

## OVERCOMING CHEMORESISTANCE IN NON-HODGKIN LYMPHOMA PRELIMINARY STUDIES OF APOPTOSIS AND NECROSIS BY P-GLYCOPROTEIN REVERSAL AGENTS

BEHZAD FOROUTAN<sup>\*1</sup>, NASRIN RAZAVIANZADEH<sup>2</sup> DIANA ANDERSON<sup>3</sup>

<sup>\*1</sup>Department of Pharmacology, School of Medicine Shahroud University of Medical Sciences Shahroud, IR Iran, <sup>2</sup>Department of Internal Medicine School of Medical Sciences, Islamic Azad University, Shahroud Branch Shahroud, Iran, <sup>3</sup>Department of Biomedical Sciences School of Life Sciences University of Bradford Bradford, United Kingdom.  
Email: behzad\_foroutan@hotmail.com

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### ABSTRACT

**Objective:** The *in vitro* measurement of drug-induced apoptosis provides a mechanism-based test for the chemosensitivity of tumor cells isolated from a patient or from a specific cell line. The goal of this study was to investigate the effects of p-glycoprotein reversal agents on apoptosis and necrosis in Burkitt lymphoma cells. These effects were determined by microscopic observation and by electrophoretic separation of DNA fragments.

**Methods:** We demonstrated induction of apoptosis in Burkitt lymphoma Raji Thymidine Kinase<sup>+</sup> (TK<sup>+</sup>) and TK<sup>-</sup> cells using different subclasses of p-glycoprotein reversal agents. A low dose of doxorubicin was also used. The selective clonal expansion of mutant lymphocytes is based upon the phenotypic properties of TK-deficient cells. The first phase of the present study involved morphological analyses and DNA degradation on agarose gel electrophoresis. The second phase analyzed DNA damage using the Comet assay and tail moments calculated with Komet imaging software.

**Results:** Electrophoretic separation resulted in a ladder pattern, indicating that the p-glycoprotein reversal agents were able to induce apoptosis and necrosis. The morphological frequency of apoptosis and necrosis in the cells was significantly increased. Most p-glycoprotein reversal agents showed an increase in tail moments in the Comet assay.

**Conclusion:** The results indicate that indomethacin and quercetin may help to overcome chemoresistance in Burkitt's lymphoma.

**Keywords:** Apoptosis, Necrosis, P-glycoprotein reversal agents, NHL cell-line model, Burkitt's lymphoma.

### INTRODUCTION

The term "apoptosis," introduced by Kerr in 1972 [1]. It refers to the morphological formation of apoptotic bodies in a cell [2]. Many diseases involve excessive apoptosis, such as neurodegenerative diseases, Parkinson's disease, Alzheimer's disease, spinal muscular atrophy, and acquired immunodeficiency syndrome (AIDS); or too little apoptosis, such as cancer (either by virus infection or by DNA mutations in genes such as p53, mdr-1, etc.) or autoimmune diseases (diabetes type 1, encephalomyelitis) [3]. Hemotherapeutic agent, alcohol and oxidative stress could also trigger apoptosis [4]. Wild type p53 and Bcl-2 proteins are two major endogenous regulators of apoptosis [5]. Although the proposed mechanisms may contribute to the development of a variable degree of cellular resistance, it is possible that the cell response (i. e., DNA repair or apoptosis) following DNA damage plays a critical role in determining cellular chemo sensitivity. The preclinical observations that tumor response to effective drug treatments is associated with the induction of apoptosis support the possibility that a decreased susceptibility to apoptosis (apoptosis resistance) might be relevant to clinical chemoresistance.

Apoptosis associated with biochemical and physical changes involving the plasma membrane, the cytoplasm, and the nucleus [6]. Necrosis leads to the destruction of the cellular organelles, rupture of the plasma membrane, and leakage of the cell's contents. Necrosis could also be the final result in situations where there is too much apoptosis occurring for phagocytotic cells to cope with, especially in cell cultures [7], where phagocytotic cells are usually lacking. Apoptosis can be induced by chemotherapeutic agents [8].

