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ISSN: 1538-4047 (Print) 1555-8576 (Online) Journal homepage: http://www.tandfonline.com/loi/kcbt20

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To cite this article: Amr Y. Esmat, Catherine Tomasetto & Marie-Christine Rio (2006) Cytotoxicity of a natural anthraquinone (Aloin) against human breast cancer cell lines with and without ErbB-2: Topoisomerase II-alpha coamplification, Cancer Biology & Therapy, 5:1, 97-103, DOI: 10.4161/cbt.5.1.2347

To link to this article: http://dx.doi.org/10.4161/cbt.5.1.2347

Published online: 16 Jan 2006.

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Research Paper

Cytotoxicity of a Natural Anthraquinone (Aloin) Against Human Breast Cancer Cell Lines with and without ErbB-2-Topoisomerase IIα Coamplification

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Received 10/18/05; Accepted 11/22/05

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/abstract.php?id=2347

KEY WORDS

Aloin, breast cell lines, growth inhibition assays, flow cytometry, fluorescence microscopy, Western blot assays

ACKNOWLEDGEMENTS

The authors would like to thank Claudine Ebel for her skillful technical assistance with the flow cytometry.

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ABSTRACT

In the present study the cytotoxic activity of aloin, a natural anthracycline from Aloe plant, is reported against two human breast cancer cell lines; without (MCF-7) and with (SKBR-3) erbB-2-topoIIα coamplification. MCF-7cell line was shown to be more sensitive to aloin than SKBR-3 demonstrated by MTT and clonogenic assays, from which IC_{50} and 50% ICF values are reported to be 60 μg/ml, respectively, in the former cell line and as high as 150 and 80 μg/ml, respectively, in the latter, which are still far below the maximum tolerated dose of the compound. The effect of aloin is suggested to be brought about by more than one mechanism depending on the dose level and tumor phenotype. This was demonstrated by flow cytometric analysis, fluorescence microscopy and western blot analysis, which revealed that aloin at higher concentrations caused a reduction in the proportion of cells undergoing mitosis by induction of apoptosis, inhibition of topo II α protein expression and downregulation of cyclin B1 protein expression in MCF-7 cell line, whereas erbB-2 protein expression was not affected. Topo IIα protein expression was mildly downregulated in SKBR-3 cell line at higher concentrations only.

INTRODUCTION

In breast cancer several DNA aberrations have been recognized, including gene amplification and loss of different chromosomal regions, which affect the sensitivity of tumors to chemotherapy. c-ErbB-2 oncogene, localized on chromosome 17q12-21, is the most frequently amplified oncogene in breast cancer.[1] Initially detected as a transforming gene, neu, in rat neurogliobastoma,[2] the human analogue, erbB-2 (Her-2), was identified as closely similar to the epidermal growth factor receptor gene, which encodes for a transmembrane tyrosine kinase receptor protein.[3] This highly glycosylated protein is a member of the epidermal growth factor receptor (EGFR) family,[4] and is expressed on most cells of epithelial origin. In vitro, overexpression of c-erbB-2 in epithelial cells ultimately affects the regulation of cell proliferation, of apoptotic pathways, of motility and of adhesion.[5] c-ErbB-2 amplification in a large panel of invasive breast tumors strongly correlates to most commonly risk factors; large tumor size, lymph node positivity, advanced stage, steroid receptor negativity, aberrant DNA content and increased rate of cell proliferation.[6]

The α isoform of topoisomerase (EC5.99.1.3) is a key enzyme in DNA replication and also a target for various chemotherapeutic agents. The gene is located in close proximity to the c-erbB2 gene on chromosome 17q21 and encodes for a 170 KDa protein. The enzyme catalyzes the unwinding of the DNA to a partly uncoiled form by inducing single-stranded breaks on both DNA strands. These breaks allow the passage of double-stranded DNA through the gap.[7] TopoIIα-targeting anticancer agents are classified into poisons and catalytic inhibitors. TopoII poisons act at a stage of the catalytic cycle of the enzyme where the gate DNA strand is open or cleaved, thus stabilize a covalent topoII-DNA intermediate (the cleavable complex), such as the anthracyclines doxorubicin and daunorubicin, the epipodophyllotoxins etoposide and teniposide and amsacrine. TopoII catalytic inhibitors interact with the enzyme at a stage where there are no DNA breaks, i.e the gate DNA strand is intact without induction of cleavable complex formation, such as bisdioxopiperazines.[8] Thus, topoIIα is considered a potential predictive marker of responsiveness to these agents. Because of the physical proximity to erbB-2, copy number aberrations may also occur in the topoIIα gene. It is reported that 40% of primary breast carcinoma showed erbB-2-topoIIα coamplification[9] and 36.9% in another study,[10] as well as 42% showed a physical deletion of the topoIIα gene.[11] Since anthracyclines are topoIIα

