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Remarks in Successful Cellular Investigations for Fighting Breast Cancer Using Novel Synthetic Compounds

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1. Introduction

Breast cancer is one of the most life threatening risks in women's life. In spite of considerable progress in its understanding and challenges, treatment is not yet the correct word to apply on this disease and losing life is the most foreseeing adventure in many patients. Although new gene therapy based approaches are looking for the cure of breast malignant cells, but using cytotoxic agents is currently the main chemotherapy approach to fight this problem. Effective chemotherapy treatment of breast cancer requires targeting the pathways that support the cell growth and proliferation. A good *in vitro* investigational model is essential to understand the process of carcinogenesis, risk and hazard mechanism of carcinogens, protection from carcinogens, mode of action and efficacy of novel and even in practice chemotherapeutic agents. The main part for any of these laboratory models is suitable cell lines to properly address the problem and goal of investigation.

Estrogen Receptor (ER) is considered to cause different growth responses in ER-positive, normal, preneoplastic and neoplastic cells (DuMond et al., 2001; Roy & Cai, 2002; Welshons et al., 2003). One of the most significant researches in cancer treatment has been based on designing and studying the ER-antagonism effects of molecules on cells. This is important to select suitable cell lines for *in vitro* drug discoveries studies. Table 1 shows a list of epithelial breast cell lines with different expression in estrogen receptor.

Intracellular enzymes responsible for the different consequences of receptors stimulations and signaling cascades are also under big considerations in fighting breast cancer cells. Dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate-NADP+ oxidoreductase) is an example of pivotal importance in biochemistry and medicinal chemistry. DHFR catalyzes the reduction of folate or 7,8-dihydrofolate to tetrahydrofolate and intimately couples with thymidylate synthase (TS). Reduced folates are carriers of one-

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Cell line	Suitable growth media	Kinds of receptor	Oncogene considerations
ZR-75-1	RPMI-1640 & 10% FBS	Estrogen receptor	
MCF-7	DMEM:F12 & 10% FBS	-Estrogen receptor	
UACC-3199	Leibovitz's L-15 & 10% FBS	-Epidermal growth factor receptor expressed -Estrogen receptor negative, -Progesterone receptor negative	
HCC1954	RPMI-1640 & 10% FBS	-Estrogen receptor -Progesterone receptor	her2/neu + (over expressed)
HCC1500	RPMI-1640 & 10% FBS	-Estrogen receptor -Progesterone receptor	Negative for expression of Her2-neu, positive for expression of p53
HCC70	RPMI-1640 & 10% FBS	-Progesterone receptor negative	Negative for expression of Her2/neu, positive for expression of p53
HCC1008	DMEM:F12 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Positive for expression of Her2-neu, positive for expression of p53
HCC1143	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Negative for expression of Her2/neu, positive for expression of p53
HCC38	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Negative for expression of Her2/neu, positive for expression of p53
UACC-893	Leibovitz's L-15 & 10% FBS	-Estrogen receptor negative -Progesterone receptor negative -P glycoprotein negative	The cells exhibit a 20 fold amplification of the HER-2/neu oncogene sequence
HCC1395	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Negative for expression of Her2/neu, Positive for expression of p53
HCC1419 & HCC202	RPMI-1640 & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Positive for expression of Her2/neu, Negative for expression of p53
HCC1806 & HCC1599	RPMI-1640 & 10% FBS	-Progesterone receptor negative, -Estrogen receptor negative	Negative for expression of Her2-neu, Negative for expression of p53
HCC1937	RPMI-1640 & 10% FBS	-Estrogen receptor negative -Progesterone receptor negative	BRCA1 (mutated, insertion C at nucleotide 5382), Negative for expression of Her2-neu, Negative for expression of p53

Cell line	Suitable growth media	Kinds of receptor	Oncogene considerations
HCC2157	ACL-4 medium & 10% FBS	-Estrogen receptor negative, -Progesterone receptor negative	Positive for expression of Her2-neu , Positive for expression of p53

Table 1. List of breast cell lines with different expression in estrogen receptor.

carbon fragments; hence they are important cofactors in the biosynthesis of nucleic acids and amino acids. The inhibition of DHFR or TS activity in the absence of salvage leads to 'thymineless' death.

There are some other enzymes which came into special consideration in cancer development, particularly in the breast cancer. Cyclooxygenase-2 is an example that over expresses in several epithelial tumors including breast cancer. Preclinical evidence favors an anti tumor role for COX inhibitors in breast cancer because there is a clear relationship between tissue prostaglandin levels in human breast tumors and the development of metastasis and patient survival (Arun & Goss, 2004). Selective COX-2 inhibitors can prevent mammary tumors from developing cancer in experimental animals. Celecoxib (a COX-2 inhibitor) has proven to minimize the progression of carcinogen-induced mammary tumors (Arun et al., 2001). A good cell line to clearly address alterations in above mentioned systems is also critical for challenging breast cancer cells *in vitro*.

A trustable measurement approach to detect results of the application of under-investigation agents on cells is very much important. Different methods have been applied to investigate cell alterations and ultimately cell death resulted from cancer chemotherapy and cytotoxic agents. Each of them has advantages and disadvantages in different situations and for different purposes. Misuse of any of these methods for the detection of the cytotoxicity of different agents on different cell lines is one of the main problems of many publications for years. These techniques usually look at the viability, morphology and/or biochemical function of various cellular functions. Table 2 lists some of the most popular methods used to measure the cytotoxicity of agents in cellular experiments.

