Evaluation of Antiproliferative Activity of Enoxacin on a Human Breast Cancer Cell Line

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ABSTRACT Breast cancer is the leading cause of cancer in women world wide both in the developed and developing countries. Thus effective treatment of breast cancer with potential anti-tumor drugs is important. In this study, human breast cancer cell line MCF-7 has been employed to evaluate the antiproliferative activity of fluoroquinolone antibiotic enoxacin in culture. The present investigation shows that enoxacin induced growth inhibition of MCF-7 cells at significant level. The growth inhibition is dose dependent, time dependent and irreversible in nature. Increase in population doubling time and decrease in saturation density were also observed in enoxacin treated cells. Growth inhibitory effects were also found to be independent of concentration of serum growth factors in medium. Enoxacin also altered cellular morphology in *in vitro* culture condition. After enoxacin treatment accumulation of MCF-7 cells at G_2/M phase suggested cell cycle arrest and in turn inhibition of cell growth, which needs further investigation. Thus, this study clearly demonstrates that human breast cancer cell MCF-7 is highly responsive to fluoroquinolone antibiotic enoxacin treatment.

INTRODUCTION

Breast cancer is the second most prevalent cancer in the world today (Parkin et al. 2001). In India among the urban women the breast has now become the most frequent cancer site and the number of cases is increasing annually, due to both to aging of the population and increase in age–specific incidences (Sen et al. 2002). Case control studies in Mumbai and Chennai have identified nullparity, late age at marriage and late age at first pregnancy as important risk factors (Rao et al. 1994; Gajalakshmi and Shanta 1991). It has also been suggested that western dietary influences and the changed life style of urban women could be one of the major causes of the slowly rising incidence of breast cancer in India (Sen et al. 2002). Therefore early detection and search for potential antitumor compounds are important in the control of breast cancer.

Fluoroquinolone are commonly used broad-spectrum antibiotics, which are relatively non-toxic and inhibit type II DNA topoisomerase in

mammalian cells and bacterial DNA gyrase. This enzyme is responsible for replication, transcription, supercoiling and chromosomal separation of prokaryotic DNA (Chen and Liu 1994). Thus inhibition of this enzyme results into cytostasis and cell death. Few recent studies of fluoroquinolones have demonstrated a significant growth inhibition of some tumor cells including tranisitional cell carcinoma of bladder, colorectal carcinoma and prostate cancer cells (Aranha et al. 2000; Ebisuno et al. 1997; Herold et al. 2002; Aranha et al. 2003).

Considering the antiproliferative activity of some fluoroquinolone antibiotics on certain neoplastic cells and due to lack of such information on breast cancer cells, attempt has been made to evaluate the growth inhibitory activity of a relatively new fluoroquinolone antibiotic, enoxacin on MCF-7 cells and that may provide some new information about therapy of breast cancer.

MATERIALS AND METHODS

Cell Culture: Human breast cancer cell line, MCF-7, was obtained from National Cancer Institute, USA. The cells were maintained in RPMI 1640 medium (GIBCO, USA) containing 5% fetal bovine serum (FBS), supplemented with additional glutamine (0.03%) and 50 μg/ml gentamycin. Cells were allowed to grow in plastic

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tissue culture flasks (Corning, USA) and were kept in CO_2 incubator at 37 $^{\circ}$C in a humidified atmosphere of 5% CO_2 and 95% air. For experimental purpose cells from exponentially growing culture were used. All the experiments were repeated three times.

Drug: Enoxacin was purchased from Sigma Chemical Co., USA. Stock solution of drug was initially prepared in dimethyl sulphoxide (DMSO) and final concentrations of 10- 50 µg/ml were made by diluting the stock solution in complete growth medium. All control experiments were carried out in medium containing 0.25 % (v/v) DMSO.

Morphology: To study enoxacin induced morphological changes, $2 \text{ X } 10^4$ cells were plated on a coverslip contained in a 35mm petri dish (Corning, USA) with 1.5 ml of complete growth medium. After 24h, plates were treated with 40 µg/ml of enoxacin. After 72h of incubation, cells were fixed in Bouin's fluid, stained with hematoxylene-eosin (HE), mounted in D.P.X. and observed under microscope.