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inhibitors and it is frequently coamplified with erbB-2, thus erbB-2 may be serving as a surrogate. Contrary to erbB-2, where gene amplification is almost always correlated with protein overexpression in breast cancer, topoII gene amplification apparently does not always lead to protein overexpression. Other factors, specifically the tumor proliferation status, may interfere with topoII protein status.[10] These topoIIα gene aberrations may contribute to chemoresistance to topoIIα inhibitors that breast cancers with erbB-2 amplification are known to have.[12-14] However, there are also clinical trials reporting either no association,[15,16] or even a higher likelihood for a response in erbB-2-amplified tumors.[17,18] In an attempt to explain the putative association between erbB-2 and altered sensitivity to anthracyclines, Coon et al[19] reported that amplification of erbB-2, but not overexpression is significantly associated with a favorable local tumor response to anthracycline-based chemotherapy in breast cancer, whereas both of gene amplification and expression of topoIIα are consistent with sensitivity to anthracyclines.

Anthracyclines are one of the most widely used class of cytotoxic agents for the treatment of breast cancer. However, one of the most important long term complications associated with doxorubicin-based therapy is cardiotoxicity and the development of acute myelocytic leukemia (AML).[20] Thus, one of the main challenges in treatment of breast cancer is to find new drugs for the use in adjuvant therapy that ensures the most benefits and the least harm for the patient. Plant drugs still constitute an important part of the medicinals used today and the remarkable properties of some of these would be very difficult to replace with synthetic drugs. The principal constituent of the different aloe varieties is a pale yellow anthraquinone glycoside; aloin. The empirical formula of aloin is $C_{21}H_{22}O_9$, which supported its formulation as 10- β -D-glucopyranosyl 1, 8 dihydroxy 3 hydroxymethyl anthracene 9 one.[21] Previously, we have demonstrated the antitumor activity of aloin against experimental murine tumors in vivo (ascites and solid Ehrlich carcinoma) with no detrimental side effects to the host,[22-24] as well as human breast ($T_{47}D$) and ovarian tumour cell lines.[25] Recently, we have shown that aloin at high concentrations causes an increase in the S-phase fraction of breast and ovarian tumor cells and the appearance of cells cycling at a higher ploidy level (> G_2M), which indicates that aloin did not inhibit DNA synthesis and that cells replicated a full complement of DNA but had difficulty in mitosis.[26] Taken together, this suggests strongly that aloin exerted its cytotoxicity through catalytic inhibition of topoIIα enzyme.

This study was undertaken to assess the cytotoxic effect of aloin against human breast cancer cell lines, with or without erbB-2 and topoIIα coamplification and on topoIIα protein expression, which is considered a potential predictive marker of response to anthracyclines in breast cancer. We also sought the contribution of aloin to induction of apoptosis and inhibition of cytokinesis.

MATERIALS AND METHODS

Cells and cell culture. Two human breast cell lines were used throughout this study: MCF-7 (ER and PgR + ve), which exihibts an aneuploid karyotype with a modal chromosome number of 65-88 and SKBR-3 (ER and PgR -ve) with a modal chromosome number of 79-84. SKBR-3 has a high level of erbB-2 oncogene amplification and protein expression and a low level of topoIIα gene amplification and protein expression (nine copies of topoIIα and six copies of chromosome 17 centromere).[11] The detailed characteristics of the tumor cell lines are described elsewhere.[27] Cells were grown in Dulbeccco's minimum essential medium (DMEM) enriched with 10% fetal calf serum, 2 mM glutamine, 50 μg/ml gentamycin in an atmosphere of 5% CO_2 and 95% air at 37°C. Cell culture medium for MCF-7 was supplemented with bovine insulin (0.6 μg/ml). Subculturing was routinely carried out every week using diluted trypsin solution (0.25%) in Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (pH 7.2).

Drug treatment. Aloin was obtained in a pure powder form (MW 418.4, Macfarlan Smith LTD, Edinburgh). A stock solution (5 mg/ml DPBS) was freshly prepared and sterilized before use by filtration through 0.22 μm filters (Millex, Millipore Co.). Seven increasing drug concentrations (40, 60, 80, 100, 150, 200, 300 μg/ml) were prepared by diluting the stock solution in cell culture medium. The tested doses were chosen in terms of its LD_{50} value and maximum tolerated dose (MTD) from our previous pharmacokinetic studies.[24]

Chemosensitivity assays. The cytotoxicity of aloin against the two human breast cancer cell lines was assessed by 2 experiments: MTT and clonogenic assays.

