ORIGINAL ARTICLE

Assessment and modulation of chemoresistance by different sub-groups of chemosensitizers in a NHL cell line model

Behzad Foroutan[1,2,*] Diana Anderson [1]

ABSTRACT

Background: The development of chemoresistance represents a major obstacle in the successful treatment of cancers such as Non-Hodgkin's Lymphoma (NHL). With the recognition of important roles for both p53 and its more recently described paralog p73 in mediating the activity of anti-cancer drugs, there has been increasing recognition that cellular resistance to anthracyclines could and does arise through failure of p53 family member signalling. Despite these advances in understanding how cells respond to DNA damage in vitro, and how this is affected by molecular genetic changes which affect p53 family member signalling, the contribution of these to in vivo chemoresistance has not been definitively established. Our major task now is to determine how these changes operate individually and collectively in vivo to produce the phenotype of clinical chemoresistance, and how we can translate this knowledge into clinically useful strategies to improve the outcome of chemotherapy.

Materials and Methods: In this study we used two cell lines derived from Nigerian patients with Burkitt's lymphoma in a suspension type cell culture. The reduction of the tetrazolium salt to a blue-black formazan product by living not by dead cells can be used to measure growth inhibitory effects (cell proliferation inhibitory) of tumor cells.

Results: An alternative option for p53+ (resistant) cells is to use a PGP reversal agent in combination with DOX, but reducing the dose of DOX when combined with chemosensitizer.

Conclusion: The altered cellular dose in chemoresistant cell lines may provide a rational basis for the use of modified anthracycline based regimens in chemosensitizers, preferably non-genotoxic, in the treatment of tumors expressing chemoresistance phenotype with p53 over-expression.

Keywords: chemoresistance, chemosensitizers, NHL cell line model.

15

BCCR

1.Department of Medical Sciences, School of Life Sciences, University of Bradford, Bradford, BD7 1DP, United Kingdom

2.Department of Pharmacology, School of Medicine, Semnan University of Medical Sciences, Semnan, Iran

* Correspondence:
Behzad Foroutan
Department of Pharmacology
School of Medicine,
Semnan University of Medical Sciences, Semnan, Iran.
Fax: 0098 231 4448999
Phone: 0098 231 444898
E-mail:
behzad_foroutan@hotmail.com

زمینه و هدف: گسترش مقاومت دارویی بیانگر یک مانع عمده در درمان موفقیت آمیز سرطان ها مانند لنفومهای غیر هاچکینی است. با به رسمیت شناختن نقش های مهم p53 و پارالوگ اخیرا توصیف شده آن p73 به عنوان واسطه فعالیت داروهای ضد سرطان، شواهد فزاینده ای در دست است که مقاومت به آنتراسایکلین ها میتواند از طریق شکست پیام ها در خانواده p53 ایجاد شود. با وجود این پیشرفت ها درک اینکه چگونه در شرایط برون تن در آسیب به DNA سلولها پاسخ می دهند و چگونه این تغییرات مولکولی ژنتیکی پیام های خانواده p53 را تحت تاثیر قرار می دهند، ارتباط این عوامل در درون تن هنوز بدرستی اثبات نشده است. حالا وظیفه اصلی ما این است که تعیین کنیم چگونه این تغییرات انفرادی و در مجموع مقاومت شیمیایی را در درون تن اجرا می کنند تا فنوتیپ مقاومت شیمیایی بالینی را تولید کنند و چگونه ما می توانیم این دانش را مبدل کنیم به روش های مفید بالینی تا نتایج شیمی درمانی را بهتر کنیم.

مواد و روشها : در این مطاله ما از دو رده سلولی در کشت سوسپانسیونی که از یک بیمار نیجریه ای با لنفوم بورکیت بود استفاده کردیم. کاهش نمک تترازولیوم منجر به تولید شکل فرامازونی به رنگ آبی سیاه توسط سلولهای زنده و نه مرده میگردد که میتواند اندازه گیری شود تا آثار جلوگیری کننده از رشد سلول های سرطانی را بسنجد.