Cell Proliferation Assay: To assess the inhibitory effect of enoxacin on growth of MCF-7 cells in monolayer culture, Sulphorodhamine B assay (SRB assay) was performed as described by Monks et al. (1991) with some modifications. Briefly, MCF-7 cells were seeded in 96 well microtitre plates at a density of 3000 cells /well, allowed to adhere for 24h and were then treated with different concentrations of enoxacin (10-50 µg/ml) for 5 days. Drug treated plates were fixed at an interval of 24h in situ by 50% cold trichloroacetic acid (TCA) at a final concentration of 10 % and incubated at 4 $^{\circ}$C for 60 min. The plates were washed with deionized water, air dried, stained by 0.4% SRB dissolved in 1% acetic acid and incubated for 10 min at room tempreature. Unbound stain was removed by 1% acetic acid and air-dried. Bound stain was solublized with 10mM trizma base and optical density (OD) were measured at a wavelength of 515nm by an automated microplate reader. Finally from OD values percentage of growth inhibition was calculated.

Irreversibility of Cell Growth Inhibition: For assessment of irreversibility of enoxacin-induced growth inhibition, $4x 10^4$ cells were plated in 35mm petridish. After 24h cells were treated with enoxacin (30 µg/ml). On the third day of drug exposure medium of the control plates and half of the drug treated plates were replaced with complete growth medium only and in the remaining half the enoxacin treatment was continued for another 3 days. Cells were counted at an interval of 24h employing trypan blue dye exclusion technique by using hemocytometer. Population doubling time (Patterson 1979) and saturation densities of control and treated cultures were calculated.

Cell Cycle Analysis: MCF-7 cells were seeded at a density of $3x10^5$ in 100mm culture dishes, allowed to adhere for 24h and then treated with 40 µg/ml of enoxacin for 96h in complete medium. The cells were harvested by trypsinization, centrifuged at 1000 g for 5 min, washed in PBS and resuspended in cold 70% ethanol. After washing the cells with PBS 1µl of ribonuclease (10 µg/ml, DNAse free) per $1x10^6$ cells were added at room temperature for 5 min. The cells were then subjected to flow cytometric analysis on FACScan flow cytometer (Beckton Dickinson) after propidium iodide staining. At least 20,000 cells were analyzed using the cell quest software (Beckton Dickinson).

Role of Serum: The role of different concentrations of serum (FBS) in enoxacin induced growth inhibition was assessed by plating $4x 10^4$ cells per 35mm petridish containing growth medium. After 24h, enoxacin (30 µg/ml) was added separately to the medium supplemented with 0.5%-10.0% FBS. In control plates as well as in drug treated plates media renewal were done on 3rd day and the cell viability was checked on 5th day as described above.

RESULTS AND DISSCUSSION

The anitumor activity of fluoroquinolone has been investigated only recently. There are few reports documenting the antiproliferative effect of ciprofloxacin, fleroxacin in some tumor cells (Aranha et al. 2000; Aranha et al. 2003; Ebisuno et al. 1997; Herold et al. 2002). In the present study antiproliferative effect of fluoroquinolone antibiotic enoxacin on breast cancer cell line, MCF-7 was investigated under different growth conditions.

The fluoroquinolone enoxacin produced significant morphological alterations on MCF-7 cells in culture. Under normal growth condition (control) these cells were regular in shape and size, had eccentric nucleus and a relatively small piece of cytoplasm (Fig. 1a). After 40µg/ml of enoxacin treatment cells became irregular in shape

and size with altered nuclear: cytoplasmic ratio, increased number of nucleoli and multiple cytoplasmic vacuoles (Fig. 1b). Most of the cells had relatively flattened appearance with long multiple cytoplasmic processes forming cross bridges with neighboring cells (Fig. 1c) indicating that enoxacin may render some changes on the cell surface associated with the adherence to the substratum as a result of which treated cells tend to adhere firmly to the growth surface, an opposite behavior of the tumor cells which tend to attach loosely to the substratum both in vitro and in vivo systems (Mukherjee and Das 1995).

recent studies it has been reported that caspase-3 is essential for morphological changes related to apoptosis on MCF-7 cells in culture (Jänicke et al. 1998b). Since it was already reported that TNF or staurosporin induced-apoptosis of MCF-7 cells was accompanied with cleavage of death substrates, gelsolin and DFF-45 (Jänicke et al. 1998b; Jänicke et al. 1998a), in our study in MCF-7 cells enoxacin may also be responsible to cleave these substrates associated with apoptosis which needs further investigation.

