells ($5x10^5$) were plated into 35mm petri-dishes. Next day, the cells were treated with different drugs. Number of viable cells was determined by trypan blue exclusion test performed 24, 48 or 72 hours later. The IC_{50} value is the concentration of the drug, which reduced the viability of the treated cells to 50% of that of the control (without drugs).

The values are means of triplicate determinations.

2. *Measurement of intracellular doxorubicin concentration:* Hepatoma cells were exposed to 25 µM doxorubicin for 4 hours in the presence or absence of 25 µM verapamil and cyclosporine A. The intracellular concentration of doxorubicin was determined [54].

3. *Assays for induction of apoptosis:* $2x10^4$ cells (for cytospin) or $1x10^5$ cells (for propidium iodide) were seeded in 35 mm petri-dishes with or without coverslips and one day later treated with different concentration of drugs (vinblastine (30 ng/ml), puromycin (2 ng/ml), colchicine (70 ng/ml), doxorubicin (100-200 ng/ml and actinomycinD (10 ng/ml)). The percentage of cells with morphological characteristics of apoptosis and necrosis was determined on May-Grunwald-Giemsa stained cytospin preparation (Shandon Elliot cytocentrifuge) by counting at least 400 cells. In parallel, after exposure, cells seeded on coverslips were fixed in methanol: water (8:2) for 10 min, washed in PBS, and subsequently stained with propidium iodide (5 µg/ml) for 5 min [55]. Coverslips were mounted onto glass slides in glycerol: HBS (1:1) and examined with a Leitz fluorescence microscope. Nucleus of untreated cells revealed typical chromatin morphology with distinct organization, whereas apoptotic nuclei were highly fluorescent, condensed and displayed polarized chromatin aggregates. To determine the ratio of apoptotic and necrotic cells, a treatment of doxorubicin was given to the variants for 1, 2 or 3 days. The percentage of cells with morphological characteristics of apoptosis was determined on coverslips following propidium iodide staining and May-Grunwald –Giemsa stained cytospin preparation.

RESULTS AND DISCUSSION

The comparative analysis of antiproliferative effect of different anticancer drugs on dexamethasone sensitive H56 cells and dexamethasone-resistant variants (S-H56-26, S-H56-106, S-H56-125) is shown in Fig. 1.

It is seen that the IC50 value for all the 5 anticancer drugs used namely, Puromycin (P), Actinomycin D (A), Vinblastine (V), Colchicine (C) and Doxorubicin (D) was least in case of S-H56-106 i.e., it showed least resistant to the drugs used.

When the antiproliferative effect of different anti-cancer drugs on heat- resistant variants and their corresponding heat sensitive variants (Fig. 2 to 4) was compared it was observed that the heat resistant variants showed moderate resistant for all the drugs used. Amongst the heat resistant insert, after variants S-H56-26 (10x 80) showed resistant against all the drugs used; S-H56-106 (10x80) showed resistance to all drugs except colchicine and S-H56-125 (10x70) showed a slight resistance only for vinblastine and moderate resistance for doxorubicin.

This shows that the heat resistant variants develop multi drug resistance and the degree of resistance varies with the cell type, number of heat cycles and the anti-cancer drug used.