A precise and accurate investigation is one that selects the best possible measurement method on the best possible cell line in the most optimal situation for the best possible conclusion. Cellular investigations to look for new anti-breast cancer agents rely on these bases. MCF-7 proves to be a suitable model cell line for breast cancer investigations worldwide. This is a well known breast cancer cell line derived from a 69 years old Caucasian female. MCF-7 cell line presents most of characteristics of differentiated mammary epithelium tissues including those of expressing estradiol and estrogenic receptors features (Brandes & Hermonat, 1983). Here, we are summarizing some of our results using this cell line to search for novel anti-breast cancer agents, with emphasis and conclusive remarks on the good laboratory practice.

2. Targeting estrogen receptors

Estrogens are known to play an important role in the regulation of the development and maintenance of the female reproductive system, in particular of the uterus, ovaries and breast. Moreover, estrogens are involved in the growth and/or function of several other tissues such as bone, liver, brain, and the cardiovascular system (Ciocca & Roig, 1995).

Method	Measurement criteria	Sample methodology references
Vital dyes (Methylene blue, Trypan blue, Phenol red, …)	Cell membrane integrity	(Shirazi et al., 2005; Shokrzadeh et al., 2006)
Clonogenic assay, cell numbers	Cellular proliferation	(Shirazi & Eftekhari, 2004; Shirazi et al., 1996)
MTT and XTT	Function of mitochondrial enzymes	(Shirazi et al., 2004; Tamaddon et al., 2007)
Thymidine assay, Bromodeoxyuridine	Cellular DNA synthesis	(Hammers et al., 2002; Maghni et al., 1999; Raaphorst et al., 1998; Yokochi & Gilbert, 2007)
Blotting techniques	DNA, RNA and Protein synthesis machinery	(Ko et al., 1993; Singh et al., 2008; Skliris et al., 2002)
Flowcytometry	Population based cell cycle analysis, Individual cell content and biophysical status	(Lukyanova et al., 2009; Niknafs & Shirazi, 2002; Skliris et al., 2002;)
Light and electron microscopes	Cellular morphology and structural features	(Lukyanova et al., 2009; Russo et al., 1977; Vic et al., 1982)

Table 2. Different popular methods to measure cellular alterations after exposure to cytotoxic agents.

Figure 1 represents the general effects of estradiol (as a proliferative estrogen receptor stimulant agent) and tamoxifen (as an estrogen receptor blocking agent) on the growth curve of MCF-7 cell line. To obtain this, 50,000 cells were seeded in four series of cell culture petri dishes and incubated in phenol red-free RPMI media supplemented with 10% fetal bovine serum for 7 days. From the beginning, three different series of petri dishes were selected for the experiments; estradiol was added into the media of one series, tamoxifen was added to the media of the second series and a mix of these two agents was added to the third series of petri dishes. Cells in each perti dish were counted for seven consecutive days as the presentation of cell proliferation in control, estradiol exposed, tamoxifen exposed, and affected by both of estradiol and tamoxifen agents. As is seen in figure 1, estradiol has a significant effect to promote the growth of MCF-7 breast cancer cells compared to the control cells. MCF-7 cells, however, are arrested for at least five days before being able to start a significant proliferation after the exposure to the estrogen-blocking agent of tamoxifen. This block is effective enough to prevent the stimulating effect of estradiol when cells are exposed to both agents simultaneously. This experiment would further emphasize on the stimulating effect of estrogen receptors in breast cancer progression.

Several studies have established that estrogens are predominantly involved in the initiation and proliferation of breast cancer. Lots of efforts are now being devoted to block estrogen formation and action as an anticancer strategy (Clemons & Goss, 2001; Jensen et al., 2001; Nelson et al., 2009). This has led to the development of compounds termed Selective

Estrogen Receptor Modulators (SERMs), which function as estrogen agonists in some tissues (bone, brain and the cardiovascular system) but as antagonists in others (uterus and breast). Estrogen action is mediated through two Estrogen Receptor (ER) subtypes, ERα and ERβ, which have distinct target tissue distributions and functional activities (Gustafsson et al., 2003; Matthews & Gustafsson, 2003; Välimaa et al., 2004). ERα is predominantly found in the uterus, bone, cardiovascular tissue, and liver and is the predominant ER expressed in breast cancer. ERβ is expressed in many tissues including prostate, breast, vascular endothelium, and ovary. The precise function of ERβ and its role in breast is not clear (Fox et al., 2008; Novelli et al., 2008). Recent studies indicate that ERβ expression may have a potential protective effect on normal cells against ERα induced hyperproliferation (Bardin et al., 2004).