The effect of different concentrations of enoxacin on the proliferation of MCF-7 cells for
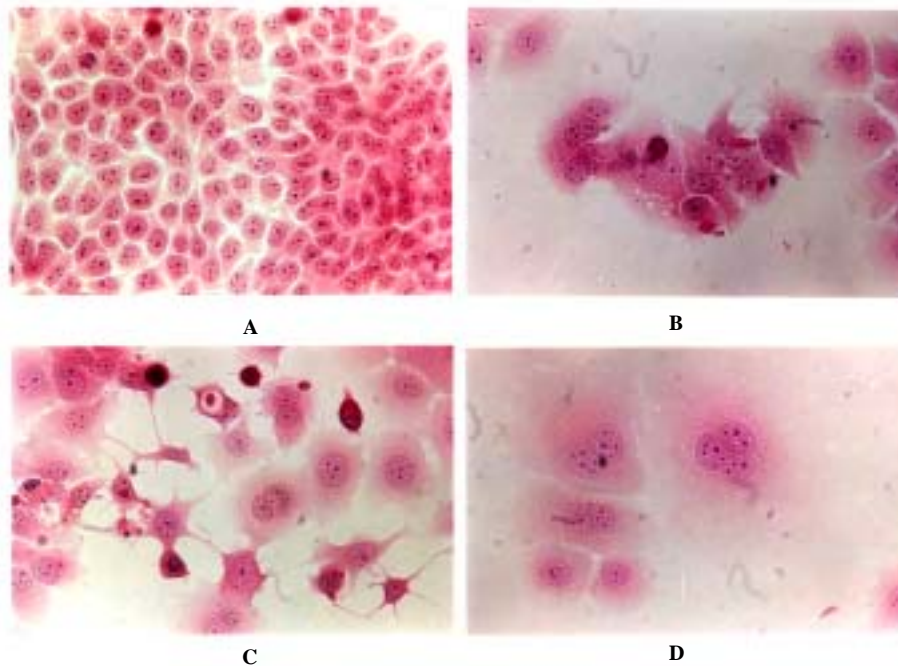

Fig. 1. Enoxacin induced morphological alterations on MCF-7 cell line. (a) Untreated control cells: regular in shape and size, eccentric nucleus and relatively small piece of cytoplasm (400X). (b-d) Enoxacin (40µg/ml) treated cells. (b) irregular in shape and size with cytoplasmic vacoules (400X), (c) flattened in appearance with cytoplasmic processes forming cross bridges (400X) and (d) appearance of multinucleated giant cells(400X).

Besides appearance of multinucleated giant cells (Fig. 1d) after enoxacin treatment indicated inhibition of cytokinesis and alteration in ploidy level which need further studies. Reports from several laboratories have shown that fluoroquinolone treated various tumor cells undergo apoptosis through mitochondrial-mediated pathway and induce morphological changes associated with programmed cell death (Aranha et al. 2000; Aranha et al. 2003; Herold et al. 2002). In some

variable periods were studied (Fig. 2). The result demonstrate that with increasing concentrations of enoxacin from 10 µg/ml to 50 µg/ml the percentage of growth inhibition of MCF-7 cells increased progressively from 55% to 92.8% after five days of drug exposure. It was also evident that at all drug concentrations the percentage of growth inhibition increased gradually with advancement of time. Fifty percent (GI50) and eighty percent (GI80) growth inhibition at 48h of
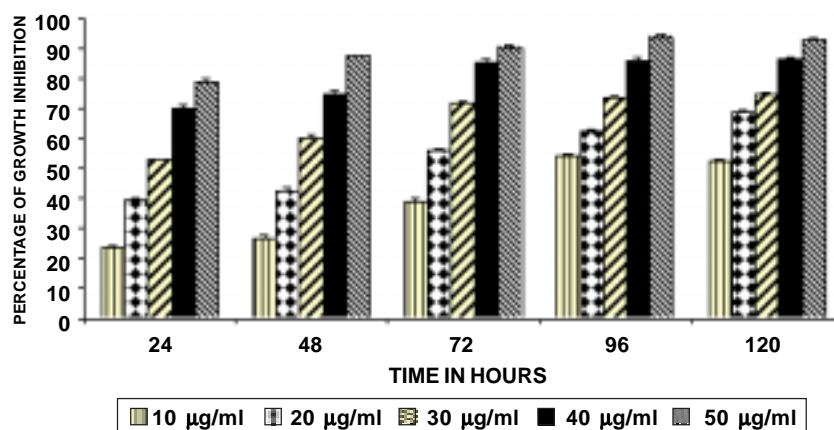
Fig. 2. Percentage of growth inhibition induced by Enoxacin (10-50 µg/ml) on MCF-7 cell line. Each point is the mean of triplicate culture, bars represent ± S. E. where bars are not shown, and they are equal to the sizes of symbols. Similar results were obtained in three independent experiments.