یافته ها: یک روش جایگزین برای سلول P53 +(مقاوم) استفاده از یک عامل معکوس کننده پی گلیکوپروتئین در ترکیب با دوکسوروبیسین، اما کاهش دوز دوکسوروبیسین با حساس کننده شیمیایی است.

نتیجه گیری : مقدار مصرف تغییر یافته در رده های سلولی مقاوم یک پایه منطقی برای استفاده از رژیم هایی با پایه آنتراسایکلینی به همراه حساس کننده های شیمیایی ترجیحا غیر سمی ژنتیکی را در درمان تومورهایی که فنوتیپ مقاومت شیمیایی را با افزایش p53 بروز می دهند فراهم می آورد.

واژه های کلیدی: مقاومت شیمیایی، حساس کننده های شیمیایی، مدل رده سلولی لنفوم غیر هاچکینی

Introduction

Chemoresistance is the major limitation to the success of CHOP in non-Hodgkin's lymphoma (NHL) patients. The well-studied form of drug resistance involving altered membrane transport is multi drug resistance (MDR) cells. MDR is a phenomenon in which a cellular phenotype develops resistance in vitro to a single cytotoxic drug, such as a vinca alkaloid, an anthracycline or a podophyllotoxin, and also becomes resistant to other structurally and/or mechanistically dissimilar drugs.[1-2] Many forms of MDR result from the expression of ATP-dependent efflux pumps with broad substrate specificity. These pumps belong to a family of ATP-binding cassette (ABC) transporters that share sequence and structural homology, and so far 48 human ABC genes have been identified. Alteration of the subcellular distribution of the drug by "trapping" in intracellular organelles is another way in which the cell can inactivate the drug. This type of resistance has been described for doxorubicin.[3] The altered cellular dose, low dose, in MDR cell lines may provide a rational basis for the use of modified anthracycline based regimens in combination with PGP reversal agents, chemosensitizers, preferably non-genotoxic, in the treatment of tumours expressing chemoresistant phenotype with p53 over-expression. Table 1 show some gain-of-function activities of mutant p53, which may contribute to chemoresistance.

Characteristic of Raji cell line:

In 1963 at the University of Ibadan, Nigeria, Pulvertaft established the first continuous human haematopoietic cell lines, a series of cell lines derived from Nigerian patients with Burkitt's lymphoma in a suspension type cell culture: Raji is the best known cell line of the panel.[13] Raji is a human B-lymphocyte-derived cell line.[14] Burkitt's and Burkitt-like lymphoma are aggressive B-cell malignancies with a high proliferative rate that may be fatal within months if not treated promptly. Therefore, the most effective treatment regimens that will continue to improve cure rates in this aggressive but treatable disease are required.[15] All transformed human Raji cells show an increased nuclear expression of p53.[16] Human B lymphoma Raji cells are resistant to nuclear apoptosis induced by various stimuli.[17-18] In the present study the cell-line Raji was used as an in vitro model for NHL with over-expression of p53 protein which is also showing apoptotic resistance.

Methods

Chemicals:

Doxorubicin, verapamil, fluphenazine, quercetin, indomethacin, Tween-20 and Triton X-100 were obtained from Sigma (Pool, Dorset, UK).

MTT assay:

The reduction of the tetrazolium salt, MTT, (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) to a blue-black formazan product by living (but not by dead cells) can be used to measure growth inhibitory effects (cell proliferation inhibitory) of tumour cells. The advantage of the MTT assay is that the results are read automatically with a microplate spectrophotometer. The methodology of the in vitro growth/growth inhibition