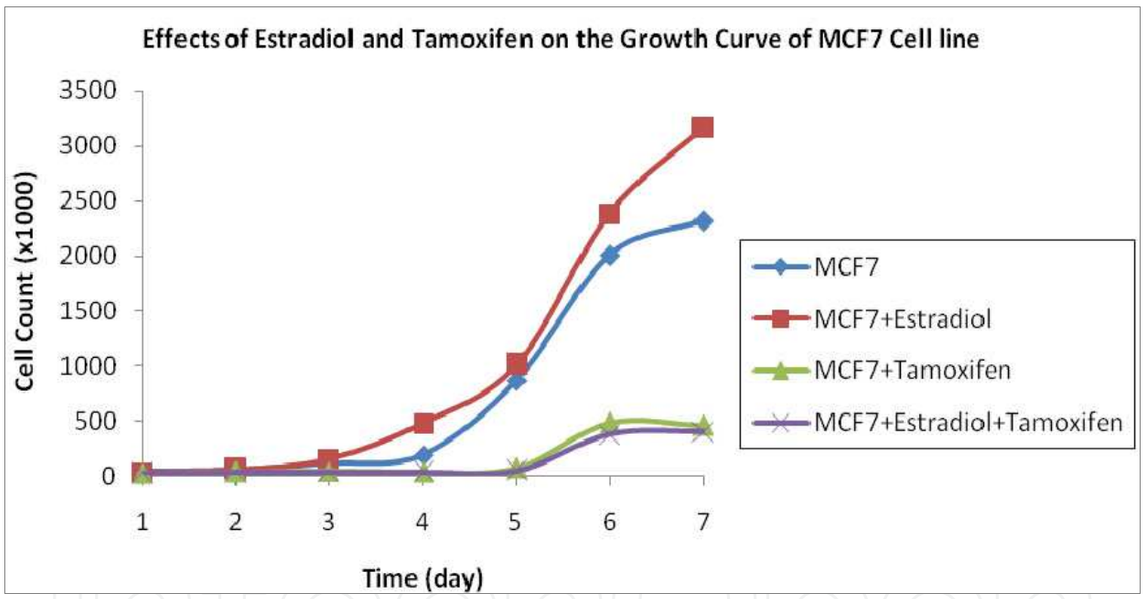


Fig. 1. Stimulation and inhibition of MCF-7 breast cancer cell line exposed to estradiol, tamoxifen and mix of these two agents for 7 days in phenol red-free RPMI media incubated in 37°C and 5% CO_2 humified incubator.

Estrogen receptors can bind a variety of steroidal and non-steroidal ligands. Tamoxifen was the first SERM approved for the treatment of breast cancer (Jordan, 1988). The search for better SERMs has driven efforts to increase the chemical diversity of these compounds, especially the non-steroidal ones (Meegan & Lloyd, 2003). Figure 2 shows the structures of tamoxifen and other known SERMs such as ralolxifen and rasofoxifen.

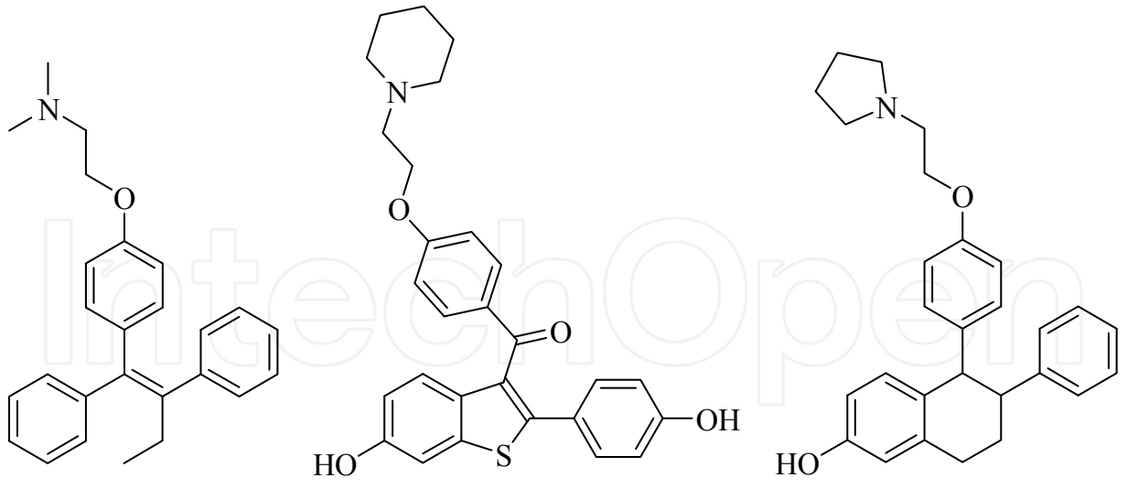


Fig. 2. Chemical structures of some known Selective Estrogen Receptor Modulators (SERMs).

Structure-activity relationship (SAR) studies and molecular modeling studies center lead to the design of novel structures containing 1,2,3-triarylpropenone scaffold to act as potential SERMs and anti breast cancer agents with a unique structure as is shown in Figure 3.

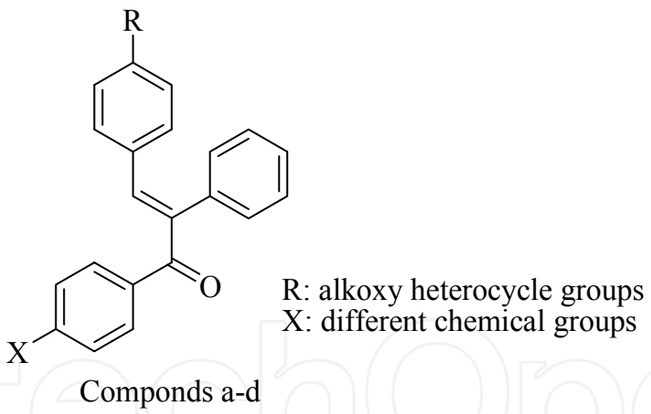


Fig. 3. The general model of 1,2,3-triarylpropenone scaffold as a novel potential SERMs and anti breast cancer agents**.**

The compounds a to d have been synthesized and undergone biological evaluations in an *in vitro* cellular system using MCF-7 breast cancer cell line as the model. The anti-proliferative activities of these compounds were determined using MTT assay. To do so, a ten thousands cells were seeded in phenol red-free RPMI-1640 medium supplemented with 10% FBS in each well of 96-well micro culture plates and incubated for 24 hours at 37 ^{o}C in a 5% CO_2 incubator. Different concentrations of each compound were added to the wells with respective vehicle control for 72 hours. Media were then removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well. Formazon crystals were dissolved in 200 µL of DMSO after 4.5 hours incubation and the dye

absorbance for each well was measured at 540 nm. A comparison of absorbance in each well containing different concentrations of each compound to the control wells could easily represent the number of live cells in that well as a result of the cell mitochondrial function (Zhu et al., 2006).