drug exposure were found to be only 25 µg/ml and 42µg/ml respectively. Thus the detailed analysis of the results clearly indicates that enoxacin causes significant growth inhibition of MCF-7 cells in dose and time dependent manner. Similar nature of antiproliferative effect by fluoro-quinolones was also demonstrated in bladder carcinoma and colorectal carcinoma cells in culture (Aranha et al. 2000; Aranha et al. 2003; Herold et al. 2002). Finally, we reported for the first time that enoxacin caused significant growth inhibition of human breast cancer cell even at 42 µg/ml concentration compared to other fluoroquinolone

reported earlier (Aranha et al. 2000; Aranha et al. 2003; Ebisuno et al. 1997; Herold et al. 2002)

In anchorage dependent growth condition the growth kinetics of MCF-7 cells in presence and absence of enoxacin was studied (Fig. 3). The results show that the growth inhibition induced by enoxacin is time dependent. From the figure number 3 it is evident that while control culture continued to grow beyond 6th day, number of cells of treated cultures grew slowly upto 5th day and then started to decline gradually. Initially after 24h of drug exposure insignificant growth inhibition of only 17 % was noticed which
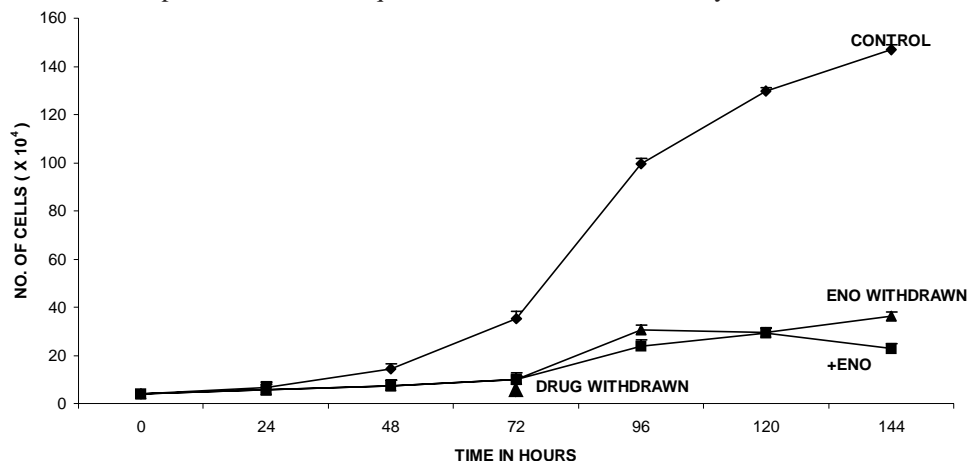

Fig. 3. Growth kinetics and irreversibility of Fluoroquinolone-induced growth inhibition of MCF-7 cells. Assays were performed as described in Materials and Methods. Each point is the mean of cell numbers from triplicate plates. Bars represent ± S.E. Where bars are not shown they are equal to the sizes of the symbols. Similar results were obtained in 3 independent experiments.

reached at moderate level of 48% after 48h and became significantly high resulting into 72%, 76%, 78% and 84% growth inhibition after 72, 96, 120 and 144 h respectively. In respect to the initial cell seeding density after 120h while in control cultures 33-fold increase of cell density was recorded, treated cultures increased only up to 7.4-fold during the same period. It was also noticed that minimum population doubling time (17h) in control cultures was increased to 28h in treated cultures. Thus as a consequence of enoxacin treatment increase of population doubling time and decrease of saturation density of MCF-7 cells can be considered as the effect which can be regarded as density dependent growth control (Mukherjee and Das 1995). In the above study, after 72h treatment when drug containing medium from treated cultures was replaced by normal growth medium slight decrease in the percentage of growth inhibition was noticed initially but with the advancement of time the rate of inhibition again increased to 75% which was little lower than that of the treated cultures. As the percentage of growth inhibition even after drug withdrawal remained significantly high, the enoxacin-induced growth inhibition can be stated as to be irreversible in nature, which was similar to the observation of ciprofloxacin-induced damage in bladder cancer cells (Aranha et al. 2000). These observations suggest that after treatment with enoxacin once breast cancer cells are programmed to die, they cannot respond to normal growth conditions.