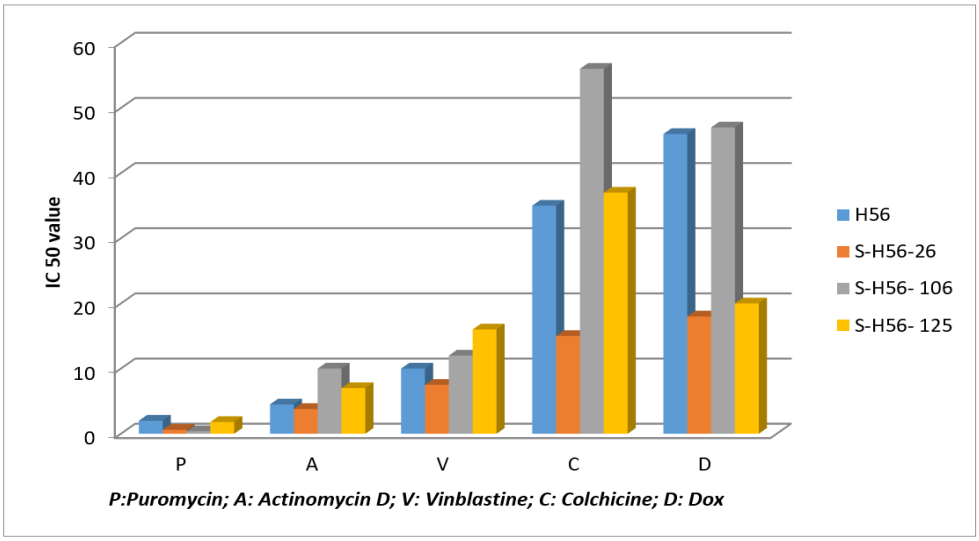

Fig. 1. *Comparison of antiproliferative effect of different anticancer drugs on dexamethasone sensitive H56 cells and dexamethasone-resistant variants (S-H56-26, S-H56-106, S-H56-125).*

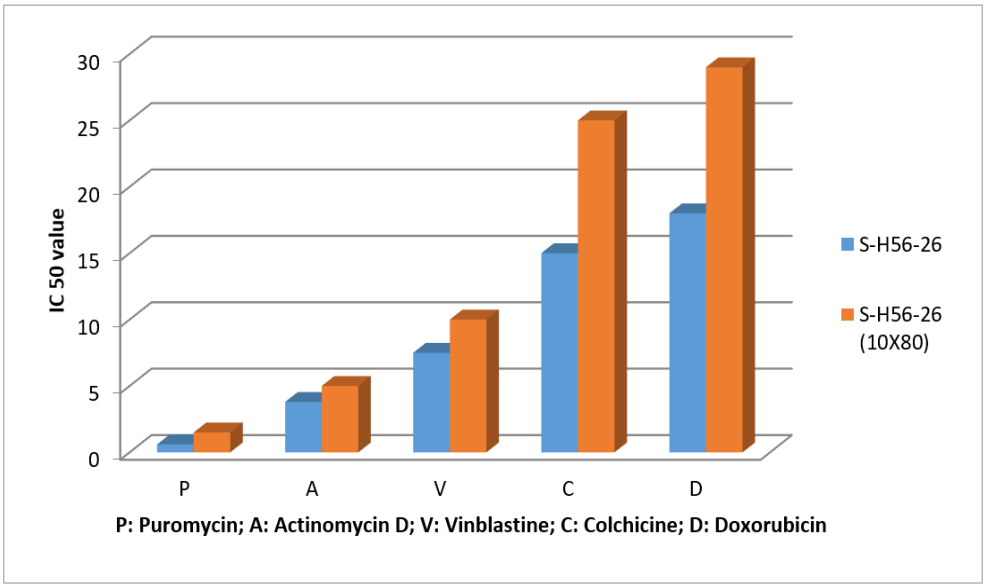

Fig. 2. *Comparison of antiproliferative effect of different anticancer drugs on heat-resistant variant S-H56-26 (10x80) and heat - sensitive variant S-H56-26.*

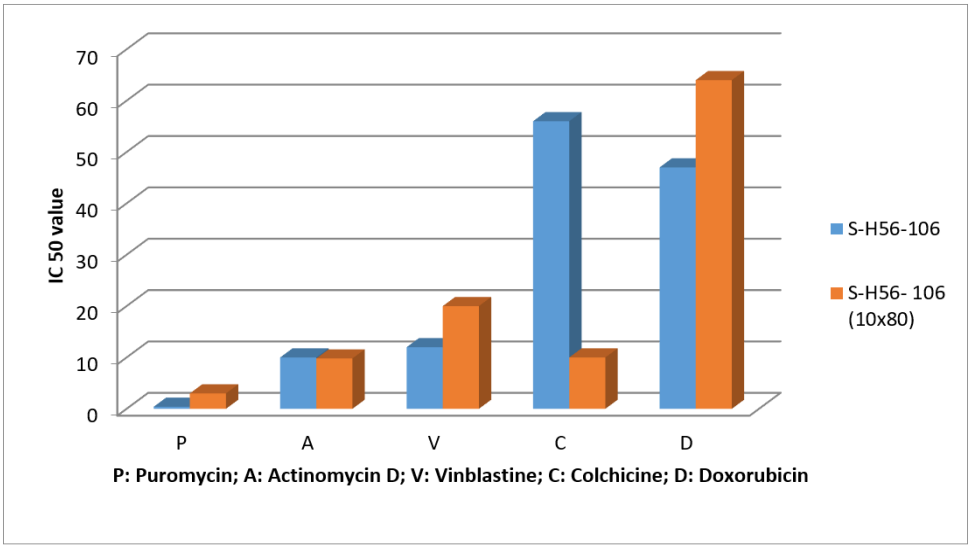

Fig. 3. *Comparison of antiproliferative effect of different anticancer drugs on heat-resistant variant S-H56-106 (10x80) and heat - sensitive variant S-H56-106.*

