

Anticancer activity of some commercial antihypertensive drugs by Neutral Red assayFathia Z.El Sharkawi¹, Hany A.El Shemy² and Hussein.Khaled³¹Biochemistry Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt.²Biochemistry Department, Faculty of Agriculture, Cairo University, Giza, Egypt³Medical Oncology Department, National Cancer Institute (NCI) Cairo University, Egyptfelsharkawi@gmail.com

Abstract: Lisinopril, propranolol and nifedipine are three commercial drugs used clinically for the management of hypertension, angina pectoris, and cardiac arrhythmias. It has been reported that these drugs have inhibitory effects on some cancer cells. In the current study the cytotoxicity of these drugs was evaluated against HeLa, HepG2, MCF-7 and EACC transformed cell lines using Neutral Red and Trypan Blue assay methods. The three drugs showed a cytotoxicity against HeLa, HepG2 and MCF-7 cells with different potentiality. Lisinopril was the most potent cytotoxic drug against HepG2 cells with $IC_{50} = 33.8 \pm 88.4 \mu\text{g/ml}$ at the concentration of 300ug/ml; while Nifedipine was the most active one against HeLa cells with $IC_{50} = 130 \pm 58.4 \mu\text{g/ml}$ at a concentration of 300ug/ml. Propranolol was the most active against MCF7 cells IC_{50} of $78.0 \pm 121.4 \mu\text{g/ml}$ at a concentration of 3000ug/ml. The three used drugs inhibited the growth of EACC cells and propranolol showed highest inhibitory activity; it inhibited 97.7% of cell growth at a concentration of 300 ug/ml and 100% inhibition at a concentration of 3000 ug/ml. Lisinopril and nifedipine showed a lower rate of growth inhibition of 18.28% and 11.40% respectively at a concentration of 3000ug/ml. **In conclusion:** At these high concentrations, the three tested drugs are lethal *in vitro* to cancer cells of endometrial, cervical, hepatic, and breast origin. Further animal studies are required to confirm this conclusion.

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1. Introduction:

Cancer rates are set to increase at an alarming rate, from 10 million new cases globally in 2000 to 15 million in 2020.(1) Many treatment techniques and modalities are now made available. most of the traditional cancer drugs are mainly cytotoxic drugs which were empirically developed based mainly on their capacity to inhibit cancer growth in experimental systems regardless of their nature and potential mechanism of action(2). At the same time, they also affect normal cells to cause serious adverse effects, such as bone marrow function inhibition, nausea, vomiting and other disturbing toxicity (3).

An alternative drug development strategy is the exploitation of established drugs that have already been approved for treatment of non-cancerous diseases and whose specific cancer cellular targets are known. The major advantage of this approach is that the pharmacokinetic, pharmacodynamic, and toxicity profiles of these drugs are in general well known; thus, their rapid translation into clinical phase II and III studies is feasible (4).

Among these drugs are antihypertensive and antianginal drugs. Lisinopril is an angiotensin converting enzyme (ACE) inhibitor drug indicated for hypertension, heart failure and acute myocardial infarction. Inhibition of ACE results in decreased plasma angiotensin II which leads to decreased

vasopressor activity and to decreased aldosterone secretion (5).

Propranolol is a beta-adrenergic blocking agent that is used for treating hypertension, angina, cardiac arrhythmias and some neurologic conditions. Nifedipine belongs to the class of medications called calcium channel blockers. It is also used to treat hypertension and angina. It was reported that lisinopril, propranolol and nifedipine had anticancer activities against scalp cancer cells (6).

So the aim of the present study is to evaluate the anticancer activity of lisinopril dihydrate, propranolol and nifedipine against four different solid tumor cell lines including HeLa, HepG2, MCF-7 and EACC cell lines.

2. Material and Methods

Neutral Red assay was used to assess the cytotoxicity of these three compounds against HeLa, HepG2 and MCF-7 cell lines. Data was represented by graphPad prism version 3.0. IC_{50} was calculated from the linear regression of the appropriate part of the percent viability curve using the least square method.

Chemicals and drugs

RPMI-1640 media, fetal bovine serum and other cell culture materials were purchased from Fisher Scientific Cell Culture (Houston, TX, USA). Neutral

Red was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents were of the highest analytical grade available.

Lisinopril, propranolol and nifedipine were obtained from Sigma, Kahira and Epico Pharmaceutical companies respectively.

Cell culture

Human transformed cell lines, from liver (hepatocellular; HepG2), breast (MCF-7) and cervical (HeLa) cell lines were obtained from Vaccera (Giza, Egypt). Cells were maintained in RPMI-1640 supplemented with 100 µg/ml streptomycin, 100 µg/ml penicillin and 10% (w/v) heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO₂ atmosphere at 37°C.