Necrosis is associated with the release of lysosomal proteases, which causes proteolysis of nuclear histones, leaving "naked" stretches of DNA not protected by histones. Electrophoresis of DNA from necrotic cells results in a smear pattern [9]. In addition, necrotic cells enhance pro-inflammatory responses of activated macrophages, whereas apoptotic cells profoundly inhibit these macrophages [10]. On a morphological level, apoptosis is

distinguished by unique features, including cell shrinkage, the formation of pyknotic nuclei, and double-stranded DNA fragmentation. Double-stranded DNA fragmentation is one of the most widely accepted criteria used to characterize apoptotic cell death because it is discernible from the random single-stranded DNA breaks produced by necrosis. DNA "laddering" is the most common assay for visualizing the double-stranded oligonucleosomal DNA fragments on agarose gels; however, this technique is difficult to quantify and not particularly sensitive as an extensive number of cells (greater than 10<sup>6</sup> cells/sample) must be caught in synchronous fragmentation to produce the oligonucleosomal ladders. The TUNEL assay also suffers from lack of specificity and cannot distinguish apoptosis from necrosis unless a second marker is used

The single cell gel electrophoresis (Comet) assay is a technically simple and fast method that detects genotoxicity in virtually any mammalian cell type without requirement for cell culture. Reports from biomonitoring studies show that the basal level of DNA damage in leukocytes is influenced by a variety of lifestyle and environmental and experimental exposures. Although not all types of carcinogenic exposures should be expected to damage DNA in leukocytes, the comet assay is a valuable method for detection of genotoxic exposure in humans. However, the predictive value of the comet assay is unknown because it has not been investigated in prospective cohort studies. Also, it is important that the performance of the assay is investigated in multi-laboratory validation trials. As a tool in risk assessment the comet assay can be used in characterization of hazards.

### Relationships among apoptosis, p53, and p-glycoprotein

Loss of function of p53 is a frequent and important event in chemoresistance [11]. p53 induces apoptosis through both transcription-dependent and independent pathways [12]. It also induces transcription of genes encoding pro-apoptotic factors that are not part of the Bcl-2 family [13], the Fas death receptor [14], or mitochondrial proteins involved in the generation of reactive oxygen species [15]. In the transcription-independent pathway, p53 induces

apoptosis by causing the Fas receptor [16]. Loss of susceptibility to apoptosis signals is a crucial step in carcinogenesis. Therefore, sensitization of tumor cells to apoptosis is a promising therapeutic strategy. This sensitizes cells to Fas-induced apoptosis through the extrinsic death pathway [16]. In contrast, p-glycoprotein (P-gp) protects cells from apoptosis [17].

Despite the fact that drug influx often suppresses drug efflux, P-gp positive cells are often protected from cell death [18, 19]. The conceptually simplest mechanism of chemoresistance is one that reduces intracellular xenobiotic accumulation, which could be effected by P-gp. P-glycoprotein is an ATP-binding cassette transporter that confers multidrug resistance in cancer cells. It also affects the absorption, distribution and clearance of cancer-unrelated drugs and xenobiotics

In the present study to induce apoptosis, the first strategy used to inhibit P-gp function, relied on the identification of non-chemotherapeutic agents as competitors [20]. Thep53 is often mutated in BL cell lines. Raji cells are stably infected with a non-productive EBV strain.

## MATERIALS AND METHODS

### Chemicals

Verapamil, doxorubicin, fluphenazine, indomethacin, quercetin, tween-20, triton X-100, dimethyl sulfoxide, sodium chloride, ethylene diamine tetra acetic acid, ethidium bromide and trypan blue 0.4% were purchased from Sigma. The Raji cell sub-lines [21] were obtained from the European Collection of Cell Cultures (ECACC). RPMI 1640, EDEM, penicillin/streptomycin, glutamine and fetal bovine serum were purchased from GIBCO.

### Demonstration of apoptosis and necrosis by morphological studies

Lehne [22, 23] described a therapeutic option that was addressed in the present study. The difference is that the cell sub-lines used in this study showed p53 protein over-expression.

The classical features of apoptosis and necrosis are best seen by electron microscopy but can also be observed at the light microscopic level using nucleic acid binding dyes [24].

To investigate the effects of chemosensitizers on aggressive non-Hodgkin's B lymphoma cell lines, we selected Raji cells as a chemoresistance model with p53 over-expression.

### Experimental design and procedure

Raji cells were seeded in 96-well microplates at  $2 \times 10^3$  cells per well in 100  $\mu$ l of the complete growth media. This cell concentration ensured logarithmic cell growth during the assay. The two far left and right columns were filled with cell culture media alone to reduce evaporation. The remaining inner columns used tetraplicate for control, control plus chemosensitizer, and low-dose doxorubicin alone.