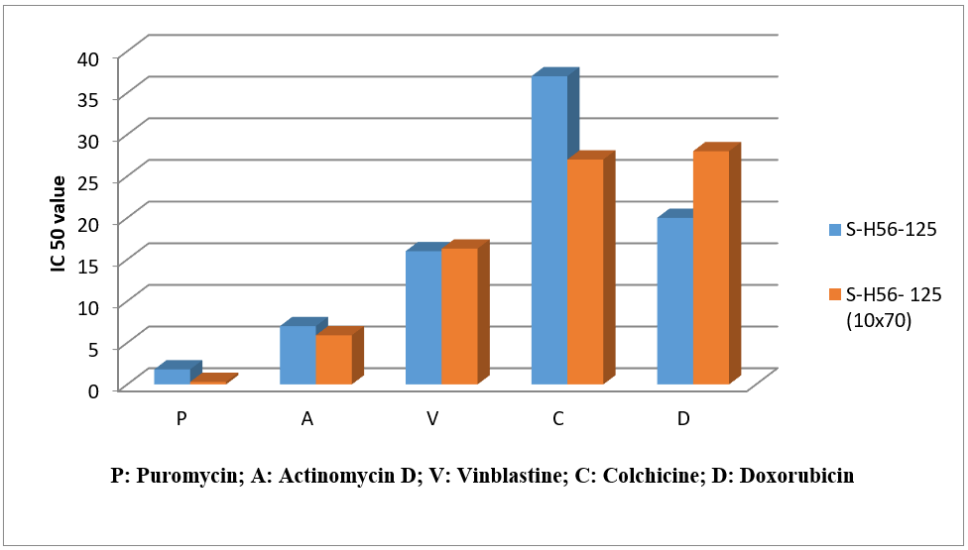

Fig. 4. *Comparison of antiproliferative effect of different anticancer drugs on heat-resistant variant S-H56-125 (10x70) and heat-sensitive variant S-H56-125*

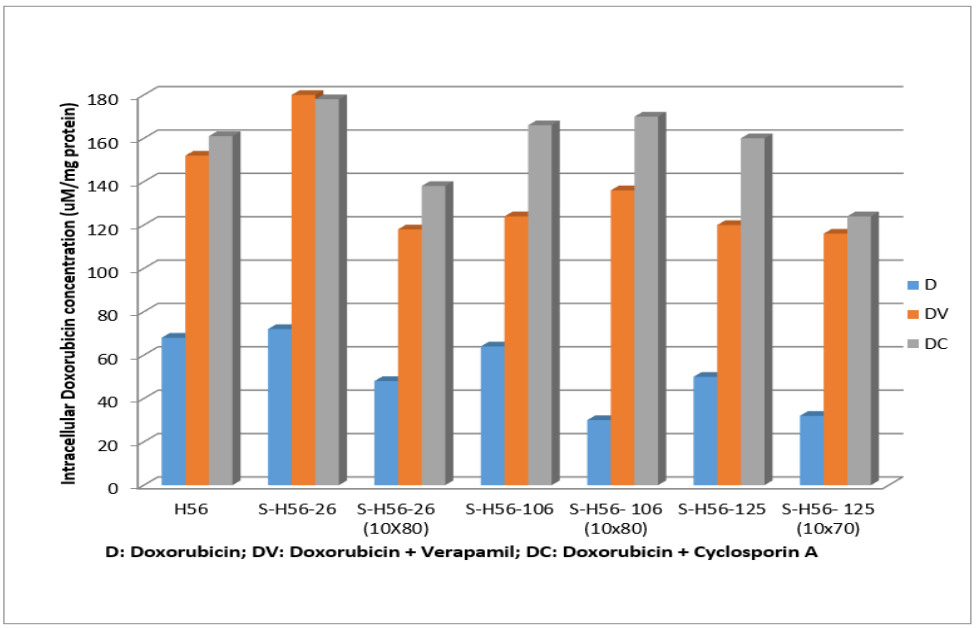

Fig. 5. *Effect of verapamil and cyclosporinA on doxorubicin retention in all the variants under study.*