The results of anti-proliferative MTT assays of compounds a to d on MCF-7 breast cancer cells are shown in the graphs below (Figure 4). Start point (time 0) is shifted in each set of figures for a better clarification of the shape and trends of graphs in case of compounds a, b and d. These graphs show the comparative cytotoxic and antiproliferative effects of all of these compounds on MCF-7 cell line.

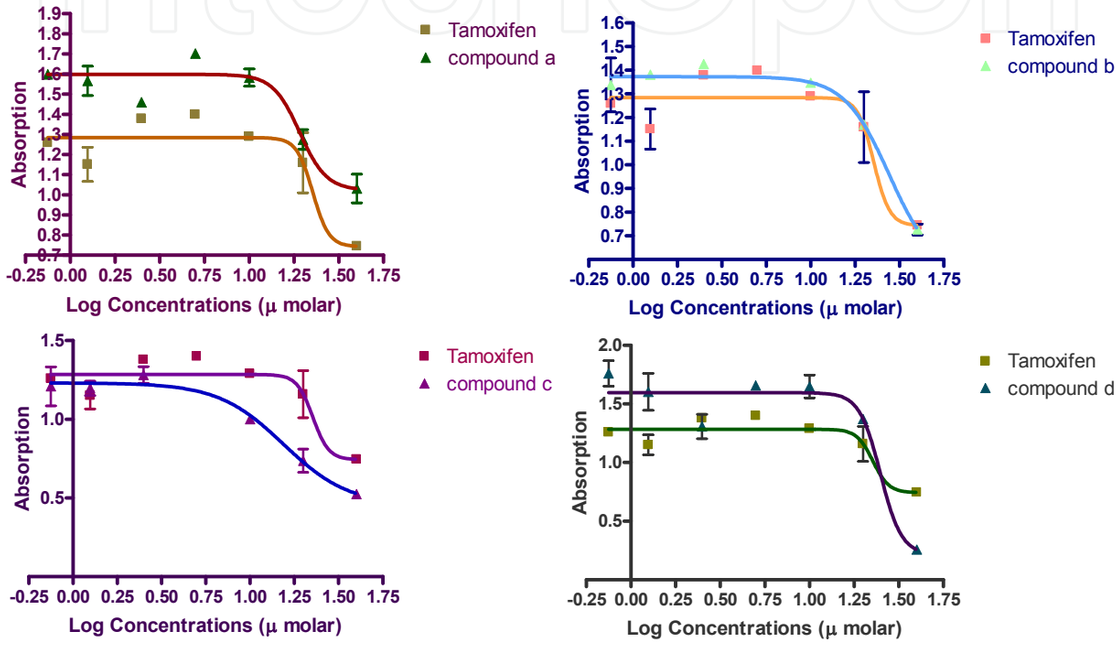


Fig. 4. Cytotoxic effects of tamoxifen and compounds a to d on MCF-7 cell line presents a comparable antiproliferative effects on cancer cells.

Estrogen receptor binding studies were carried out for the compounds with ERα and ERβ using a fluorescence polarization procedure to prove the stimulatory and inhibitory mechanism being through the estrogenic receptors (data not shown). The compounds were active on ERα at nanomolar concentrations and on ERβ at micromolar concentrations. Therefore compounds a to d selectively bind to ERα.

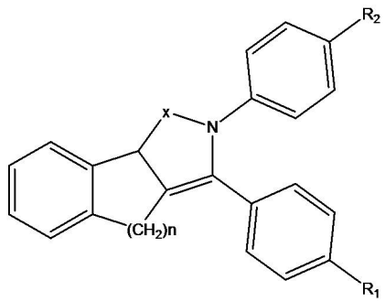
Interestingly, clonogenic assays on MCF-7 cell line after exposure to these compounds fail to present solid and reliable growth inhibitory effects. Figure 5 shows some graphs resulted from the same exposure strategy of above-mentioned compounds on MCF-7, but using the clonogenic methodology to compare the results. A clear weakness is evident in these graphs preventing from any conclusive interpretation of results. We will further discuss this finding at the end of this chapter.

3. Targeting COX-2 enzyme

There is considerable evidence to suggest that prostaglandins play an important role in the development and growth of cancer. The enzyme cyclooxygenase (COX) catalyses the

conversion of arachidonic acid to prostaglandins (Abou-Issa et al., 2001). There has been a considerable amount of interest in recent years to take advantage of COX inhibitors specifically COX-2 inhibitors in prevention and treatment of malignancies (Talley et al., 2000; Zarghi et al., 2006). Majority of COX-2 inhibitors belong to a class of diaryl heterocycles that possess vicinal diaryl substitution attached to mono, bicyclic or tricyclic central rings (Penning et al., 1997; Prasit et al., 1999; Riendeau et al., 2001).