MTT assay. For each cell line, exponentially growing cells were enzymatically detached and a single tumor cell suspension in culture medium at a density of 30 x 10^3 cells/ml was prepared. Cells were seeded in 96-well microtiter plates (200 μl /well) and allowed to attach for 24 h under the previous specified conditions. Culture medium in each well was aspirated and replaced with fresh culture medium containing the variable drug concentrations and allowed to grow for a further 96 h. Five wells were used for controls and each dose level. The cell viability was then determined by the MTT assay[28] with minor modifications. In brief, 100 μl of MTT (2 mg/ml in DPBS) (3-(4,5dimethylthiazol-2-yl) 2,5 diphenyl-tetrazolium bromide) were added and the plates were incubated at 37°C for 2 h in the dark. This assay is based on the cleavage of the tetrazolium salt by viable cells and the accumulation of a water insoluble formazon salt proportional to the number of living cells in the well. After careful aspiration of the culture medium 150 μl of DMSO were added to each well and the plates were incubated for a further 1 h. The plates were read at wavelength 595 nm with reference to the appropriate blank (DMSO only) in a microplate spectrophotometer (Biorad laboratories). The percentage of cell viability was calculated by multiplying the ratio absorbance of the sample versus the control by 100. Drug IC_{50} was determined as aloin concentration showing 50% cell growth inhibition as compared with control cell growth.

Clonogenic assay. Cells were seeded in 24-well plates (1ml/well) at the previously mentioned conditions. The cell density of the suspension used for plating was adjusted to 3000 cells/ml for MCF-7 cell line and 15000 cells/ml for SKBR-3 cell line (owing to the slower growth rate of the latter). After 24 h of plating, the culture medium was removed and replaced with 1 ml of fresh culture medium containing the different drug concentrations. Multiple plates were prepared to establish five replicates for controls and each drug dose level. The medium was replaced twice a week. At the end of the treatment period (two weeks), colonies were fixed in absolute methanol for 20 min., stained with 2% crystal violet and counted. At least 200 tumor cell colonies per flask were required in the control wells to assure an adequate range for measurement of drug effect. The mean colony count for control was taken as 100% survival (0% inhibition) and the percent inhibition of colony formation (ICF%) in drug-treated wells was calculated.

Flow cytometry. Cell apoptosis by the propidium iodide method was quantified by FACscan flow cytometer (Becton Dickinson). In brief, cells (3.5 x 10^6 cells/100 ml/dish) were harvested by trypsinization at 96 h after treatment with two concentrations of aloin (300 μg/ml and IC_{50} value). The cells were washed twice with DPBS and centrifuged (1200 rpm for five min). Cells were fixed in 70% ice cold ethanol overnight at 4°C. Samples were subsequently washed twice with DPBS, resuspended in DPBS containing 0.2 mg/ml RNase A and 0.02 mg/ml propidium iodide, gently vortexed and incubated at 4°C in the dark for at least half an hour before analysis.[29] For each sample, an average of 10 x 10^3 cells were acquired. Apoptotic cells appeared as a broad hypodiploid DNA peak before G_1 phase of the cell cycle.

Fluorescence microscopy. Induction of apoptosis or loss of cell viability after aloin treatment was verified by Olympus BX50 epifluorescence microscope equipped with a dual band pass fluorescence filter after double staining with ethidium bromide and acridine orange solution.[30] This method depends on the loss of plasma membrane integrity as cells die. Acridine

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orange and ethidium bromide excite a green and orange fluorescence, respectively, when they are intercalated into DNA. Acridine orange is a vital dye and can cross the plasma membrane and thus will stain the viable cells uniformely green and early apoptotic cells, which have still intact plasma membranes green with yellowish dots as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore, stain orange with condensed and often fragmented nuclei. Cells were plated and incubated with aloin exactly as in FACs analysis, except that adherent cells were harvested by scrapping in 1 ml of DPBS. Single cell suspension was obtained by repeated syringing then an aliquot of 25 µl was mixed well with 1 µl of AO/EB solution (100 µg/ml,1:1) and examined immediately.

Western blot analysis. Cells were plated and incubated with the increasing aloin concentrations as previously mentioned then adherent cells were harvested by scrapping in DPBS. Three plates were set up for control and each dose level. Pooled cell suspension was centrifuged at 3000 rpm for three min at 4°C and the supernatant was discarded. Cells were lysed in a hypotonic ice cold lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA,1%Triton X-100) containing diluted protease inhibitor cocktail (x 1000) (10 µl/ml lysis buffer). After three freeze/thaw cycles in liquid nitrogen, lysates were centrifuged at 10,000 rpm for 10 min at 4°C. Protein concentration was determined in the cytosolic samples of whole cell lysates by the Bio-Rad dye reagent. Extracts were then diluted in lysis buffer to equal protein concentrations and stored at -80°C until analysed. SDS-PAGE was performed according to Laemmli.[31] Proteins (50 µg) in sample buffer were heated at 90°C for 5 min then loaded on 10% gels (75 µg protein on 7% gel in case of topoIIα only), electrophoresed and electrotransferred onto nitrocellulose membranes. Transfer efficiency was routinely monitored by staining the membranes with PonceauS. Nonspecific antibody bindings were blocked by preincubation of the membranes for 1 h with 5% skim milk suspension, then the membranes were incubated for 16 h at 4°C with the following diluted antibodies: monoclonal mouse anti-erbB-2 (1:1000), polyclonal rabbit anti-topoIIα (1:200), polyclonal rabbit anti-cyclin B1 (1:500) and polyclonal rabbit anti-p53 (1:500). All antibodies were provided from Santa Cruz Biotechnology, except for erbB-2 (Calbiochem Biosiences). The membranes were furtherly incubated for 1 h at 4°C with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:10000, Santa Cruz Bio-technology. (After each incubation the membranes were washed thrice (PBS,0.1% Tween-20), the peroxidase-catalyzed reaction was visualized with enhanced chemiluminescence (Pierce, Perbio Science) and the membranes were exposed to x-ray films. All experiments were made in triplicates.