Cytotoxicity assays

The cytotoxicity of lisinopril, propranolol and nifedipine was tested against MCF-7, HeLa and HepG2 cells by the Neutral Red (NR) assay as previously described (7).

Exponentially growing cells were collected using 0.25% Trypsin-EDTA and plated in 96-well plates at 1000-2000 cells/well. Cells were exposed to each test compound for 72 hrs in appropriate condition 5% (v/v) CO₂ atmosphere at 37°C. After several washings, cells were exposed to 0.0075% NR solution for 2 hours in humidified atmosphere of 5% (v/v) CO₂ at 37°C subsequently washed with PBS. Ethanol/water/acetic acid (50:49:1) solution was used to dissolve the NR-stained cells and color intensity was measured at 540 nm. in a micro-plate reader.

Determination of Cell Viability by Trypan Blue Assay

Cytotoxicity of the three compounds mentioned above was tested against Ehrlich Ascites Carcinoma cells (EACC) was employed as a representative of animal tumor cell lines. Animals were transplanted with EACC from an immortal culture obtained from National Cancer Institute, Cairo University, and maintained at mice transplanted line. International protocols governing the ethical treatment of animals were followed. EACC cell counts were adjusted to 10⁶ cells/1 ml (counting both mature and immature cells). The cytotoxicity of each compound against EACC cells was determined by the Trypan blue exclusion test. The cell counts were adjusted to (110 5 cell/ 0.1 ml). Next, 0.1 ml of the cell suspension containing 10⁵ cells/0.1 ml was added to each of four 1.8 ml screw-caps sterile Eppendorf tubes. Ten concentrations of the tested compounds were added, three replicas each. For each compound one tube served as negative control, where

culture medium was added instead of the active compound. The tubes were incubated at 37 °C in the presence of 5% (v/v) CO₂ for 24 hrs (dark condition, humidified air). After overnight incubation, cells were stained with Trypan blue (0.2 %) dissolved in PBS, and the number of viable (unstained) versus dead (stained) cells was estimated. Two hundred cells were counted for EACC tube (8)

3. Results

Lisinopril dehydrate, propranolol and nifedipine showed cytotoxicity against the three solid tumor cell lines. HepG2 was the most susceptible cell line to lisinopril dehydrate than the other two cell lines with IC₅₀ = 33.8±88.4 µg/ml and 95% Confidence limit (CL) of 0.79 to 66.8 at the concentration of 300ug/ml.(Figure 1) . Also IC₅₀ of lisinopril against the other two cell lines was 1449±45.6 µg/ml and 95% CL of 1449 to 1488 for HeLa cells and 2306±45.9 µg/ml with 95% CL of 2287 to 2326 for MCF7 cells at the tested dose.

On the other hand Propranolol showed more potent significant inhibitory activity against MCF7 cells with IC₅₀ of 78.0± 121.4 µg/ml and 95% (CL) of 26.6 to 129.3 at a tested concentration of 3000ug/ml (Figure2) , while its IC₅₀ on HeLa and HEPG-2 cells was 108.8± 149.6 and 236± 178 with 95% (CL) of 45.4 to 172.1 and 161 to 311 respectively.

Nifedipine affected HeLa cells with IC₅₀ = 130±58.4ug/ml and 95%CL = 101 to 159 more than the other two cell lines at which IC₅₀ of the drug was 147±108 and 216± 94.4 and 95%CL of 93 -201 and 165 - 266 against MCF-7 and HEPG-2 respectively at a concentration of 300ug/ml (Figure 3).

Table 1 shows the different cytotoxicity effects of the three tested compounds against the three types of cell lines used ; from this table it was shown that Lisinopril was significantly more potent against HEPG-2 than the other two cell lines. Also Propranolol was significantly more potent against MCF-7 than against HEPG-2 cell lines. Nifedipine was significantly more potent against HeLa than against HEPG-2 cell lines. On the other hand it was found that Lisinopril was significantly less potent against both HeLa and MCF-7 cell lines than both Propranolol and Nifedipine.