In order to see whether moderate drug resistance of heat- resistant variants is correlated with functional increased P-glycoprotein activity, the cellular accumulation of doxorubicin, known to be transported by P-glycoprotein, was measured. The moderate drug resistance of the heat-resistant variants was found to be correlated with decreased accumulation of doxorubicin (46-64%) in the variants studied. Addition of verapamil (a known calcium channel blocker) or cyclosporin A (a broad spectrum MDR modulator) known to reverse multidrug resistance by inhibiting P-glycoprotein function [56, 57, 58] led to a significant increase in cellular doxorubicin accumulation in all the variants under study. These results show that functional P- glycoprotein is over expressed in all variants tested, although this over expression is less pronounced that it was detected in the moderately multidrug-resistant derivatives of clone 2. Similar reports of doxorubicin retention have been known [59, 60].

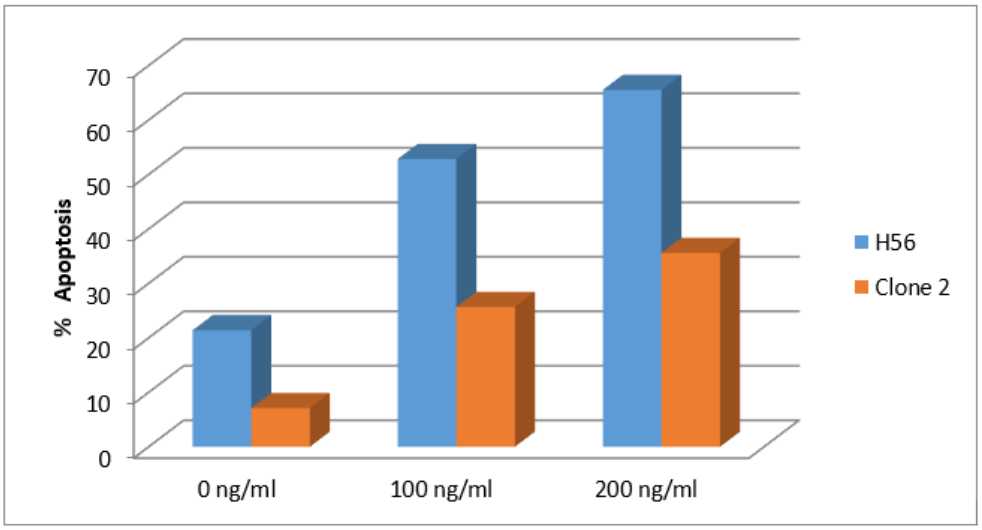

Fig. 6. *Average % Quantitation of apoptotic cell death in H56 and clone 2 cells treated with 0 ng/ml, 100 ng/ml and 200 ng/ml doxorubicin for 1, 2 and 3 days*

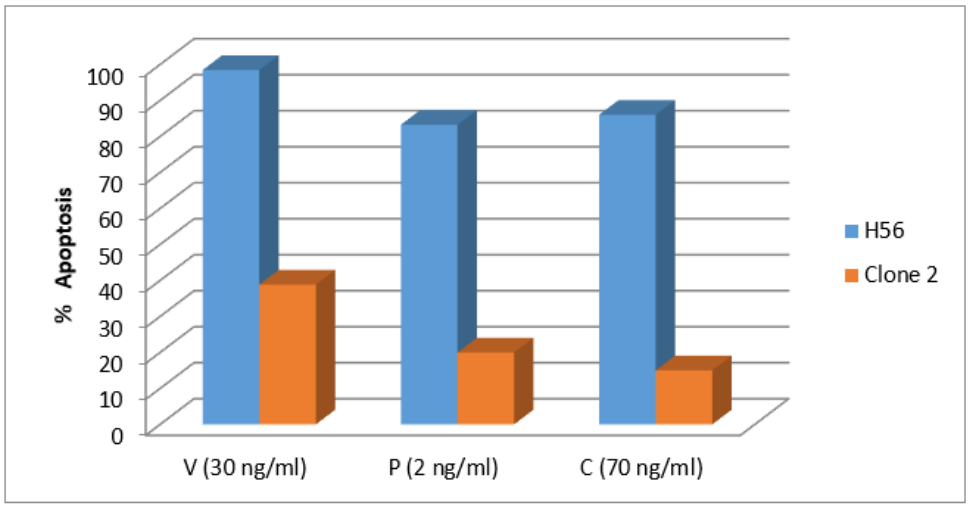

Fig. 7. *% Quantitation of apoptotic cell death in H56 and clone 2 cells treated with vinblastine (V), puromycin (P) and colchicine (C) for 3 days*

In an attempt to characterize the mechanisms responsible for the different cytotoxicity of doxorubicin on H56 and clone 2 cells, the possible apoptosis inducing effect was tested. Treatment of hepatoma cells with doxorubicin induced typical apoptotic morphology in both cell lines (Fig. 8).