Table 1. Some gain-of-function activities of mutant p53	
Activity	**Reference**
Increase in expression of multidrug resistance-associated protein (MRP1)	Tsang et al., 2003 [4]
Activation of DuTPase expression	Pugachava et al., 2002 [5]
Down-regulation of CD95(Fas/APO-1)	Zalcenstein et al., 2003 [6]
Induction of gene amplification	El-Hizawi et al., 2002 [7]
Complex formation with TAp73	Marin et al., 2000 [8], Strano et al., 2000 [9], Bergamaschi et al., 2003 [10], Monti et al., 2003 [11]
Complex formation with TAp63	Strano et al., 2002 [12]

MTT assay described below represents a modification of the original MTT colorimetric assay described by Mosmann.[19]

The modification of the original in vitro assay procedures has been described (Scudiero et al., 1988). In the present study, 2 x 103cells/100µl Raji cells sub-lines (TK+ and TK-) were harvested from exponential-phase maintenance cultures (75-90% confluence in T-25 cm2 flasks, Corning, NY, USA). The cells were counted by trypan blue exclusion dye using a haemocytometer and a light microscope (Nikon, Japan) and dispensed as replicated cultures in multi-well cell culture microplates (Corning, NY, USA) for 24 hours. Following incubation in 5% CO2 at 37°C, 100µl of cell culture medium alone, cell culture medium containing chemosensitizers, cell culture medium containing doxorubicin and cell culture medium containing chemosensitizers in combination with doxorubicin were dispensed within the appropriate wells and incubated for 48 hours. A stock solution of MTT was prepared as follows: 5mg MTT/ml in PBS was prepared under sterile conditions and stored at 4°C for a maximum of one month and at -20°C for more than one month. A working solution of MTT was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in pre-warmed standard culture medium. Fifty microlitres of this solution were added to each well (resulting in 50µgMTT/250µl total medium volume), and microplates were incubated at 37°C for 4 hours. At the end of this period, the microplates were centrifuged (500g) for 10 minutes and then the upper media removed by aspiration from each well by pippetting out (just 10-20µl left in the bottom of each well). Then 150µl DMSO was added to each well. The dark blue crystals, which were the products of living cells, were dissolved overnight at 37°C, and then the microplates were read on a microplate reader, using a wavelength of 570nm. From the beginning, the microplates were normally read within 45-60 minutes of adding the DMSO. Optical densities (OD) were obtained through the computer programme and the results printed out and kept for data analysis after being transferred to Excel 2003 spreadsheet. The value for the blank (DMSO) was subtracted from the raw absorbance values. All samples were analysed in quadruplicate and all procedures were carried out in the Type 2 cabinet hood (except during centrifugation and reading step of microplates) to pre-vent and avoid any possible contamination.

Stock solutions of the drugs:

Doxorubicin, verapamil and fluphenazine (always freshly prepared) were dissolved in dH2O at a final concentration of 1mM, was aliquoted and stored at -20°C. Indomethacin dissolved in ethanol and quercetin dissolved in DMSO at a final concentration of 1mM aliquoted and stored at -20°C until further usage.

Doubling time of the Raji cells sub-lines:

The number of cells aliquoted to each well was determined as a function of the population doubling time of each sub-line using the linear relationship between absorbance and the number of cells in the growth medium during the incubation time. The basis of this test is that any drugs, regardless of site or mechanism of action will interfere with the growth of each cell sub-line and there would be a reduction of the growth rate reflected by cell number. The growth characteristics of Raji sub-lines were studied by counting the number of the cells over a 5-day period from an initial plating density 2 x 103 cells/well. This ensures that each chemosensitivity study was carried out during the logarithmic growth phase of each Raji cells sub-line. Table 1 shows the results.

Treatment of Raji cells:

Raji cells were seeded in 96 well microplates at 2 x 103cells/well in 100µl of the complete growth medium. This cell concentration ensured logarithmic cell growth during the assay. The two far left and right columns were filled with cell culture medium alone to reduce evaporation and avoid any possible contamination from the remaining inner columns. Quadruplicate wells were used for the control, control plus chemosensitiser, doxorubicin alone, doxorubicin in combination with the chemosensitiser. The mean concentration was determined in three independent experiments, each performed in quadruplicate, as mentioned above.