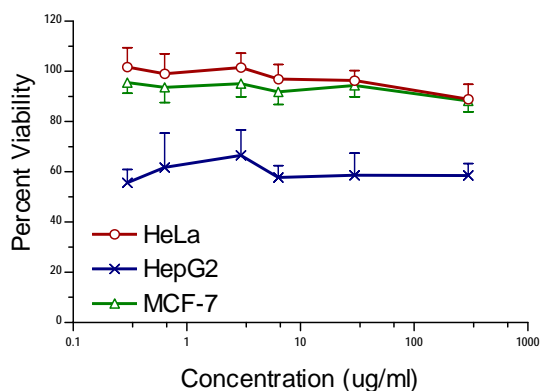
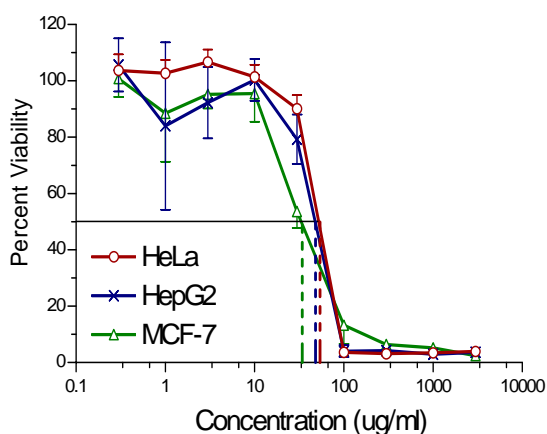
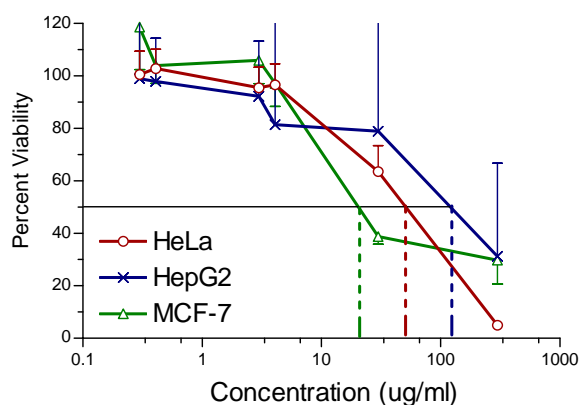
Trypan blue assay was used for the evaluation of anticancer activity of lisinopril , propranolol and nifedipine against EACC cell line. Propranolol showed 97.7 % inhibition of cell growth at a concentration of 300 ug/ml and 100% inhibition at a concentration of 3000 ug/ml. Lisinopril showed 18.28% inhibition of cell growth at a concentration of 300 ug/ml .Nifedipine inhibited the growth of EACC cells by 11.40 % at a tested dose of 300 ug/ml. (Table 2) .

Table 1- Cytotoxic effects of lisinopril, propranolol, nifedipine against HeLa, HEPG-2 and MCF-7 cell lines (Mean \pm SD).

Compound		HeLa	HEPG-2	MCF-7
Lisinopril	IC ₅₀ \pm SD, μ g/ml	1449 \pm 45.6	33.8 \pm 88.4	2306 \pm 45.9
	95% CL, μ g/ml	1449 to 1488	0.79 to 66.8	2287 to 2326
Propranolol	IC ₅₀ \pm SD, μ g/ml	108.8 \pm 149.6	236 \pm 178	78.0 \pm 121.4
	95% CL, μ g/ml	45.4 to 172.1	161 to 311	26.6 to 129.3
Nifedipine	IC ₅₀ \pm SD, μ g/ml	130 \pm 58.4	216 \pm 94.4	147 \pm 108
	95% CL, μ g/ml	101 to 159	165 to 266	93 to 201

Table 2- Cytotoxicity of propranolol, lisinopril and nifedipine against EACC cell line at fixed concentration of 300 μ g/ml

Compound	Concentration μ g/ml	Cell Viability %	\pm SD
Propranolol	300	2.285608	3.958789
Lisinopril	300	81.62038	6.41795
Nifedipine	300	88.60813	11.94706

**Figure 1:** Effect of Lisinopril on HeLa, HepG2 and MCF7 cell lines.**Figure 2:** Effect of Propranolol on HeLa, HepG2 and MCF7 cell lines.**Figure 3:** Effect of Nifedipine on HeLa, HepG2 and MCF7 cell lines.

4. Discussion

Pharmacological treatment of cancer passed into two subsequent eras; the pregenomic era where most anticancer drugs were mainly cytotoxic, and post-genomic era-type drugs referring to rationally based designed agents. An alternative drug development strategy is the exploitation of established drugs that have already been approved for treatment of non-cancerous diseases and whose cancer target(s) have already been discovered.

The current study has been carried out to test the ability of three antihypertensive commercial drugs (lisinopril, propranolol and nifedipine) to inhibit the proliferation of four types of cancer cells HeLa (cervix), HepG2 (liver), MCF-7 (breast) and EACC (endometrium) cell lines by using the neutral red and trypan blue assays methods. The neutral red assay is one of the most used cytotoxicity tests which provides a quantitative estimation of a number of viable cells in a culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes (7).