RESULTS

Effect of aloin treatment on cell growth inhibition. Representative cytotoxicity profiles for aloin against MCF-7 and SKBR-3 cell lines are shown in Figure 1A–C). The addition of increasing concentrations of aloin (40–300 µg/ml) in the culture medium of MCF-7 and SKBR-3 cells (96 h in MTT assay and two weeks in clonogenic assay) reduced the percentage of cell viability and increased the percentage of inhibition of colony formation, in a dose-dependent manner, in both cell lines. MCF-7 cell line was shown to be more sensitive to aloin (IC_{50} value and 50% ICF at 60 µg/ml, respectively) than SKBR-3 cell line (IC_{50} value at 150 µg/ml and 50% ICF at 80 µg/ml). Complete inhibition of colony formation was attained at aloin concentrations of 100 and 150 µg/ml in MCF-7 and SKBR-3 cell lines, respectively and so forth (Fig. 1C). At these specified concentrations, MTT absorbances in MCF-7 and SKBR-3 tumor cells leveled at 0.38 ± 0.02 and 0.65 ± 0.07 absorbance units, respectively, which represents 36.20 and 41.40 % of the absorbances obtained for respective control cells (Fig. 1A). This means that although cell growth was completely halted, yet these ceased dividing cells were viable and had the ability to reduce MTT.

Effect of aloin on induction of apoptosis in MCF-7 and SKBR-3 cell lines by FACs and fluorescence microscopy. Cell cycle analysis of MCF-7 cells in absence of aloin showed that 61.51% of cells were in G_1 phase, 28.89% in S phase and 9.6% in G_2M phase. Incubation of MCF-7 cells with the highest concentration of aloin (300 µg/ml) produced an apoptotic rate of 32.75%, a decrease in the percentage of cells at G_1 phase and in contrast an increase in the percentage of cells cycling in S and G_2M phases (50.30, 33.02 and 16.69% in G_1, S, G_2M phases, respectively). Addition of aloin at 60 µg/ml (dose which produced IC50 value) showed a parallel cell distribution in the cell cycle phases, compared to control cells (63.56 % in G_1 phase, 26.50% in S phase and 9.94% in G_2M phase), with no evidence of apoptosis (Fig. 2A–C). On the other hand, flow cytometric assay of SKBR-3 control cells showed that 77.3, 12.97 and 9.73 % of cells were in G_1, S and G_2M phases, respectively. A less notable apoptotic rate of 14.33% was noticed in SKBR-3 cells after incubation with aloin at 300 µg/ml. Exposure of SKBR-3 cells to 300 µg/ml and 150 µg/ml (IC_{50} value) caused a dose-dependent increase of cells cycling in S and G_2M phases (71.88& 73.29%, 14.09 &13.78% and 14.04&12.93% in G_1, S and G_2M phases, respectively) (Figs. 3A-C). Fluorescence microscopy evaluation of MCF-7 and SKBR-3 cells following exposure to aloin at the highest concentration (300 µg/ml) revealed late apoptotic features in the former cell line (stained orange) (Figs. 4A and C) and early apoptotic features in the latter (stained green) (Figs. 4B and D), characterized with an increase in cell size, blebbing of cytoplasm, chromatin condensation and nuclear fragmentation. These observations could account for the enhanced mitochondrial reduction of MTT at high concentrations.

Effect of aloin on topoIIα, p53 and cyclin B1 protein expressions in MCF-7 cells. No immunoreactive bands were seen in Western blots for topoIIα at high concentrations of aloin (300 and 200 µg/ml), whereas faint bands appeared at lower concentrations (150 and 100 µg/ml) then persistent bands at low concentrations (80, 60 and 40 µg/ml), compared to control (Fig. 5A). As to cyclin B1protein expression, it was found to be strongly downregulated at concentrations range of 80–300 µg/ml, while moderately affected at 60 and 40 µg/ml, compared to the control (Fig. 5C). In contrast to topoIIα and cyclin B1, strong bands for p53 protein overexpression were identified at the three highest concentrations (300, 200 and 150 µg/ml), which were less stronger at dose levels of 100 and 80 µg/ml and normal at 60 and 40 µg/ml (Fig. 5B).