Results

The results showed that DOX was able to inhibit proliferation of the Raji cell sub-lines alone and in combination with different sub-groups of chemosensitizers. How-

ever, Tween-20 and Triton X-100 significantly enhanced the inhibitory effects of DOX in combination indicating the disruption of cell membranes. Both cell sub-lines behaved in a similar manner.

Figure 1 Treatment of Raji (TK+) cells with different subgroups of p-glycoprotein reversal drugs after 48 hours incubation at 37°C and 5% CO2. Black columns show the cells alone, white columns show the cells which were treated with a chemosensitiser, black columns with white dots show the cells treated with 25nM DOX and white columns with black dots show the cells which were treated with 25nM DOX with chemosensitiser. Means of optical densities were determined and analysed by a two tailed Student t-test and bars indicate mean ± SE for two independent experiments.

Figure 2 Treatment of Raji (TK-) cells with different subgroups of p-glycoprotein reversal drugs after 48 hours incubation at 37°C and 5% CO2. Black columns show the cells alone, white columns show the cells which were treated with a chemosensitiser, black columns with white dots show the cells treated with 25nM DOX and white columns with black dots show the cells which were treated with 25nM DOX with a chemosensitiser. Means of optical densities were determined and analysed by a two tailed Student t-test and bars indicate mean ± SE for two independent experiments.

Discussion

The NHL in vitro model was able to demonstrate manipulation and modulation of cells in the presence of a chemosensitiser to overcome the chemoresistance obstacle, when using a low dose of DOX. It is clear that the p53 tumour suppressor gene is vital in assuring genome stability.[20] DNA double-strand breaks represent chromosomal lesions that can lead to mutation, tumour transformation, or apoptosis. Double-strand breaks can occur as a result of chemotherapy. For example, the cytotoxic activity of DOX appeared to correlate more closely with the formation of DNA double-strand breaks than with single strand lesions.[21] Mammalian cells possess potent and efficient mechanisms to repair double strand breaks, and thus complete normal development as well as mitigating oncogenic potential and preventing cell death. When the repair process of double-strand breaks fail, chromosom-

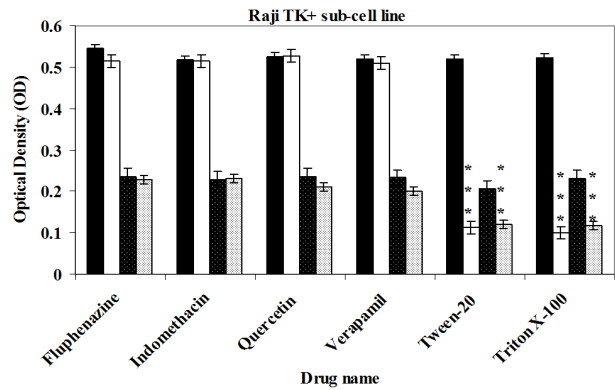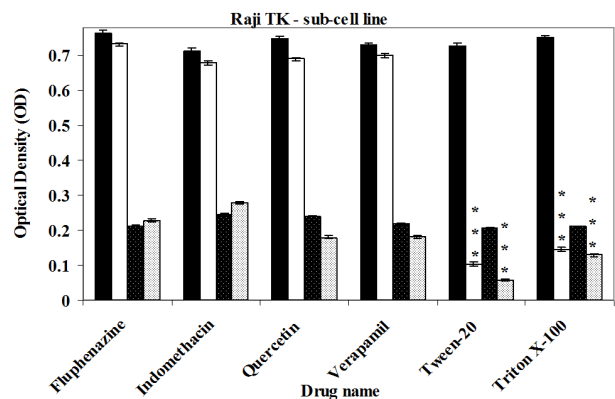| Table 1. Logarithmic growth phase of each Raji cells sub-line	
Cell sub-line (name)	Doubling time (hour)
Raji TK+	23 ± 1.5
Raji TK-	20 ± 2

Figure 1:

Figure 2:

al instability results and can be associated with tumour formation or progression. This is possibly what has happened in NHL patients during chemotherapy, in particular in p53+ patients, due to the lack of repair in these patients.