The three tested antihypertensive drugs are belonging to different sub-classes; ACE inhibitors (lisinopril), beta-adrenergic blockers (propranolol), and calcium channel blockers (nifedipine). Babu *et al.*, reported that lisinopril, propranolol and nifedipine had a significant inhibitory effects on carcinoma of cervix cells in a concentration dependent manner and propranolol was found to be the most active drug with 39.57% inhibitory activity against HeLa cells at a dose of 256ug/ml (9). These results are in agreement to some extent with the present study since the most active drug against cervix cancer cells (HeLa) was nifedipine; it had 95.02% inhibitory activity against HeLa cells at a dose of 300 ug/ml.

In another study done by Latha *et al.*, it was found that nifedipine also had anticancer activity against carcinoma of scalp at a dose of 256ug/ml (6). Nifedipine was shown to induce apoptosis and decrease cellular proliferation in many cancer cell lines *in vitro* and *in vivo* by yet an undefined mechanism that may or may not depend on blocking any ionic channels (1). It has been shown that the rise in cytosolic-free calcium activates protein kinase C (10) catalyzes the phosphorylation of a number of cellular proteins necessary for proliferation (11). In addition, transient rises in cytosolic calcium have been shown to initiate activation of the calcium receptor calmodulin, which may also play an important role in the regulation of cell proliferation (10). Tumors are generally recognized as possessing unusually high calcium levels which may be due to either excessive influx of extracellular calcium or the ability of neoplastic mitochondria to retain higher calcium concentrations (12). Regulation of intracellular calcium is an important signaling mechanism for cell proliferation in both normal and cancerous cells. In normal epithelial cells, free calcium concentration is essential for cells to enter and accomplish the S phase and the M phase of the cell cycle. In contrast, cancerous cells can pass these phases of the cell cycle with much lower cytoplasmic free calcium concentrations, indicating an alternative mechanism has developed for fulfilling the intracellular calcium requirement for an increased rate of DNA synthesis and mitosis of fast replicating cancerous cells. However, there is a growing body of evidence that suggests the T-type Ca²⁺ channel is abnormally expressed in cancerous cells and that blockade of these channels may reduce cell proliferation in addition to inducing apoptosis. Recent studies also show that the expression of T-type Ca²⁺ channels in breast cancer cells is proliferation state dependent, therefore selectively blocking calcium entry into cancerous cells may be a valuable approach for preventing tumor growth (13).

It was found that propranolol potentiates the anti-angiogenic effects and anti-tumor efficacy of chemotherapeutic agents thus implicating it in breast

cancer treatment (14). Also propranolol inhibited pancreatic cancer cell proliferation by blocking signaling through the beta-adrenoceptor and through induction of apoptosis(15). The present study, like that of Babu *et al.*, (9), also demonstrated that propranolol showed anti-proliferative activity against the HeLa cells but it was significantly more potent against MCF7 cells. However, in the present study, Lisinopril dehydrate showed different results than that demonstrated by the same authors as it was significantly the less potent cytotoxic drug against HeLa and MCF7 cell lines.

Angiotensin II (AngII), the biologically active peptide of the renin-angiotensin system (RAS), is also recognized as a potent mitogen that participates in various pathological situations involving tissue remodeling. The role of AngII in cell proliferation and migration, as well as in several experimental angiogenesis models, suggests that the RAS system may be involved in tumorigenesis. Recent studies have revealed local expression of several RAS components in various cancer cells and tissues, including brain, lung, and pancreatic cancers, as well as breast, prostate, skin, and cervix carcinomas (16). The idea that ACE inhibitors might play a protective role in cancer was suggested by observations of reduced incidence of breast and lung cancer in patients undergoing long-term treatment with the captopril, lisinopril, or enalapril (17). Also, Waker *et al.*, reported that the anticancer effects of ACE inhibitors are through anti-angiogenic activity and inhibition of liver cancer growth in rodent models (18).

The range of effective *in vitro* concentrations of the tested 3 drugs in the current study are far higher than the known therapeutic plasma levels achieved in treating other diseases. This would prevent their practical applications in clinical trials on human beings. However, it remains to be seen that using either the metronomic way of giving these drugs, i.e. giving low doses on prolonged schedules, or using them as potentiating agents to other anticancer medications, might be useful.

Conclusion

Lisinopril, propranolol and nifedipine may have anti-proliferative activities against HeLa, HepG2, MCF-7 and EACC cell lines; lisinopril was the most active drug against HepG2, propranolol against MCF7 and EACC and nifedipine against HeLa cell lines. Further investigations on wider range of different cell lines e.g. NCI-60 panel and on animals are needed to confirm these results.

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