Forty eight hours after treatment, an alkaline SCGE assay was performed, and apoptotic cells were scored under a fluorescent microscope following staining with ethidium bromide. Morphological studies were done by counting the cells under the microscope after completing the cells' 48-hour incubation period with different sub-groups of P-gp reversal agents.

The control cells, not exposed during the incubation period, provided the results for the numbers of apoptotic, necrotic, and intact cells, which were compared with the exposed cells.

### Single-cell gel electrophoresis (Comet) assay

Rydberg and Johanson [25] [25] (Rydberg & Johanson, 1978) (Rydberg and Johanson, 1978) (Rydberg and Johanson, 1978) (Rydberg & Johanson, 1978) [25] [25] (Rydberg and Johanson, 1978) introduced the Comet assay [25], and it was further developed by Ostling and Johanson [26]. All of the experiments involving SCGE in this study used the alkaline version (pH  $\geq$  13.0) of the assay, which measures DNA damage (primarily DNA strand breaks and alkali labile sites), as

well as the method of Singh et al. [27], as adapted by Tice et al. [28]. Evaluations were carried out with a computerized image-analysis system (Komet 4.0; Kinetic Imaging, UK) attached to a fluorescence microscope (20 $\times$  objective), which was equipped with a BP546/10 excitation filter and a 590 nm barrier filter (Leica, Germany). All of these steps were conducted under dimmed light to prevent the occurrence of additional DNA damage. In each experiment, 300 cells were counted from two slides, and the experiment was repeated (i. e., 600 cells in total).

### Cell viability

The viability of Raji cells was measured by Trypan blue dye exclusion, indicating intact cell membranes (Phillips, 1973). 10  $\mu$ l of 0.05% Trypan blue was added to 10  $\mu$ l of cell suspension, and the percentage of cells, excluding the dye, was estimated using a Neubauer improved haemocytometer [29]. Viability was generally greater than 92%, but always was greater than >80% well above the cut-off point for cytotoxic doses in geno toxicity experiments [30].

### Apoptotic and necrotic detection

Typical apoptotic cells under a light microscope show small, highly condensed chromatin bodies. Each apoptotic body has a fragmented piece of nucleus surrounded by a viable cell membrane. This can be easily differentiated from a normal cell under a light microscope [31]. Necrosis usually involves groups of contiguous cells and leads to swelling of the cytoplasm and irreversible failure of cell organelles.

Following these criteria, a total of 600 randomly selected cells from each sample of Raji subcell lines were observed for apoptosis using a fluorescent microscope. To confirm morphological assessments of apoptosis and necrosis, smears were stained with ethidium bromide. The cells were centrifuged, and pellets were re-suspended in minimum volumes of PBS.

Smears were made on microscopic slides and air-dried. After 4 minutes, the slides were rinsed in water and air-dried. Observations were made after mounting the slides. This procedure was a modification of Singh's method [32].

### DNA ladder assay

A simple method for detecting apoptosis is the DNA ladder assay. After induction of apoptosis, cells were washed twice with PBS (approximately 0.4 to 0.5  $\times$  10<sup>6</sup> per sample) and centrifuged (500 g). The pellets were then lysed with 150  $\mu$ l hypotonic lysis buffer (10 mM EDTA, 0.5% Triton X-100 in 1 mM Tris-HCL, pH = 7.4) for 15 minutes on ice and were precipitated with 2.5% polyethylene glycol and 1 M NaCl for 15 minutes at 4°C. After centrifugation at 15,000 g for 10 minutes at room temperature, the supernatant was transferred to a clean Eppendorf and incubated with RNaseI for 1 hour at 37°C; then in the presence of proteinase K (0.3 g/l) at 37°C for 2 hours; and finally DNA-precipitated with isopropanol (0.6-

0.7 v/v) at -20°C for 1 hour. After centrifugation (13,000 g at 4°C for 15 minutes), the supernatant was carefully decanted, and the Eppendorfs were left upside-down for 1 hour at room temperature. Finally, each pellet was dissolved in 10  $\mu$ l of 1 X Tris-EDTA (10 ml of 1M Tris-HCL, pH = 7.6 and 2 ml of 0.5M EDTA, pH = 7.6 was added to 988 ml of dH<sub>2</sub>O filtered with 0.5-micron filtered and autoclaved). Also, to each pellet 1  $\mu$ l of dye was added and electrophoresed on a 1.5% agarose gel containing ethidium bromide (final concentration 0.5  $\mu$ g/ml). A constant voltage of 90V was used for 70 minutes. Two microliters of different bp size DNA marker ladder were used in the left and right side of the gel as the controls. Ladder formation of DNA was detected under ultraviolet light and photographed.