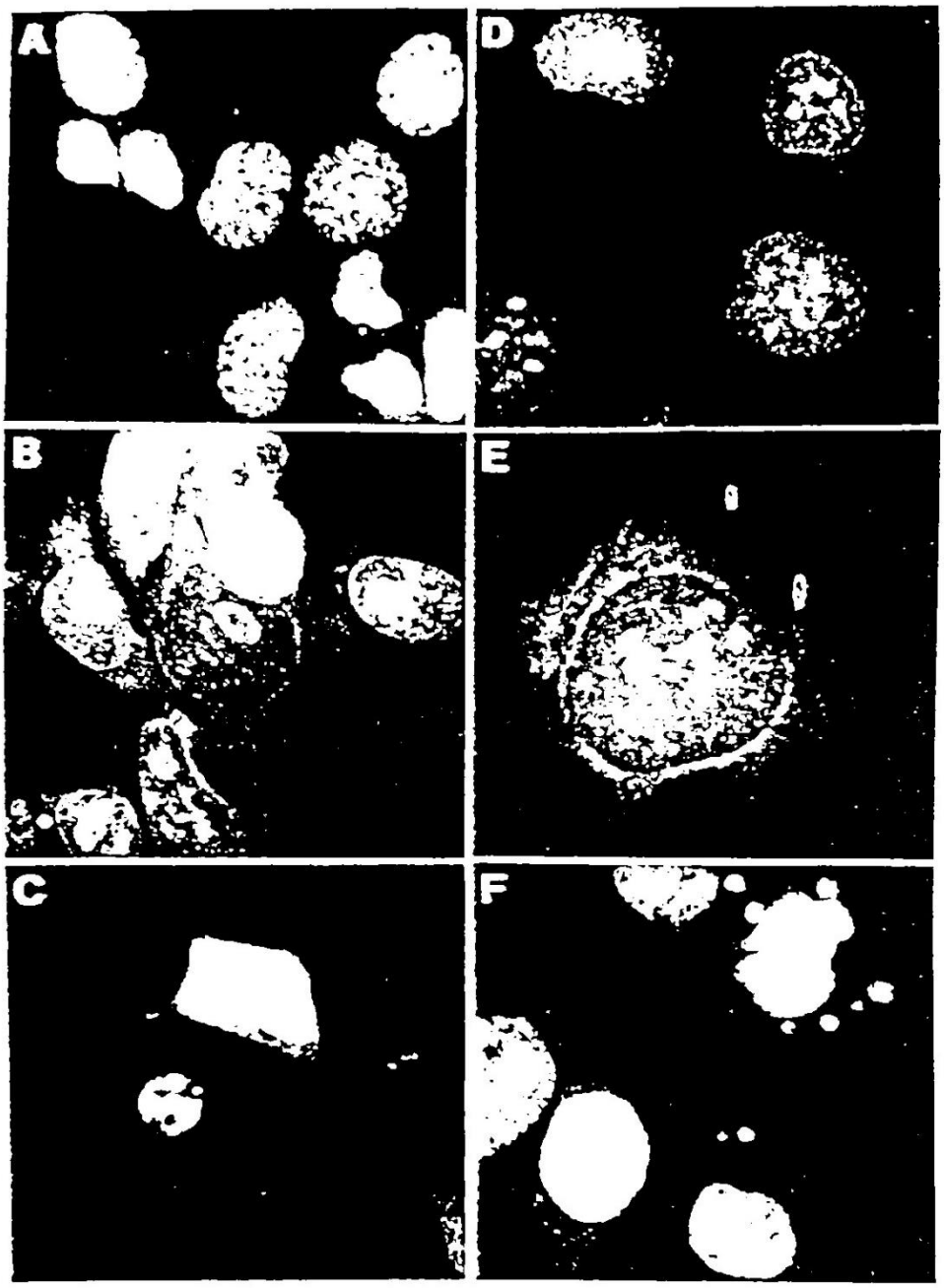

Fig. 8. *Morphological appearance of apoptotic cells induced by doxorubicin treatment in H56 and clones 2 cells. Apoptotic nuclei are highly fluorescent and condensed, apoptotic bodies can also be seen (B, C, E, F). (A and D: control cells)*