Effect of aloin on ErbB-2 and topoIIα protein expression in SKBR-3 cells. Strong immunoreactive bands for erbB-2 protein expression were identified at 124 kDa in control and aloin-treated cells at the variable concentrations (Fig. 6A). One immunoreactive band was seen in the western blot at 170 KDa, which is the known molecular mass of topoIIα, at all tested aloin concentrations (Fig. 6B), however the band appeared less dense at the two highest doses (300 and 200 µg/ml) compared to control and other tested doses (150,100 and 80 µg/ml). This indicates that aloin had the ability to fairly downregulate topoIIα protein expression at high concentrations only.

DISCUSSION

In this article we have demonstrated the potential antitumor activity of the natural anthraquinone compound; aloin against two different human breast tumor cell lines; MCF-7 (no erbB-2 gene amplification and a normal copy number of topoIIα gene) and SKBR-3 (with erbB-2 and topoIIα coamplification). MCF-7 appeared to be more sensitive to aloin than SKBR-3 demonstrated by MTT and clonogenic assays, from which both of IC_{50} and 50% ICF values are reported to be 60 µg/ml, respectively, in the former cell line and as high as 150 and 80 µg/ml, respectively, in the latter (Figs. 1B and C). Likewise, colony formation was completely halted up in both cell lines at 100 µg/ml in MCF-7 and 150 µg/ml in SKBR-3. The less sensitivity of SKBR-3 to aloin is in harmony with previous studies, which consistently linked coexpression and coamplification of erbB-2 and topoIIα genes with adverse prognosis and sensitivity to anthracycline drugs.[19,32,33] In addition, it has been reported that cell lines transfected with erbB-2 and then exposed to doxorubicin in vitro does not show enhanced sensitivity to

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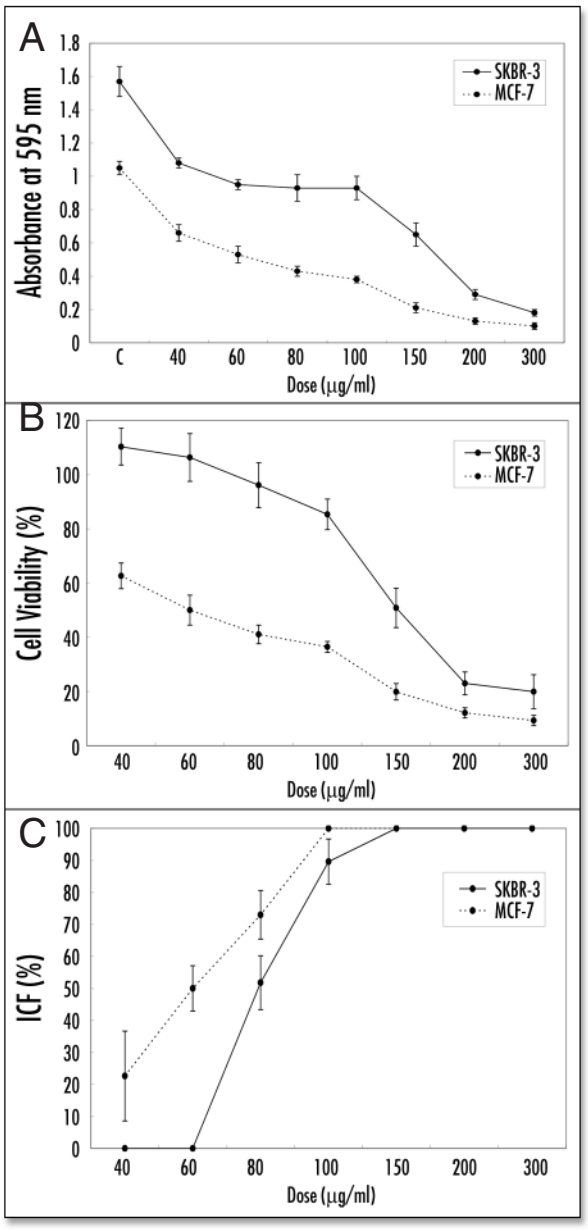

Figure 1. Effect of exposure of aloin at variable concentrations for 96h on the growth of attached MCF-7 and SKBR-3 cells. (A)MTT growth inhibition assay (B)Viability of aloin-treated MCF-7 and SKBR-3 cells (measured by MTT assay). (C) Colony formation assay after growth for two weeks. Error bars shown represent S.D. of five wells.