### Data analysis

To investigate cytotoxic effects, different subclasses of P-gp reversal agents were used. The results gained from cells exposed and not exposed to P-gp reversal agents and from cells exposed to doxorubicin were compared by the Chi-square test, and the null hypothesis was assumed at p < 0.05. For a positive inducer of apoptosis, 25nM of DOX was used. In each experiment, 300 cells were counted from two slides, and the whole experiment was

repeated (i. e., 600 cells in total) to ensure the reproducibility of the results. It cannot be ruled out that some cells lysed completely, and if this were the case, they would not have been available for counting. The tables in this article are presented with this limitation.

**RESULTS**

**Morphological studies**

Table 1 and 2 shows the morphological results from an analysis of 600 cells. Results had shown the significance of examining TK+ vs. TK- cells as showed in tables 1 and 2. Positive controls

(Tween-20, Triton X-100, and doxorubicin) also significantly induced apoptosis and necrosis. The apoptosis of a great number of cells, after applying p-glycoprotein reversal agents, suggests that apoptosis induction does not depend on the cell cycle. All happens as if the cell was programmed for the activation of the final phase of apoptosis, probably by the activation of an endonuclease. The alteration of the genetic program of the cell undergoing differentiation or the change of signals sent to the cell can modify cellular sensitivity to apoptosis induction. The capacity of tumor cells to enter apoptosis becomes the key of the response to the tumor disease treatment.

**Table 1: Morphological frequency of apoptosis and necrosis in Raji (TK+) cells with no treatment (control) and after treatment with different sub-groups of P-gp reversal agents, Six hundred cells were examined under the microscope from two independent experiments and data were analyzed by the chi-square test ( $\chi^2$ )**

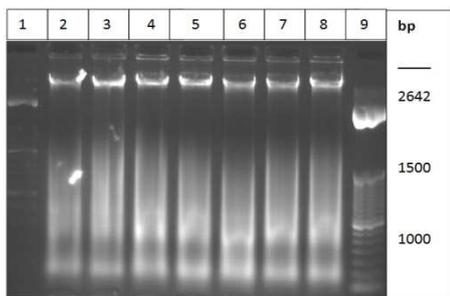
Name of drug	Apoptosis	Necrosis	Intact cells	p_value
Control (no treatment)	61	47	492	-
Fluphenazine (3 $\mu$ M)	65	98	437	<0.001
Indomethacin (5 $\mu$ M)	134	240	226	<0.001
Quercetin (10 $\mu$ M)	82	198	320	<0.001
Verapamil (6 $\mu$ M)	60	96	444	<0.001
Tween-20 (0.001% v/v)	168	352	130	<0.001
Triton X-100 (0.001% v/v)	40	446	114	<0.001
Doxorubicin (25nM)	258	261	81	<0.001

**Table 2: Morphological frequency of apoptosis and necrosis in Raji (TK-) cells before treatment (control) and after treatment with different sub-groups of P-gp reversal agents. Six hundred cells were examined under the microscope from two independent experiments and data were analyzed by the chi-square test ( $\chi^2$ )**

Name of drug	Apoptosis	Necrosis	Intact cells	(p_value)
Control (no treatment)	69	56	475	-
Fluphenazine (3 $\mu$ M)	79	130	391	<0.001
Indomethacin (5 $\mu$ M)	161	132	307	<0.001
Quercetin (10 $\mu$ M)	95	135	370	<0.001
Verapamil (6 $\mu$ M)	75	268	407	<0.001
Tween 20 (0.001% v/v)	162	414	58	<0.001
Triton X-100 (0.001% v/v)	55	464	71	<0.001
Doxorubicin (25nM)	454	62	84	<0.001

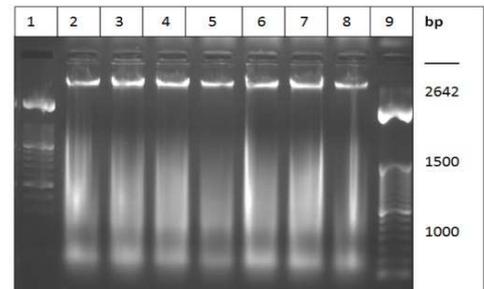
**DNA laddering**

A characteristic feature of apoptosis is DNA fragmentation. Wyllie described, in association with apoptosis, nucleosomal fragmentation which can be seen by agarose electrophoresis with a ladder of DNA bands representing 180-200 base pairs [24]. Results of DNA fragmentation of the Raji cell sub-lines after exposure to the P-gp reversal agents are shown in Fig. 1 and Fig. 2.