As a part of ongoing program to design new types of selective COX-2 inhibitors, our center has synthesized novel COX-2 inhibitor derivatives having a new tricyclic central ring scaffold and different substituents at the N-3 as is shown in figure 5.



R1=SO2Me
R2=H,F,Cl
X=N,NH

Fig. 5. Central structure of novel COX-2 inhibitors.

The nature and size of substituent attached to N-3 influenced both selectivity and potency for COX-2 inhibitory activity. Two different compounds of C1 and C2 with different N-3 substituent have been applied to MCF-7 cell line for the evaluation of anticancer effects, using clonogenic assay. MCF-7 cells were seeded for the clonogenic assay in 12-well plates at 150 cells per well for 24 hours. These cells were then exposed to C1 and C2 derivatives for 24 hours. Media was then changed to fresh media without these compounds and plates remained in incubator for couple of days until most of colonies in the control wells contained more than 50 cells. Media was then excluded and cells were fixed with 96% ethanol and stained using trypan blue. Plates were washed and percentages of colonies in different wells were compared to controls (Shirazi et al., 2005).

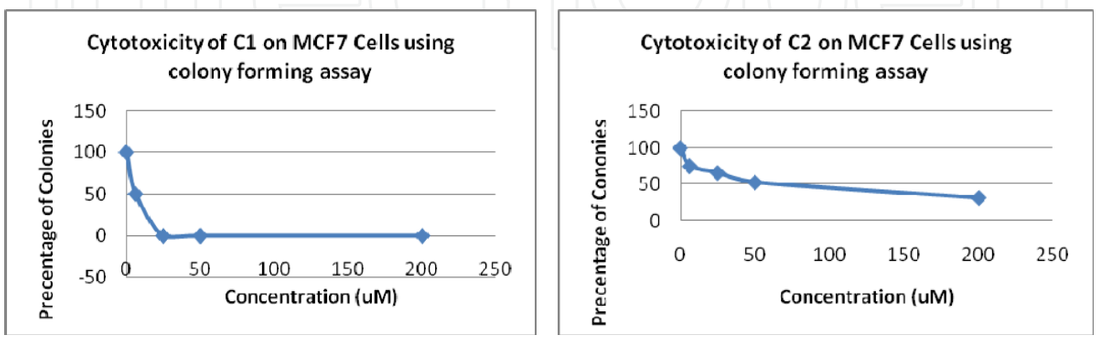


Fig. 6. Cytotoxicity of two novel COX-2 inhibitors of C1 and C2 on MCF-7 cell line using clonogenic assay.

As is shown in figure 6, both compounds have acceptable cytotoxicity effects with C1 being stronger. However, the same experiment has been conducted using the same cell line and the same concentrations of C1 and C2 compounds but using MTT assay. MTT failed to present any cytotoxicity for these compounds on MCF-7 cell line as is shown in figure 7.

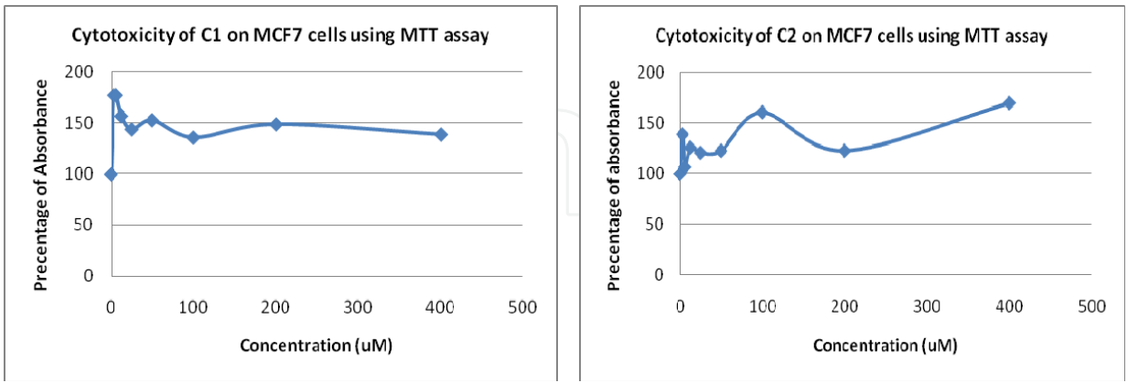


Fig. 7. MTT based cytotoxicity measurement of two novel COX-2 inhibitors of C1 and C2 on MCF-7 cell line.

Failure of one experiment using a technique in spite of success for the other technique in acquiring result is a considerable phenomena in cellular investigation on cytotoxic agents and will be discussed later on in this chapter.

4. Targeting dihydrofolate reductase (DHFR) enzyme

Inhibitors of DHFR are classified as either 'classical' or 'non-classical' antifolates. The 'classical' antifolates are characterized by a *p*-aminobenzoylglutamic acid side-chain in the molecule and thus closely resemble folic acid itself. Methotraxate (MTX) is the most well known drug among the 'classical' antifolates. Compounds classified as 'non-classical' inhibitors of DHFR do not possess the *p*-aminobenzoylglutamic acid side-chain but rather have a lipophilic side-chain. MTX serves as an antimetabolite, which means that it has a similar structure to that of a cell metabolite, resulting in a compound with a biological activity that is antagonistic to that of the metabolite, which in this case is folic acid (Barnhart et al., 2001; Takemura et al., 1997).