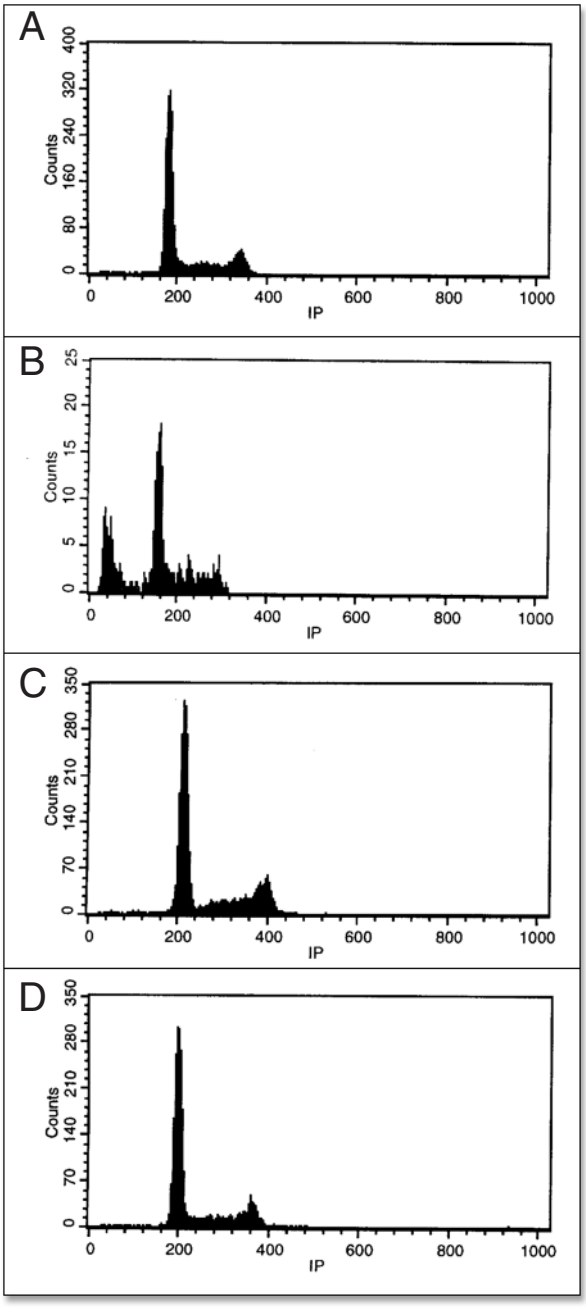

Figure 2. Analysis of the cell cycle patterns of MCF-7 cells treated with aloin. (A) Control cells. (B)Aloin-treated cells at 300 μg/ml. (C)Aloin-treated cells at 60 μg/ml (IC_{50} value).

chemotherapy relative to parent cell lines.[34] Recently, Fritz et al.[35] affirmed that patients lacking both erbB-2 and topoIIα overexpression have the best long-term survival, whereas patients overexpressing both erbB-2 and topo IIα show the worst disease outcome ($P < 0.0001$). The authors concluded that treatment with anthracyclines was not capable of reversing the negative prognostic impact of topoIIα or erbB-2 overexpression. Also, our findings are in line with other reports that positively correlated between the dose intensity of doxorubicin-based chemotherapy and response rates in patients with metastatic breast cancer and overexpressing erbB-2, which suggest that erbB-2 mediated drug resistance may be overcome by full doses of doxorubicin (overview in ref. 20).

It is of interest to note that cytotoxicity of aloin is mediated by more than one mechanism depending on the dose level and tumor phenotype. On the basis of dose level, this is well demonstrated by the western blots of MCF-7 cell line, which indicate clearly that maximal cytotoxic activity of aloin, which was attained at concentration 100 μg/ml and upward, was more likely due to inhibition of topoIIα protein expression and downregulation of cyclin B1 protein expression, which was not the case for lower concentrations (40–80 μg/ml)(Figs. 5A and C). TopoIIα is reported to be produced primarily in late S phase and during the G_2M phase of the cell cycle as it plays an essential role in cell cycle progression, with mutants incapable of

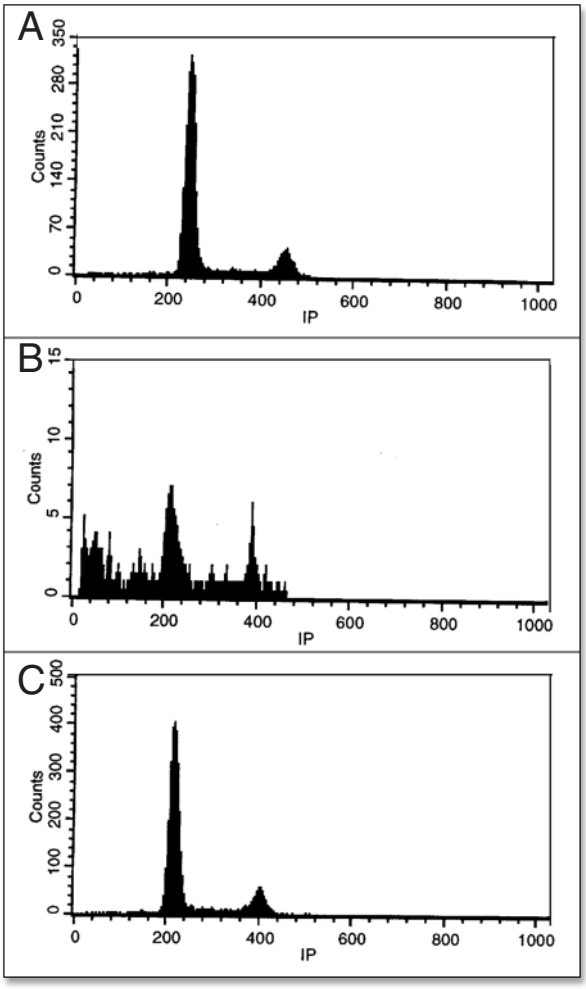

Figure 3. Analysis of the cell cycle patterns of SKBR-3 cells treated with aloin. (A) Control cells. (B)Aloin-treated cells at 300 µg/ml. (C)Aloin-treated cells at 150 µg/ml (IC_{50} value).