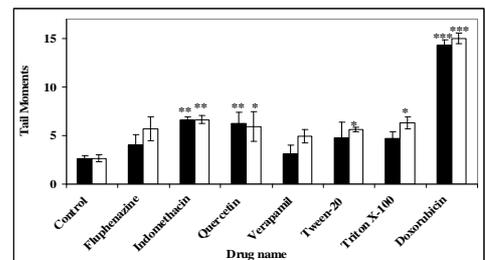


**Fig. 1**

The DNA ladder did not show the formation of DNA fragmentation, which was isolated from both cell sub-lines. In control cells, the DNA ladder was shorter than that of cells exposed to different drugs, indicating that less-fragmentized DNA existed when compared to the cell sub-lines that were exposed to the P-gp reversal agents. However, all lanes were similar, possibly indicating that the DNA ladder assay did not discriminate properly in this situation. It may be necessary to use another technique, such as DNA end labeling.



**Fig. 2**



**Fig. 3: Effects of PGP reversal agents on DNA after long-term incubation with P-gp reversal agents. Black columns are the results of TK+ cells and white columns are the results of TK- cells. Means and medians were determined and data were analysed by the Mann-Whitney test. Bars indicate standard errors for two independent experiments showing reproducibility.**

**SCGE results**

Using a modification of the SCGE assay, the formation and unhooking of DOX-induced breaks in cell lines were measured. To determine the induction of DNA damage, the SCGE was conducted, and tail moments were calculated automatically via the Komet 4 program. Fig. 3 shows the results of the two independent experiments.

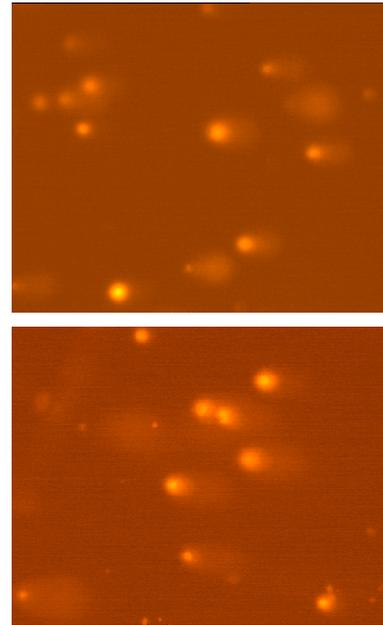
Data were analyzed through SPSS 13.0 for Windows and were compared by the Mann-Whitney test. Low damage induction to DNA occurred, which was measured by the SCGE assay (fig. 3). Except for indomethacin and quercetin, which showed significant differences, the other P-gp reversal agents showed limited DNA damage when compared with control cells. Doxorubicin increased the tail moments in both cell sub-lines ( $p < 0.001$ ), indicating the effects of DOX in a nanomolar dose scale, and also inhibited the growth of both cell sub-lines, indicating the inhibitory effects of the drug.

For all p-gp reversal agents, the frequencies of apoptosis and necrosis were increased. However, this was not the case for genotoxicity, suggesting that apoptotic and necrotic events are independent of genotoxicity in both cell sub-lines. Plaumann reported that flavonoids activate wild type p53 [33]. Because of this ability, it should be beneficial in resistant patients to activate the remaining function of p53 activities in response to DNA damaging agents [34].