Studies of mice deficient in various components of the non-homologous end joining pathways of repair of double-strand breaks have revealed key roles in both the developmental programme of B- and T-lymphocytes as well as in the maintenance of general genome stability.[22]

The current thinking about double-strand breaks and their lack of repair is that they cause chromosomal instability resulting in a MDR phenotype. Instead of making the genome more unstable (by continuing chemotherapy for resistant patients), it may be better to keep the genome stable in NHL patients. According to the present study, it appears that stability can be maintained by reducing the dose of DOX and combining it with a PGP reversal drug such as Tween-20 or Triton X-100 in resistant patients with p53 over-expression. In such resistant NHL patients at least 50% of their cells are not operating effectively (normally). Our earlier results have shown that p53+ NHL cells with alkylating damage are irreparable (23). Continuing conventional chemotherapy is probably not very beneficial for resistant patients due to their lack of repair, which might also associated with MGMT depletion. This could increase the risk of further mutation, which could cause even more chromosomal lesions as an additive result of the p53 deficient repair machinery.[24] Hence it may be more appropriate to modulate the effects of DOX in combination with ideally non-genotoxic chemosensitizers in the presence of the mutant p53 to overcome the phenomenon. Therefore, using some of the PGP reversal agents could be a promising way forward not only for NHL resistant patients, but also for the other human tumours resistant to the standard regimens. With regards to alkylating damage to DNA, a study of cyclophosphamide induced DNA damage and repair occurring in vivo was conducted in the brown Norway rat myelocytic leukaemia model by Wang and his colleagues.[25] DNA single-strand breaks, DNA-DNA interstrand cross-links, DNA-protein cross-links, and DNA double-strand breaks were measured by alkaline and neutral elution in their study. No SSB or DSB were observed at 4 h after the cyclophosphamide injection over the dose range of 15-250 mg/kg, but a low level of SSB was observed at 18-28 h after cyclophosphamide treatment. These data suggest that the cytotoxic effects of cyclophosphamide were mediated mostly by DNA interstrand cross-links and DNA-protein cross-links. Single strand breaks appearing late after cyclophosphamide injection in vivo may be a reflection of repair of DNA-DNA interstrand cross-links and DNA-protein cross-links and an indication of the optimal timing for administration of DNA-repair inhibitors. This observation is of interest because DSB can potentate the

instability of the genome of the NHL patients. Therefore the therapeutic benefit of cyclophosphamide in this model, when it is given during the CHOP regimen might help resistant patients. In contrast the study by Valenzuela and her colleagues revealed that DOX induced DSB. They evaluated their results by using pulsed-field gel electrophoresis and compared with cellular effects, PGP expression and intracellular glutathione levels.[26] Nevertheless, the lack of repair capacity which has been demonstrated in p53+ NHL patients makes the cyclophosphamide option of limited benefit.

Based on the present results, an alternative option for p53+ (resistant) cells is to use a PGP reversal agent in combination with DOX, but reducing the dose of DOX when combined with such an agent as suggested by the results which have been achieved in the present study. Tween-20 and Titon-X100 showed highly significant differences in growth arrest in both cells sub-lines alone and a combination with DOX suggesting the use of liposomal doxorubicin delivery as a new administration for p53+ (resistant) NHL patients. This should be an effective strategy for standard regimens based on doxorubicin (doxorubicin-based regimens). The results also suggest a new effective strategy for pharmaceutical industries. Another limitation factor in NHL resistant patients reflects the fact that there is also some evidence that chemotherapy can produce secondary cancers.[27] The most serious side effect is the possibility of developing a second tumour known as acute myeloid leukaemia. This complication is quite rare, and the importance of chemotherapy in treating non-Hodgkin's lymphoma in children is more important than the risk, which means that cyclophosphamide might be useful in young resistant patients and perhaps not elderly patients. Therefore liposomal doxorubicin might help in elderly patients with p53 over-expression. Thus the identification of novel early biomarkers from drug-induced toxicity could aid drug discovery by improving the toxicity prediction process. However, the question is how to minimize the cardiotoxic effect but maintain the antitumor effect of DOX.[28-29] It is well known that the lack of a complete and long-lasting response to chemotherapy is a main drawback limiting the clinical potential of DOX in NHL treatment. Proteomic techniques have identified novel biomarkers with the potential to enable prediction of response to anticancer therapy, particularly chemore-