New, more lipophilic antifolates have been developed in an attempt to circumvent the mechanisms of resistance, such as decreased active transport, decreased polyglutamation, DHFR mutations and so on (Assaraf, 2007; Gangjee et al., 2006; Takemura et al., 1997). In a series of synthesized compounds for this purpose in our center the pyrimidine ring remained (figure 8) and the side-chain attachment at the position 2 was replaced with different substituent.

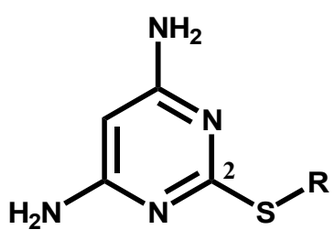


Fig. 8. The central structure of novel DHFR inhibitors.

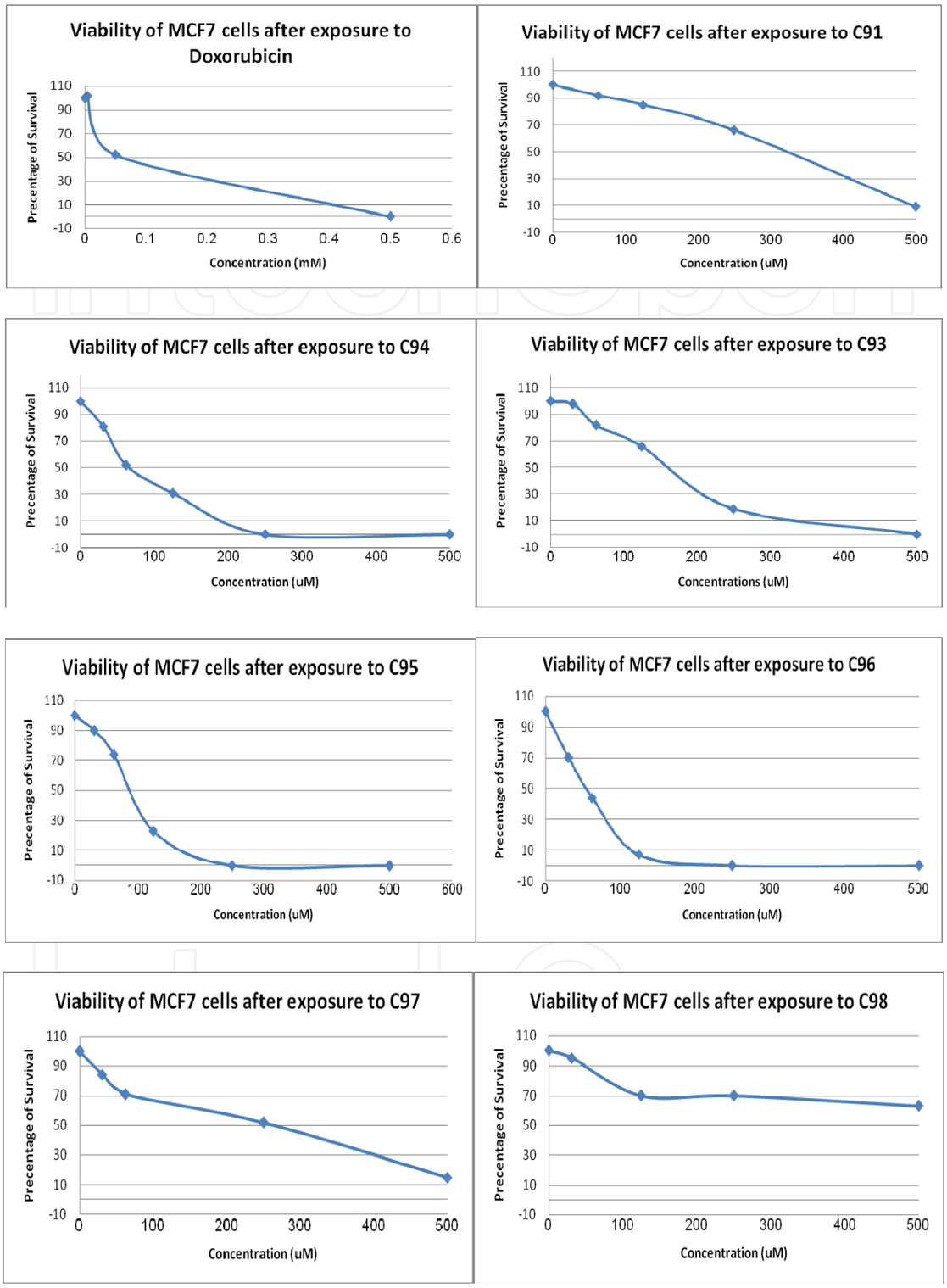


Fig. 9. Cytotoxicity measurement of seven selected DHFR inhibitors on MCF-7 cell line resulted from clonogenic assay.

These modified antifolates differ from the traditional 'classical' analogues by increased potency, greater lipid solubility, or improved cellular uptake. Although being very effective as inhibitors, problems still remain with respect to the issue of toxicity due to the lack of selectivity (Cody et al., 2003; Graffner-Nordberg et al., 2004; McGuire, 2003). To evaluate the cytotoxic potency of these compounds, we have used the clonogenic assay. MCF-7 cells were plated in 6-well plates (200 cells/well) for 24 hours before treatment with the test compounds to allow the attachment of cells to the wells surface. Seven different concentrations of each compound, doxorubicin (as reference), and 0.5% DMSO (applied solvent to dissolve the compound) were added to the monolayer cells in triplicates. The plates were then incubated for 10 days at 37 ^{o}C in atmosphere of 5% CO_2. The media were removed after 10 days and the colonies were stained with a solution of 0.5% crystal violet in ethanol for 10 minutes and the number of colonies containing more than 50 cells was counted under microscope. The relation between the number of the colonies (as a percentage to the control containing 0.5% DMSO) and the concentrations of each compound were plotted to get survival curve of the tumor cell line and IC_{50} values were calculated. Cellular viability test results for some examples of this series of novel DHFR inhibitors are presented in figure 9.

