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Antimicrobial, antitumor and side effects assessment of a newly synthesized tamoxifen analogue

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Abstract

Tamoxifen citrate is a very prevalent drug marketed under several trade names like Apo-Tamox, Nolvadex, Tamec, Tamizam and Tamoplex. This molecule is approved by the FDA for breast cancer treatment. Some studies have shown that tamoxifen has anti-tuberculosis and anti-parasitic activities. Like any drug tamoxifen possesses side effects, more or less dangerous. Basically, this work is a comparative study which aims to: Primarily compare the

antimicrobial and antitumor activities of tamoxifen and a newly synthesized tamoxifen analogue. Secondly, determine the molecule with lesser side effects. Three groups of mice were injected with tamoxifen citrate and compound **2** (1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-phenyl-but-1-ene dihydrochloride) at doses corresponding to C1 (1/10), C2 (1/50), C3 (1/100) to compound **2** lethal dose ($LD_{50} = 75$ mg/kg) administered for adult mice. A group of not injected mice has served as a study control. Experimental results suggest that compound **2** have better antitumor and antimicrobial activity than tamoxifen citrate besides its lower toxicity effects.

Keywords: tamoxifen citrate, 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-phenyl-but-1-ene dihydrochloride, antimicrobial activity, antitumor, toxicity, mice.

1. Introduction

Nowadays humans are exposed to a variety of diseases, with a degree of dangerousness and virulence that continues to grow each year. This effect can be detected by the emergence of diseases that are resistant to treatments previously discovered and approved by agencies such as the Food and Drug Administration (FDA). This resistance is underlined in the cases of infectious diseases, cancer and several other diseases. Bacteria[1, 2], viruses[3] and malignant cancer cells adapt to therapies and find ways to escape the treatment[4, 5].

Many organizations like United Nations (UN) and WHO sounded the alarm on the inefficiency of older treatments against new emerging bacterial strains, which can be catastrophic because it can lead to a human powerlessness against a possible outbreak if emergency measures are not taken, like the discoveries of new active molecules and new treatments to combat multidrug-resistant strains[6].

For this purpose, research laboratories and pharmaceutical companies have focused on this subject. Several research strategies have been developed to search for effective products: natural way, by analysis of plant extracts, or synthetic way. In this paper, we will focus about chemically synthesized molecule [7, 8].

Tamoxifen (ICI46,474) is an example of “magical” molecule, with a very remarkable story. Essentially synthesized by Imperial Chemical Industries ICI (AstraZeneca) as part of a fertility control program, this selective estrogen receptor modulator has failed on the goal in which it was designed. After the establishment of links between estrogen and hormone dependent breast cancer in 1960, tamoxifen has been clinically tested for breast cancer disease where it has shown encouraging results[9].

In 1973, tamoxifen has been marketed in the UK as a breast cancer treatment and in 1975 as an ovulation inductor. The FDA approved tamoxifen as treatment of metastatic breast cancer in 1977[10].

Thereafter, in 1986 it was approved as adjuvant together with chemotherapy. Later between 1988 and 1990 it was recognized and allowed to be used alone for patients with: node-positive post-menopausal and pre or post-menopausal node-negative estrogen positive disease. In the same period, tamoxifen was approved as a risk reducer for high-risk women for breast cancer. Three years after, men with breast cancer was treated with it (1993). In 2000, tamoxifen treatment *in situ* of ductal carcinoma (DCIS) was approved[11].

Like any drug tamoxifen has side effects, more or less dangerous. The percentage of occurrence, frequency and the manifestation of these effects are variable, and depend mostly on two factors: the dose administered and the subject receiving the treatment. According to the guide of use and labels approved by FDA for tamoxifen commercialization and some others studies, with tamoxifen the incidence of uterine malignancies (uterus-endometrial cancer and uterine sarcoma) is increased by 50% [12,13]. Tamoxifen also causes gynecologic symptoms like pelvic pain, menstrual irregularities, and vaginal bleeding[14]. The thrombo embolic events were reported (pulmonary embolism and deep vein thrombosis) [15]. Liver can be affected with tamoxifen and some severe hepatic abnormalities including fatty changes in the liver, cholestasis, hepatitis, hepatic necrosis (some fatal) and liver cancer were reported [12]. Hypercalcemia, visual disturbance, nausea, depression in addition of abnormality in hematology and lipid profiles are mentioned [12].

Recent innovative studies have shown that tamoxifen has activity against bacteria even those that present a problem of resistance such as tuberculosis; In addition to the activity against leishmaniosis that has been indicated [16, 17].

Ferrocenyl or phenyl derivatives of diphenyl butene series, that are tamoxifen analogs, are known for their anti-proliferative activity on breast cancer cells and leukemia, as well as for their excellent antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae*, *P. savastanoi* pv. *Savastanoi*, *A. tumefaciens*, *F. Solani*, *Listeria monocytogenes* and *Salmonella enterica* [18-24].

The focus of this study is based on the comparison of two molecules: tamoxifen citrate and newly synthesized Compound **2**. This comparison is made on two parts: a microbiological aspect corresponding to the test of the activities of these two molecules against Gram-negative and Gram-positive bacteria as first part, and the evaluation of toxicological effects on mice as second part. Compound **2** is the dihydrochloride version of compound **1** and was used in place of **1** in order to make it soluble into water.