The kinetics of the cell cycle distribution of MCF-7 cells treated with enoxacin in shown in table 1. The typical DNA histograms of progressive cell cycle changes are shown in figure 4. The untreated control cells showed progressive accumulation of cells from 35.3% to 56.6% in G_0/G_1, with a parallel decrease of the population from 51.7% to 35.1% in G_2/M phase with increasing culture time from 48 to 96h, reflecting the effect of growth factor depletion on progressively confluent cultures. However, in enoxacin treated cultures the relative number of cells in G_0/G_1 phase decreased to 25,32 and 33% and in G_2/M phase the cell number increased to 59,55.5 and 53.4% after 48,72 and 96h of treatment respectively (Table 1). These data provide evidence for cell cycle arrest induced by enoxacin and in turn, the inhibition of cell growth. Similarly fluoro-quinolone induced cell cycle arrest in bladder carcinoma, prostrate cancer and colorectal carcinoma was also reported previously (Aranha et al. 2000; Aranha et al. 2003; Herold et al. 2002). Accumulating evidences also indicate that down regulation of different cell cycle molecules are involved in fluoroquinolone mediated cell cycle arrest (Aranha et al. 2000; Aranha et al. 2003). Thus in our cell system further investigation in this direction is needed.

Effect of serum growth factors on the *in vitro* proliferation of MCF-7 cells after enoxacin treatment was studied (Fig. 5). The result shows that at all serum concentrations ranging from 0.5% to 10% growth of enoxacin treated cells were significantly retarded as compared to that of the control cultures. The highest 86% of growth inhibition which was recorded after 5 days of drug treatment at 10% concentration of serum, almost remained unaffected up to 3% serum concentration and beyond this slight decrease of growth inhibition was noticed. Thus the results clearly demonstrate that the enoxacin-induced growth inhibition of breast cancer cells is independent of variations of serum growth factors. Accumulating evidence indicate that topoisomerase II level of HeLa cells remain less affected with changing of serum concentration (Hwang and Hwang 1994). It was also reported that the nuclear topoisomerase II level correlate with the sensitivity of the topoisomerase II targeting drugs (Davis et al. 1988). Thus in our cell system the possibility of presence of almost same level of topoisomerase II enzyme at different serum concentrations may exist and as a consequence

Table 1: Cell cycle distribution of MCF-7 cells by FACS analysis after treatment with enoxacin (40 µg/ml).