5. Discussion

Human mammary gland adenocarcinoma MCF-7 cell line (ATCC HTB-22™) is proven to be a good breast tissue model for anticancer drugs investigations in our experiments. However, selection of a suitable cell line is only a part of a successful and meaningful *in vitro* cellular examination of potential anticancer agents. Many different factors might very much influence the final outcome of the evaluation of a medication in a cellular experiment, among them are the cell culture media and its components during the time of drug exposure and afterward, exposure time, drug solvent, volume of drug solution to be added to the cell culture media, the proper use of agonists and antagonists for the purpose of elaborations on the results and making a meaningful conclusion, methodology of cellular viability assessment, and the most important factor; the personnel who run the experiment. We are not going to extensively discuss all of these parameters and their specific influences on the final result and conclusion, but the limited examples presented in this chapter may be sufficient to raise awareness for a good cellular practice.

The importance of a suitable protocol for the measurement of survival percentage (live versus death) of cells is underestimated in many of experiments. Selection of the method in many instances is easily a matter of facility, budget and distributing companies' advertisements in the region. However, one should notice that for many known and unknown reasons, various methods of MTT, XTT, SRB, fluorescence dye staining and so on might work or not for different experiments. The main reason might well be the cellular measurement criteria for any of these methods. One should keep in mind that although mitochondria is the heart of cellular energy system, but MTT and XTT experiments would only measure the functionality of a mitochondrial enzyme (Cody et al., 2003; Marshall et al., 1995; Scudiero et al., 1988) and would not necessarily reflect the cell viability. The same is very much true for many of staining methods e.g Annexin V which is an indication of cell membrane flip-flop that would most properly occur during the process of apoptosis (Kolodgie et al., 2003; Van Heerde et al., 2000). Both of these methods are extensively used for the measurement of the cytotoxicity of many different agents. The chemical structure of

under investigation compound, its solvent, its cellular site of action, the exposure time, the lag time from the beginning of exposure to the start of measurement, and even the selection of cell line might dramatically alter the final survival curve. Methotrexate is a good example of MTT limitation in cytotoxicity measurement (Haber et al., 1993) and colleagues have shown that MTT protocol is not able to assess the cytotoxicity of this anticancer agent on various cells including MCF-7. Our experiments on other novel DHFR inhibitors have also proven the same conclusion when MTT results were not conclusive while the clonogenic g assay could easily provide a meaningful dose-response result. Figure 10 shows a comparison of MTT versus clonogenic assay for the measurement of methotrexate as well as some other novel DHFR inhibitors. As is shown in this figure, clonogenic assay was more successful in determining the LD_{50} of these compounds in MCF-7 cell line, but not the MTT protocol. Alteration of the exposure time and lag time between the addition of drugs and start of MTT assay, media components and calculation method were not helpful to provide a conclusive survival curve using this method (Data are not shown).

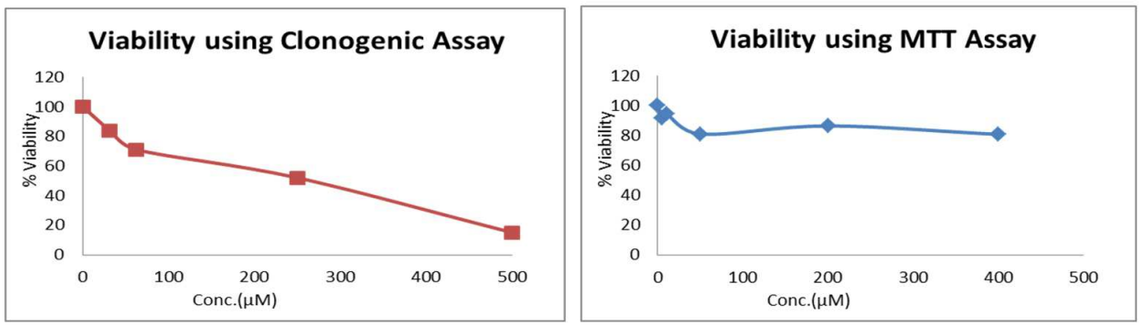


Fig. 10. Comparison of clonogenic versus MTT assays for the measurement of methotrexate and some other novel DHFR inhibitors.

Clonogenic assay is usually considered as a final answer for drugs cytotoxicity because of its long waiting time to acquire result. A minimum of five to six cellular doubling times to look at results in clonogenic assay might well overcome all cellular adventures of arrest, repair, detoxification and exertion pumps influences on drug cellular mortality which might affect the result of cross sectional measurement methods like MTT and Annexin V. Figure 7 is another example of the limitation of these type of experiments in some instances in comparison with the clonogenic assay. Clonogenic assay, however, would surprisingly fail to present a meaningful graph of cytotoxicity after exposure to some compounds.