Necrosis occurred at much lower rates than apoptosis in all doxorubicin treated cells. The ratio of apoptotic cells was significantly lower in clone 2 cells treated with different concentrations of doxorubicin (100-200 ng/ml) than in H56 cells (Fig. 6). One day treatment with 200 ng/ml doxorubicin induced 52% of apoptosis in H56 cells, and 16% in clone 2 cells, for example, while the ratio of necrotic cells was about the same (less

than 20%). 70% of apoptosis was achieved in H56 cells following treatment with 200 ng/ml doxorubicin for 3 days, while in clone 2 cells only 42% was detected.

The apoptosis inducing effect of other drugs (vinblastine, puromycin, colchicine) were also examined. We found that 3 days treatment with 30 ng/ml vinblastine resulted almost 100% apoptosis in H56 cells, while less than 40% in clone 2; 2µg/ml puromycin induced 83% apoptosis in H56 and about 20% in clone 2, 70ng/ml colchicine induced 86%apoptosis in H56 cells and about 15% in clone 2. In conclusion, all examined drugs induced apoptosis in H56 and clone 2 cells, however H56 cells are prone to undergo apoptosis than clone 2 cells.

H56 is a dedifferentiated, dexamethasone-sensitive, while clone 2 is a dedifferentiated, dexamethasone-resistant hepatoma clone. Both clones were found to be sensitive to anticancer drugs and expressed no detectable levels of P- glycoprotein. The detailed comparison of the two clones however revealed, differences in their drug sensitivities. Interestingly, H56 was found to be more sensitive to several anticancer drugs than clone 2.

To understand better the mechanisms responsible for the different drug-induced apoptosis rate in H56 and clone 2 cells, a preliminary study was done to examine the expression of p53 tumor suppressor gene in these cells. Using p53 specific antibody and immunocytochemistry significant differences were found in the expression of p53. While strong specific p53 staining was observed in the cytoplasm and in the nuclei of H56 cells, much less intense staining was seen in clone 2 cells.

It was shown that stable heat- resistant variants of clone 2 cells became moderately multidrug-resistant. The situation found in the case of the heat-resistant derivatives of dexamethasone-resistant H56 cell is different, because these cells became moderately resistant only to certain drugs.

The results of the present study showed that severely and repeatedly heat-treated hepatoma cells became resistant to either certain anticancer drug (heat-resistant derivatives of dexamethasone–resistant H56 cells), or became multidrug-resistant (heat-resistant variants of clone 2) and over expressed functional P-glycoprotein. It is not known yet whether the difference in the profile of drug resistance found in the derivatives of H56 and clone 2 is due to the different degree of heat resistance (the variants isolated from H56 are less heat-resistant than variants of clone 2 or other factors.

Perhaps the most interesting of the experimental results is that the severely heat-treated H56 cells became resistant only to certain drugs. Functional P-glycoprotein was over expressed in all these cells. Thus, the results of present study are in concordance with earlier reports which state that cell lines with acquired MDR overexpress the P-glycoprotein [61, 62, 63, 64]. It was also found out that anticancer drugs induce apoptosis in H56 and clone 2 cells; however, H56 cells are more prone to undergo apoptosis than clone2 cells, which correlates with significant differences in expression of p53.

CONCLUSION

The comparison of IC50 values following 3 days treatments with different drugs revealed that the most heat–resistant clones, S-H56-106 (10x80) and S-H56-26(10x80), became moderately resistant to certain drugs whereas S-H56-125(10x70), the least heat-resistant cell, showed slight increase in resistance to only two drugs. Interestingly, doxorubicin resistance was increased in all the three variants.

Severely and repeatedly heat-treated hepatoma cells became resistant to either certain anticancer drug (heat-resistant derivatives of dexamethasone–resistant H56 cells), or became multidrug-resistant (heat- resistant variants of clone 2) and over expressed functional P-glycoprotein. H56 cells are more prone to undergo apoptosis than clone2 cells. Thus, it is concluded that thermotolerance leads to MDR.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept: S.J., Design: S.J., Data Collection or Processing: S.J., Analysis or Interpretation: S.J., G.N., Literature Search: S.J., G.N., Writing: S.J., G.N.

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