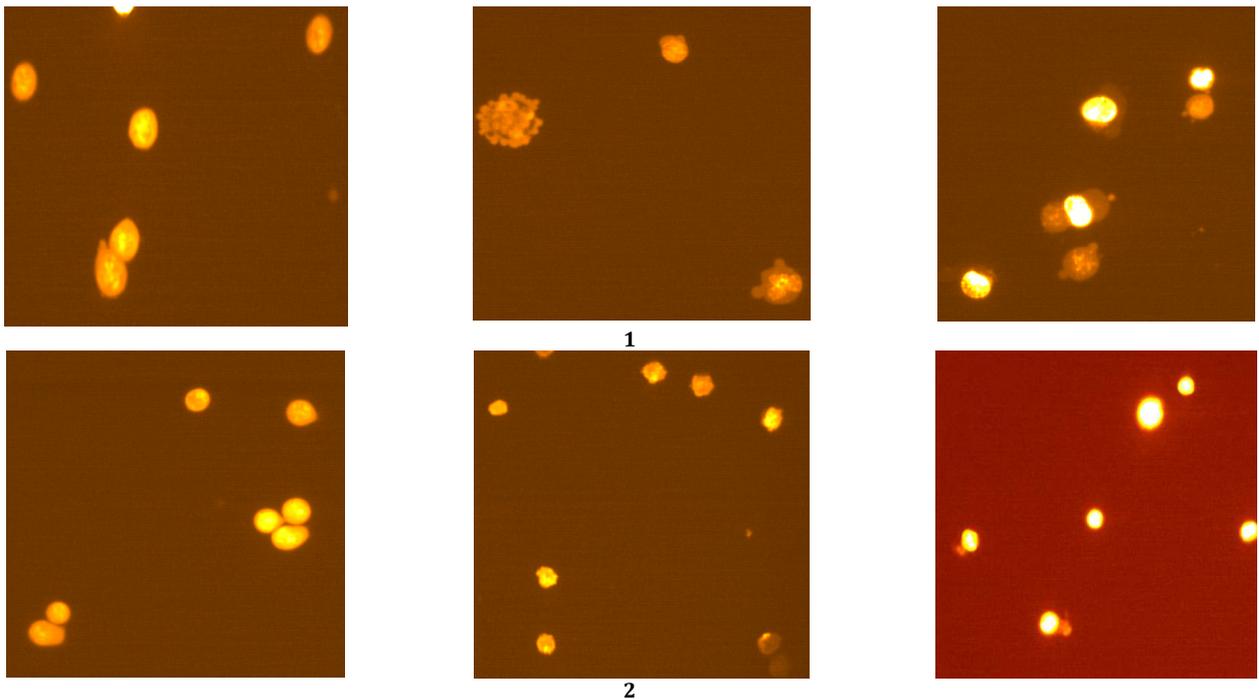
The results showed that most of the P-gp reversal and cell membrane perturbation agents induced apoptosis, except for fluphenazine and verapamil, indicating the lack of effectiveness of these drugs. However, quercetin and indomethacin also exhibited effects by increasing the DNA damage induction by increasing the tail moment levels. It was concluded that in the Raji cell-line, which over-expressed p53 protein, indomethacin did not over-promote proliferation signals but promoted apoptosis. These results were promising for chemoresistant patients with p53 protein over-expression (p53<sup>+</sup>). Tween-20 and Triton-X100 are detergents that lyse cells with no direct effect on proliferation. They serve as positive controls for loss of membrane integrity. This could be achieved by reducing the dose of chemotherapeutic agents in combination with a cell membrane perturbation agent, such as Triton X-100 or Tween-20. The results also showed an increased effect for these P-gp reversal agents, when compared with DOX and with DOX in combination with membrane perturbation agents (Tween-20 or Triton X-100).

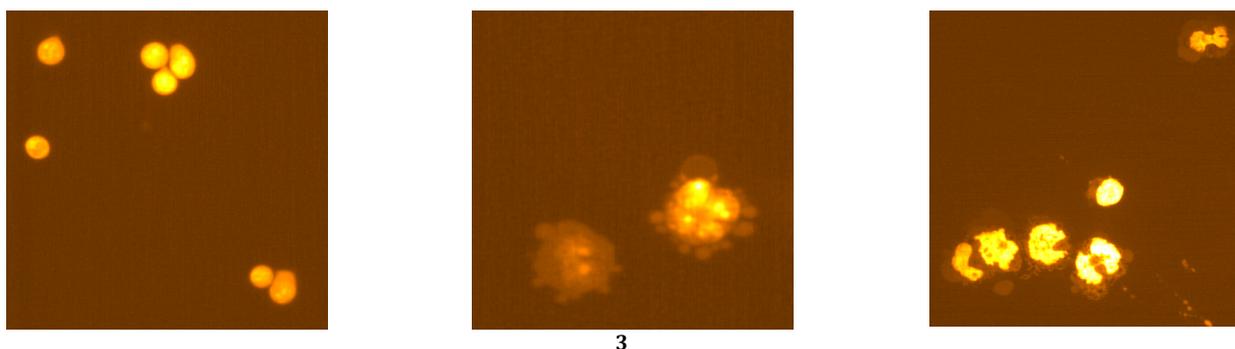
**Morphological features of apoptosis and necrosis**