There are many different mechanisms which might cause these differences in the result of the viability measurement using different methodologies. Cellular target of the test compound and the cellular repair system are two of the most possible explanation. Rosenberg confusion about the effects of electric field on the cells resulted in cisplatin identification and later use as a very important and most used anticancer drug in many different kinds of malignancies including the breast cancer (Rosenberg et al., 1969). Cells in Rosenberg set up did not die, rather changed shape and remained alive for a long time (Rosenberg, 1985, 1977). Cisplatin, like many other anti-mitotic agents, does not kill cells right after exposure. Its principle mechanism of action is on the DNA and thus while stopping DNA synthesis and cell proliferation, won't affect the mitochondrial action and cell membrane integrity. That is why, while the thymidine assay and cell cycle progression

based techniques like the flowcytometery, as well as proliferation based measurements like the clonogenic assay present good results, cell membrane integrity and mitochondrial enzyme function based assays have a significant lag time before the presenting of measurable alterations. A successful cellular repair event during this lag time may change the final conclusion dramatically. One needs to be aware of these possibilities in interpretation of cytotoxicity test results. Figure 11 represents the measurement of cisplatin cytotoxicity effect on MDCK cell line using MTT assay. As is shown in this figure, a 48 hours exposure time difference is needed to acquire a reasonable survival curve using this method.

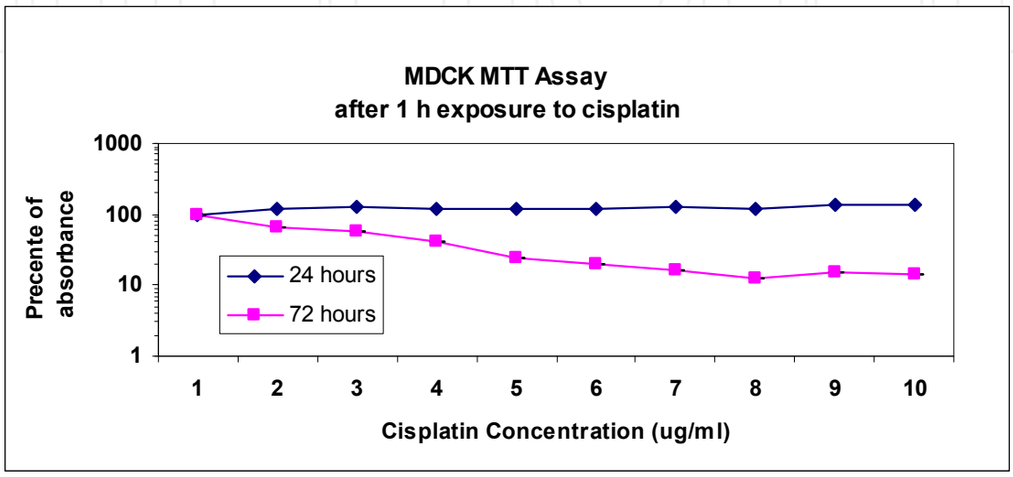


Fig. 11. The lag time required to get a good MTT result on the cytotoxicity of cisplatin on MDCK cell.

A discrepancy analysis to measure the cytotoxicity of many of novel anticancer drugs developed in our center under the same condition on the same cell line using two different methods of clonogenic assay and the neutral red assay, did not show agreement with a clear horizontal line and 95% confidence interval of about 1. It would further prove the importance of the selection and application of a suitable survival measurement system in the analysis of various anticancer candidates.

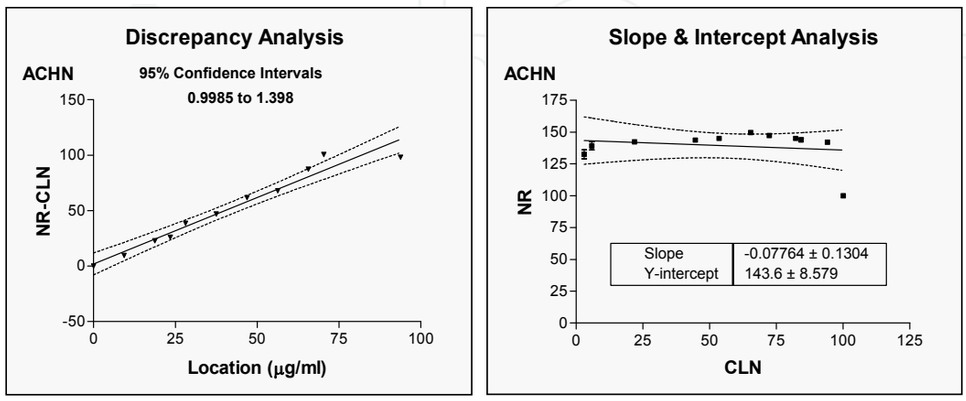


Fig. 12. A comparison of clonogenic versus neutral red assay for the measurement of cell survival after exposure to various novel anticancer drugs, using a discrepancy analysis method.

A good cellular practice on anticancer drugs requires the best selections of cell line and model system, the best matched measurement methodology, and the most optimized lag time to look at the result for acquiring the most precise and accurate conclusion.

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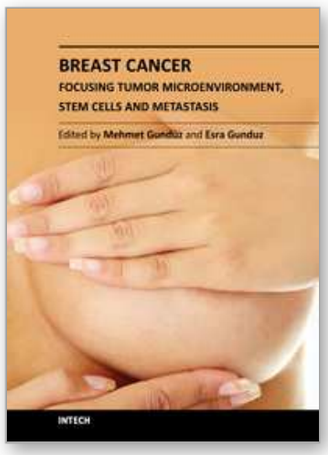

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed characteristics of breast cancer cell, role of microenvironment, stem cells and metastasis for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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