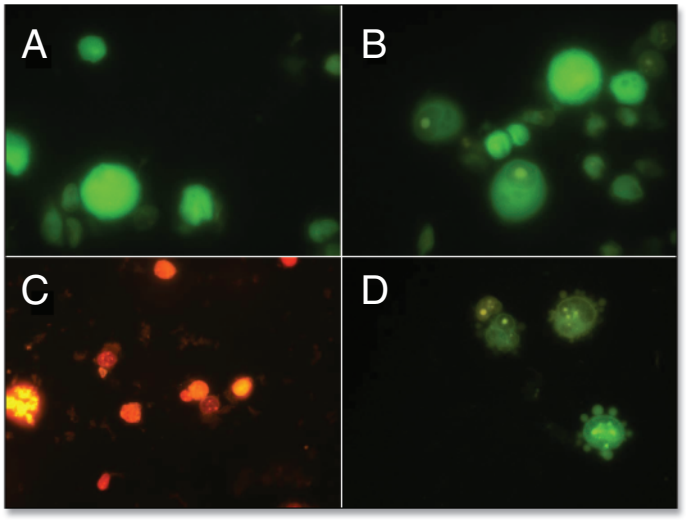

Figure 4. Fluorescent photomicrographs of control and aloin-treated MCF-7 and SKBR-3 cells for 96h at 300 µg/ml after staining with AO/EB solution (× 63). Control MCF-7 and SKBR-3 cells (stained green) (A and B). (C) Aloin-treated MCF-7 cells showing late apoptosis (stained orange with yellowish dots) with nuclear fragmentation. (D) Aloin-treated SKBR-3 cells showing early apoptosis (stained green with yellowish dots) with nuclear fragmentation and blebbing of cytoplasm.

chromosome segregation during mitotic division.[36] Also, it is known that cyclin B1 is important in G_2M progression and that reduced expression could result in accumulation of inactive Cdk1-cyclin B1 kinase complex, which when active regulates eukaryotic G_2M progression.[37] This suggests that the cytotoxic action of aloin at higher concentrations is brought about by inducing apoptosis and cell cycle arrest at G_2M. This postulation is emphasized by the subdiploid peak and the more pronounced increase in the number of cells cycling in S and G_2M phases (Figs. 2B and C), overexpressed p53 protein (Fig. 5B) and the late apoptotic features in cells stained with ethidium iodide and acridine orange mixture (Figs. 4A and C). The role of aloin in upregulating the intracellular apoptotic signaling events (caspases) is warranted since apoptosis is one of the most important mechanisms of chemotherapy-induced cytotoxicity. Anthracyclines are known to induce apoptosis through p53 protein and experimental tumors with a mutated p53 (inactive) are resistant to anthracyclines.[20] Also, Berruti and colleagues[38] reported an association between wild-type p53 protein and response to neoadjuvant doxorubicin-based chemotherapy. Thus, it may be assumed that one of the modes of action of aloin is its direct binding to the enzyme or causing deletions in topoIIα gene. This assumption is in line with the previous reports on in vitro model of lung cancer, in which topoIIα gene deletions were acquired during exposure to doxorubicin, which led to decreased topoIIα mRNA expression.[39,40]

On the basis of tumor phenotype, it was observed that although colony formation was completely halted at concentration 150 µg/ml and so forth in SKBR-3 cell line (Fig. 1C), yet topoIIα protein overexpression was not apparently affected by aloin treatment at the range of concentrations used, except for the first 2 high dose levels (Fig. 6B) and an increase in the percentage of cells cycling in S and G_2M phases was recorded at the highest concentration (300 µg/ml) (Fig. 3B). This demonstrates that the amount of the enzyme determines the amount of drug-stimulated inhibition of enzyme expression and also confirms that aloin may induce toxicity against SKBR-3 tumor cells via other non topoIIα-dependent mechanisms. A prospective study on a human breast cell line with topoIIα gene deletion (e.g., MDA-361) may be needed to further resolve this suggestion. Also, immunoblotting of erbB-2 shows that aloin has no direct effect on erbB-2 protein overexpression (Fig. 6A), which indicates that erbB-2 protein as a growth factor receptor does not interact with aloin physically like other topoIIα inhibitors. Since aloin is an anthraquinone like doxorubicin, then it may act as a strong chelating agent, i.e. able to remove metal ions from the active sites of metalloenzymes that are involved in cell growth and proliferation and thus could contribute, in some part, to its overall toxicity.[41]