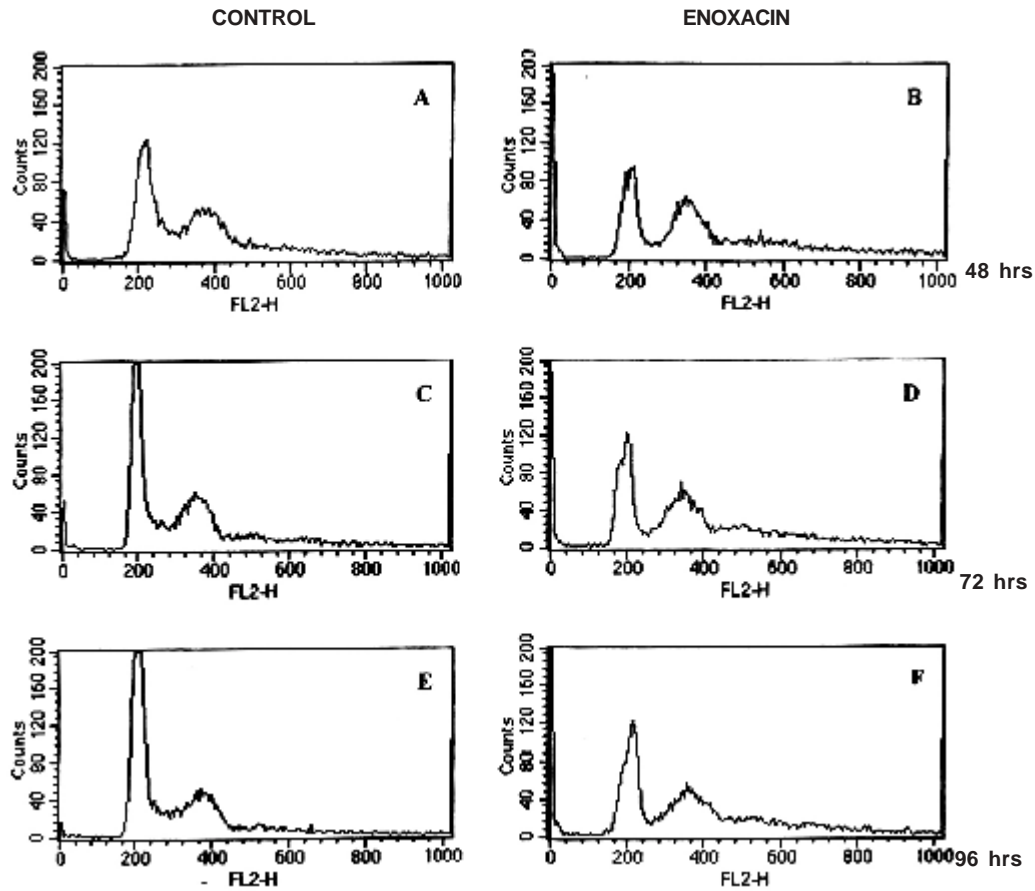| Drug exposure (hours)	% of cells at different phases of cell cycle							
	Control				Enoxacin			
	Sub G_1	G_0/G_1	S	G_2/M	Sub G_1	G_0/G_1	S	G_2/M
48	1.07	35.25	5.99	51.65	2.63	25.01	2.74	58.59
72	1.07	47.05	4.59	42.83	2.58	31.72	3.25	55.46
96	0.75	56.62	4.78	35.10	3.40	32.82	2.61	53.37

Fig. 4. DNA histograms of MCF-7 cell showing progressive cell cycle changes after 48, 72, and 96 hours of treatment with enoxacin (40µg/ml).
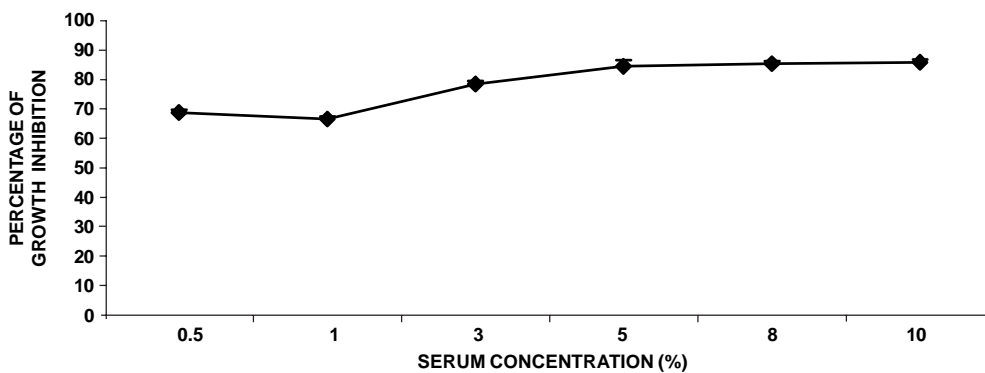

Fig. 5. Effect of serum (FBS) concentrations in growth medium on the proliferation of MCF-7 cells after enoxacin (30µg/ml) treatment. Assays were performed as described in Materials and Methods. Each point is the mean of cell numbers from triplicate plates. Bars represent ± S.E. Where bars are not shown they are equal to the sizes of the symbols. Similar results were obtained in 3 independent experiments.

of these facts percentage of growth inhibition of this breast cancer cells almost remained unchanged at different serum concentrations.

The above results clearly demonstrate that the fluoroquinolone antibiotic enoxacin induced significant antiproliferative activity on breast cancer cells and thus it is suggested that breast cancer cells may be responsive to the treatment with fluoroquinolone compound enoxacin. Moreover, the risk of drug toxicity as well as development of drug resistance may also be minimized due to the use of lower concentration of drug like enoxacin. Further investigations in this direction are required.

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