Fig. 4 through 6 show the morphological features of Raji cell sub-lines exposed to DOX, indomethacin, or verapamil. Some of the reversal agents significantly increased the frequency of apoptotic cells compared with control cells. Thus, the P-gp reversal agents were able to overcome apoptotic resistance in the NHL cell-line model. Apoptotic cells in the controls contributed approximately 10% of the whole population (i. e.,  $61/600 \times 100 = 10.16\%$  for TK<sup>+</sup> and  $69/600 \times 100 = 11.5\%$  for TK<sup>-</sup>). After exposure to the P-gp reversal agents, apoptotic cells increased to 22.3% with indomethacin and to 43% with doxorubicin in TK<sup>+</sup> cells. In TK<sup>-</sup> cells, apoptotic cells increased from 11.5% to 26.8% and 75.7% with indomethacin and doxorubicin, respectively.

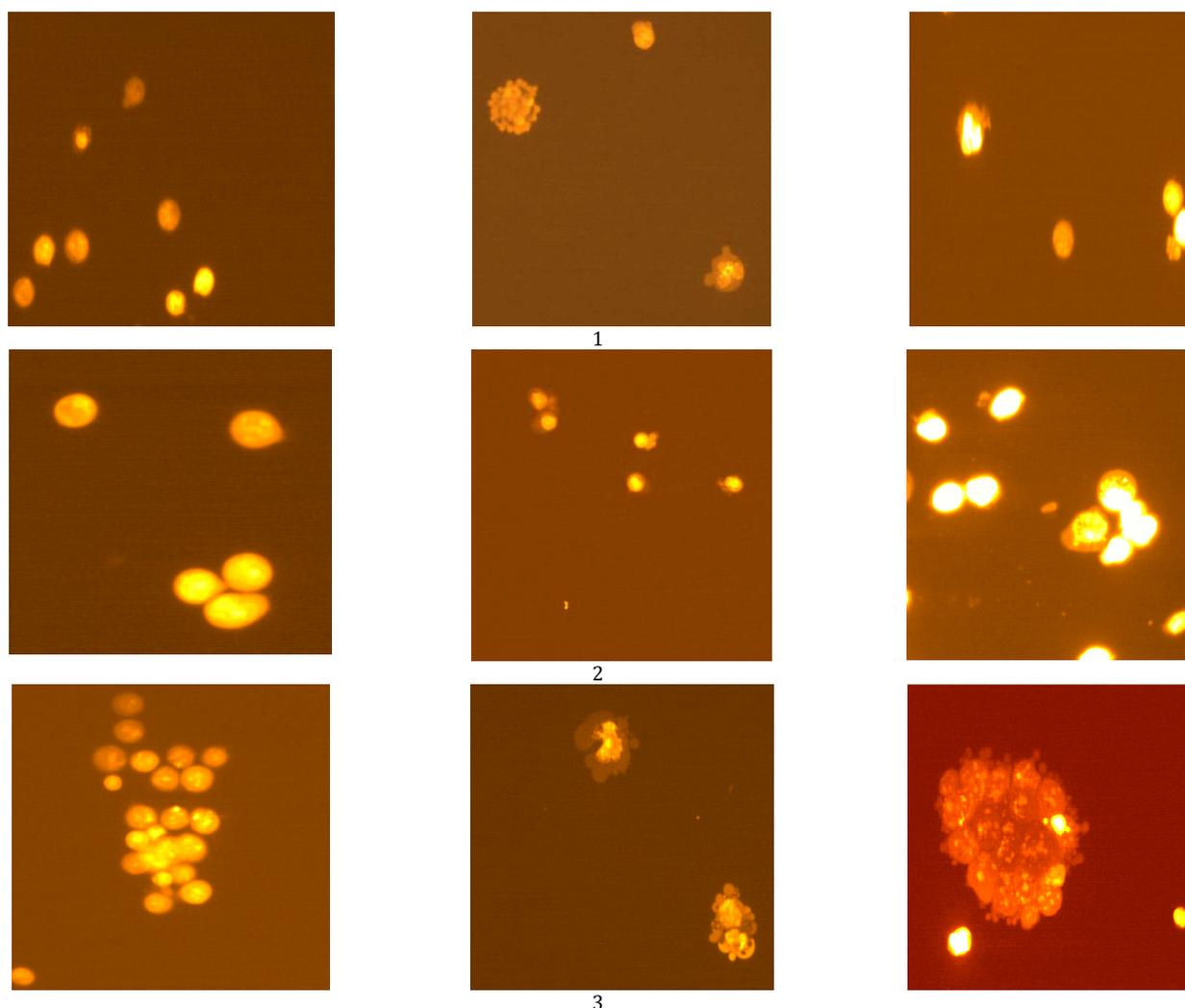


**Fig. 4: Comet images of the Raji cell sub-lines after treatment with 25 nM DOX for 48 hours in 37°C and 5% CO<sub>2</sub>. TK<sup>+</sup> treated cells (left). And TK<sup>-</sup> treated cells (right), as photographed with the Leica microscope below X 200**