It is of relevance to note in this connection that the reduced sensitivity of SKBR-3 cell line to aloin than MCF-7cell line may be correlated to alteration in topoIIα protein or in its regulation and this contrasts with the previous findings which have correlated erbB-2 amplification exclusively to chemoresistance to topoIIα inhibitors on one hand,[12,13,14] and with those that have implicated quantitative reduction in topoIIα protein expression in the drug resistance phenotype on the other hand.[42] However, it correlates well with our previous work,[25] in which the $T_{47}D$ breast tumor cell line, which contains less topoIIα protein expression than MCF-7 and SKBR-3,[43] showed a higher sensitivity towards aloin on continuous

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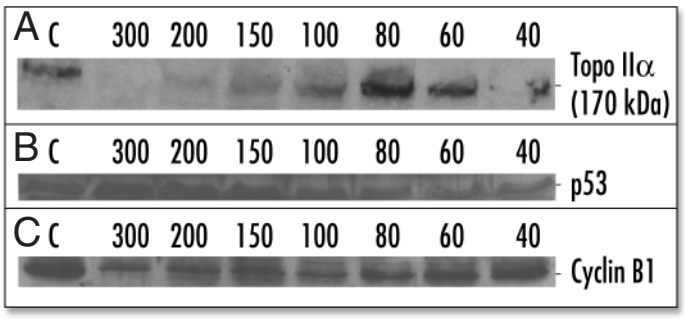

Figure 5. Western blots of cell extracts from aloin-treated MCF-7 cell line at variable concentrations (40–300 μg/ml) for 96 h representing protein expression of topollα(75 μg protein) (A), p53 (50 μg protein) (B) and Cyclin B1 (50 μg protein) (C).

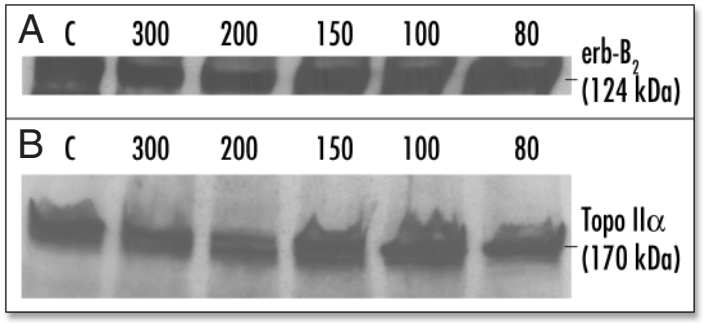

Figure 6. Western blots of cell extracts from aloin-treated SKBR-3 cell line at variable concentrations (80–300 μg/ml) for 96 h representing protein expression of topollα (75 μg protein) (A) and c-erbB-2 (50 μg protein) (B).

exposure (IC_{50} and 50% ICF values of only 20 μg/ml). Accordingly, our results are compatible with the former concept that amplification of target genes of the cytotoxic drugs is a common mechanism by which cancer cell lines confer resistance to cytotoxic drugs in vitro. For example, the cytotoxic actions of antitumor agents as coformycin and methotrexate can be overcome by the amplification of the genes (adenylate deaminase 2 and dihydrofolate reductase), which metabolize these drugs, in cancer cell lines.[44] Consistent with our findings, Houlbrook and his coworkers[43] affirmed that MCF-7 is more sensitive to topoIIα inhibitors, such as intercalating (adriamycin and mitoxantrone) and non intercalating topoIIα (epipodophyllotoxins or etoposide) inhibitors than SKBR3. Another factor that could contribute to the variable sensitivity of tumor cell lines to aloin and can not be disregarded is the differences in the proliferation rates of tumor cells. MCF-7, which was more sensitive to aloin than SKBR-3, was the fastest growing cell line (SPF was 28.89 and 12.97%, respectively)(Figs. 2A & 3A). This suggestion is consistent with the notion that high S phase fraction (SPF) of tumor cells increases the responsiveness to chemotherapy and consequently survival.[20]

In conclusion, the cell growth inhibitory activity of the natural anthracycline, aloin is reported in breast tumor cell lines with and without erbB-2 and topoIIα coamplification. However, it possesses a more pronounced impact on tumor without erbB-2 and topoIIα coamplification (MCF-7), confirmed with our previous work on other breast cell line ($T_{47}D$), which necessitates higher doses to attain the same effect produced in a tumor with erbB-2 and topoIIα coamplification (SKBR-3). Its dose- dependent cytotoxic action is mediated by inducing apoptosis and reducing the proportion of cells undergoing mitosis. Due to multiple modes of action, it could be suggested that aloin may be used in treatment of tumors with or without mutant p53, but it would be more useful in treating tumors without defects in their apoptosis-transduction machinery. The results of this exploratory study, in accordance with previous clinical studies, highlight the importance of FISH assay in evaluating the erbB-2 and topoIIα status of breast tumors in order to obtain the favorable response to anthracycline-based therapy. Assessment of aloin treatment on heart function in vivo is underway to verify the cardiotoxicity of aloin compared to doxorubicin.

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