sistance. Before these "potential prognostic markers" are applied clinically, however, further studies are required to validate whether these alterations are the cause or the result of resistance to anticancer therapy.[30]

References

1. Lum BL, Fisher GA, Brophy NA, Yahanda AM, Adler KM, Kaubisch S, et al. Clinical trials of modulation of multidrug resistance. Pharmacokinetic and pharmacodynamic considerations. . Cancer. 1993;72(11 Suppl):3502-14.

2. Lum BL, Gosland MP, Kaubisch S, Sikic BI. Molecular targets in oncology: implications of the multidrug resistance gene. . Pharmacotherapy. 1993;13:88-109. .

3. Coley HM, Twentyman PR, Workman P. The efflux of anthracyclines in multidrug-resistant cell lines. Biochem Pharmacol. 1993;Oct 19;46(8):1317-26. .

4. Tsang WP, Chau SP, Fung KP, Kong SK, Kwok TT. Modulation of multidrug resistance-associated protein 1 (MRP1) by p53 mutant in Saos-2 cells. Cancer Chemother Pharmacol. 2003;51:161-6.

5. Pugacheva EN, Ivanov AV, Kravchenko JE, Kopnin BP, Levine AJ, Chumakov PM. Novel gain of function activity of p53 mutants: activation of the dUTPase gene expression leading to resistance to 5-fluorouracil. Oncogene. 2002;Jul 11;21(30):4595-600.

6. Zalcenstein A, Stambolsky P, Weisz L, Muller M, Wallach D, Goncharov TM, et al. Mutant p53 gain of function: repression of CD95(Fas/APO-1) gene expression by tumor-associated p53 mutants. Oncogene. 2003 Aug 28;22(36):5667-76.

7. El-Hizawi S, Lagowski JP, Kulesz-Martin M, Albor A. Induction of gene amplification as a gain-of-function phenotype of mutant p53 proteins. Cancer Res. 2002 Jun 1;62(11):3264-70.

8. Marin MC, Jost CA, Brooks LA, Irwin MS, O'Nions J, Tidy JA, et al. A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nat Genet. 2000 May;25(1):47-54.

9. Strano S, Munarriz E, Rossi M, Cristofanelli B, Shaul Y, Castagnoli L, et al. Physical and functional interaction between p53 mutants and different isoforms of p73. J Biol Chem. 2000 Sep 22;275(38):29503-12.

10. Bergamaschi D, Gasco M, Hiller L, Sullivan A, Syed N, Trigiante G, et al. p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell. 2003 Apr;3(4):387-402.

11. Monti P, Campomenosi P, Ciribilli Y, Iannone R, Aprile A, Inga A, et al. Characterization of the p53 mutants ability to inhibit p73 beta transactivation using a yeast-based functional assay. Oncogene. 2003 Aug 14;22(34):5252-60.

12. Strano S, Fontemaggi G, Costanzo A, Rizzo MG, Monti O, Baccarini A, et al. Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. J Biol Chem. 2002 May 24;277(21):18817-26.