**Fig. 5: Morphological features of Raji TK<sup>+</sup> cells after treatment with indomethacin (1), verapamil (2) and DOX (3). In each row, left images show intact cells, middle images show apoptotic cells, and right images show necrotic cells as they appeared under the microscope X 200**



**Fig. 6: Morphological features of Raji TK<sup>-</sup> cells after treatment with indomethacin (1), verapamil (2), and DOX (3). In each row, left images show intact cells, middle images show apoptotic cells, and right images show necrotic cells as they appeared under the microscope X 200**

#### **P-glycoprotein detection**

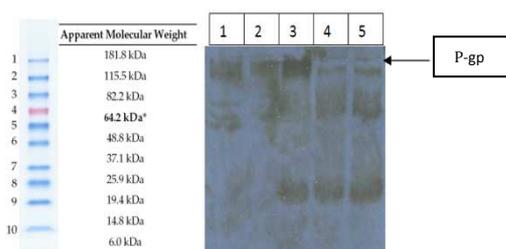
Fig. 7 shows the P-glycoprotein detection. The results showed that Burkitt's lymphoma Raji TK<sup>+</sup> and TK<sup>-</sup> cells actually express p-glycoprotein. Caco2 cells were used as a positive control for P-gp.

#### **DISCUSSION**

A 48-hour exposed time was chosen for DOX, in recognition of the long cellular half-life of DOX *in vivo* in NHL B-cells; a prolonged

exposure to DOX may be more relevant to the *in vivo* situation [35]. Low dose levels of DOX were able to increase apoptosis and necrosis in both cell sub-lines, based on morphological studies, and also increased the tail moments, as measured by SCGE assay.

However, conflicting results were achieved with DNA fragmentation, possibly because the DOX was not able to induce apoptosis through intrinsic pathways in Raji cell-lines, and/or because the DNA ladder assay did not operate appropriately.



**Fig. 7: The P-gp detection. Lane 1 = protein ladder, 2 = Raji (TK+), 3 = Raji (TK-), 4 = Caco-2 and 5 = human lymphocytes**

P-glycoprotein reversal agents, such as indomethacin and quercetin, showed an increase in apoptotic and necrotic features and increased DNA tail moments. No significant increase was recorded for tail moments following the SCGE assay with verapamil. Tween-20 and Triton X-100 showed an increase in apoptotic and necrotic features, but little effect was seen with these P-gp reversal agents in the SCGE results, indicating a lack of induction of DNA damage.

With regard to overcoming apoptotic resistance, indomethacin and quercetin appear to be the most promising agents at these doses. There is not enough convincing data to distinguish these as the most promising p-glycoprotein candidates. All p-glycoprotein agents tested activated apoptosis and necrosis (Tables 1 and 2), with minimal effect on DNA damage (fig. 3), when compared to doxorubicin as a positive control.

Perhaps the inhibitory effects of these NSAIDs on cyclooxygenases (COX-1 and COX-2) are different than their effects on p-glycoprotein. These results coincide with those of Ye *et al.* [36] and Arisawa *et al.* [37]. It seems that the combination of indomethacin with doxorubicin may have a potential for clinical application, especially in the circumvention of P-gp-mediated MDR.

Quercetin, like indomethacin, increased the apoptotic and necrotic cells, indicating the usefulness of this agent in combination with DOX, as reported by Li *et al.* [38]. A study conducted by Yasuhara *et al.* [39] confirmed the consistency of the Comet assay for the detection of apoptosis in single cells and provided evidence for its applicability as an additional method to detect apoptosis in cells. Unfortunately, the morphological frequencies of apoptotic and necrotic cells were not confirmed using apoptotic markers, such as the TUNEL assay or caspase. Also, there were no positive controls for apoptosis, as marked by the ladder. These limitations should be addressed in future experiments.

## CONCLUSION

Preliminary results indicate that indomethacin and quercetin may be the most promising agents for overcoming chemoresistance in non-Hodgkin lymphoma.

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