13. Pulvertaft JV. Cytology of Burkitt's Tumour (African Lymphoma). Lancet. 1964 Feb 1;1(7327):238-40.

14. Ngu VA. The African Lymphoma (Burkitt Tumour): Survivals Exceeding Two Years. Br J Cancer. 1965 Mar;19:101-7.

15. Evens AM, Gordon LI. Burkitt's and Burkitt-like lymphoma. Curr Treat Options Oncol. 2002 Aug;3(4):291-305.

16. Moro F, Ottaggio L, Bonatti S, Simili M, Miele M, Bozzo S, et al. p53 expression in normal versus transformed mammalian cells. Carcinogenesis. 1995 Oct;16(10):2435-40.

17. Hirokawa M, Kawabata Y, Miura AB. Dysregulation of apoptosis and a novel mechanism of defective apoptotic signal transduction in human B-cell neoplasms. Leuk Lymphoma. 2002 Feb;43(2):243-9.

18. Kawabata Y, Hirokawa M, Kitabayashi A, Horiuchi T, Kuroki J, Miura AB. Defective apoptotic signal transduction pathway downstream of caspase-3 in human B-lymphoma cells: A novel mechanism of nuclear apoptosis resistance. Blood. 1999 Nov 15;94(10):3523-30.

19. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983 Dec 16;65(1-2):55-63.

20. Griffiths SD, Clarke AR, Healy LE, Ross G, Ford AM, Hooper ML, et al. Absence of p53 permits propagation of mutant cells following genotoxic damage. Oncogene. 1997;14(5):523-31.

21. Goldenberg GJ, Wang H, Blair GW. Resistance to adriamycin: relationship of cytotoxicity to drug uptake and DNA single- and double-strand breakage in cloned cell lines of adriamycin-sensitive and -resistant P388 leukemia. Cancer Res. 1986 Jun;46(6):2978-83.

22. Zhu C, Mills KD, Ferguson DO, Lee C, Manis J, Fleming J, et al. Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations. Cell. 2002 Jun 28;109(7):811-21.

23. Foroutan B, Ali Ruf A, Jerwood D, Anderson D. In vitro studies of DNA damage and its repair in cells from NHL patients with different p53 mutant protein status, resistant (p53(+)) and sensitive (p53(-)) to cancer chemotherapy. J Pharmacol Toxicol Methods. 2007 Jan-Feb;55(1):58-64.

24. Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene. 1993 Feb;8(2):307-18.

25. Wang JY, Prorok G, Vaughan WP. Cytotoxicity, DNA cross-linking, and DNA single-strand breaks induced by cyclophosphamide in a rat leukemia in vivo. Cancer Chemother Pharmacol. 1993;31(5):381-6.

26. Valenzuela MT, Nunez MI, Villalobos M, Siles E, Olea N, Pedraza

V, et al. Relationship between doxorubicin cell sensitivity, drug-induced DNA double-strand breaks, glutathione content and P-glycoprotein in mammalian tumor cells. Anticancer Drugs. 1995;6(6):749-57.

27. Andre M, Mounier N, Leleu X, Sonet A, Brice P, Henry-Amar M, et al. Second cancers and late toxicities after treatment of aggressive non-Hodgkin lymphoma with the ACVBP regimen: a GELA cohort study on 2837 patients. Blood. 2004;103(4):1222-8.

28. Dziegiel P, Jethon Z, Suder E, Sopel M, Rabczynski J, Surowiak P, et al. Role of exogenous melatonin in reducing the cardiotoxic effect of daunorubicin and doxorubicin in the rat. Exp Toxicol Pathol. 2002 Feb;53(6):433-9.

29. Tham P, Dougherty W, Iatropoulos MJ, Gordon G, James VC, Hall C, et al. The effect of mitoxantrone treatment in beagle dogs previously treated with minimally cardiotoxic doses of doxorubicin. Am J Pathol. 1987 Jul;128(1):121-30.

30. Brady CA, Jiang D, Mello SS, Johnson TM, Jarvis LA, Kozak MM, et al. Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell. 2011 May 13;145(4):571-83.