2. Materials and methods

2.1. Chemical compounds

Tamoxifen citrate was purchased from Sigma Chemical Company (Dorset, UK). 1,1-bis[4-(3-dimethylaminopropoxy)phenyl]-2-phenyl-but-1-ene dichloride(compound **2**) was prepared (Figure 1). Compound **1** [25] (1.15 g, 2.36 mmol) was dissolved in 150 mL of diethyl ether and stirred. A 2 M solution of hydrochloric acid in diethyl ether (2.6 mL, 5.2 mmol) was added drop-wise into the solution. A white precipitate was immediately formed. After stirring for 20 min, the mixture was filtered under argon and the obtained white solid was washed with 3 x 5 mL of diethyl ether and dried under vacuum, giving compound **2** in quantitative yield. Crystals contain traces of diethyl ether that can't be removed even after several hours under high vacuum, making elemental analysis not relevant. ¹H NMR (DMSO-d₆): δ 0.88 (t, *J* = 7.1 Hz, 3H, CH₃), 2.03-2.30 (m, 4H, CH₂), 2.37-2.50 (m, 2H, CH₂), 2.75 (d, *J* = 4.4 Hz,

113 6H, NMe₂), 2.80 (d, J = 4.4 Hz, 6H, NMe₂), 3.09-3.32 (m, 4H, CH₂N), 3.94 (d, J = 5.1 Hz,
114 2H, CH₂O), 4.11 (d, J = 5.1 Hz, 2H, CH₂O), 6.63 (d, J = 8.4 Hz, 2H, C₆H₄), 6.76 (d, J = 8.4
115 Hz, 2H, C₆H₄), 6.98 (d, J = 8.4 Hz, 2H, C₆H₄), 7.07-7.29 (m, 7H, C₆H₄ + C₆H₅), 10.84-11.22
116 (m, 2H, NH⁺). ¹³C NMR (DMSO-d₆) : δ 14.3 (CH₃), 24.7 (CH₂), 24.8 (CH₂), 29.5 (CH₂), 42.8
117 (NMe₂), 42.9 (NMe₂), 54.8 (CH₂N), 54.9 (CH₂N), 65.6 (CH₂O), 65.8 (CH₂O), 114.3 (2 CH
118 C₆H₄), 115.1 (2 CHC₆H₄), 127.0 (CHC₆H₅), 128.9 (2CH_{arom}), 130.3 (2CH_{arom}), 131.1
119 (2CH_{arom}), 132.4 (2CH_{arom}), 136.4 (C), 136.7 (C), 138.4 (C), 141.4 (C), 142.9 (C), 157.1 (C),
120 157.9 (C). IR (KBr, ν cm⁻¹): 3026, 2961, 2688, 2512, 2474 (CH, CH₂, CH₃).

121 **2.2. Bacterial strains and growth conditions**

122 *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 6538, *Escherichia coli*
123 ATCC 10536, *Escherichia coli* K12ATCC 23716 were used as indicator strains. Bacteria
124 were grown aerobically in LB broth (Difco laboratories, Sparks, MD, USA) at 30°C for 24 h.

125 **2.3. Determination of the antimicrobial activity**

126 Micro dilution method on 96 well plates was used to estimate the antimicrobial effects of
127 molecules. Tamoxifen citrate and compound **2**, were dissolved in double-distilled sterile
128 water, the levofloxacin was dissolved in dimethyl sulfoxide (DMSO). A serial of two half
129 dilutions were performed for each molecule to be tested in a final volume of 100 μ L and every
130 well was inoculated with 100 μ L bacterial culture (10⁶ CFU/mL). The final concentrations of
131 each molecule were 100 μ g/mL, 50 μ g/mL, 25 μ g/mL, 12.5 μ g/mL, 6.25 μ g/mL, 3.1 μ g/mL,
132 1.55 μ g/mL and 0.75 μ g/mL. A set of wells containing 200 μ L of LB medium was used as
133 negative control and a blank for reading the optical density at 600nm. The positive control
134 contained 100 μ L of LB medium inoculated with 100 μ L of culture (10⁶ CFU/mL) obtained
135 after overnight culture of 16h shaking at 30 °C. The MIC (Minimal Inhibitory Concentration)
136 was determined as the minimal concentration of molecule where no visible growth was noted.

MTT, (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), was used to determine the MIC. A sample from each well was plated on solid LB medium and maintained overnight at 30°C to determine the MBC as the lowest concentration of molecule corresponding to the inhibition of 95% of cells growth. The IA was calculated according to the formula:

$$IA(\%) = 100 * \left(1 - \frac{[OD600(M) - OD600(i)]}{[OD600(C) - OD600(i)]}\right)$$

Where M is OD with molecule and C is OD of control culture.

2.4. Cell viability assay

MCF7 cells (5×10^4) were seeded in 96-well plates and left to grow overnight. The following day, cells were treated with increasing concentrations of Tamoxifen citrate and compound **2** (0.1µM to 1mM) in triplicate. After 24 h, cell viability was measured using the Cell Proliferation kit (MTT). Results were representative of at least three independent experiments, and were expressed as inhibition rate in reference to the cell growth without drug treatment.

2.5. Animals

Three-to-five-week-old swiss mice (≈20g) were purchased from Central Pharmacy (SIPHAT, Tunisia), quarantined for more than 1 week before starting the experiments, kept under pathogen-free conditions, and handled according to the Guidelines of the “Institute Ethical Committee Guidelines” for the care and use of laboratory animals [26], as provided by the Ethical Committee of Faculty of Sciences at University of Sfax, which approved the present animal experiments.

2.6. Experimental design

Mice were accommodated in polycarbonate cages and were daily provided with water and standard pellet diet (SNA, Sfax, Tunisia). Cages are kept in an air-conditioned room, temperature 22 ± 3 °C and 40% of relative humidity. After intraperitoneal injection, the lethal dose of compound **2** and tamoxifen citrate resulting in 50% mortality (LD_{50}) were found as 75mg/kg and 218mg/kg body weight (bw), respectively. In this study, we used the LD_{50} of compound **2** (75mg/kg) for both molecules in order to compare their effects for the same dose. Mice were divided into seven groups, counting seven mice per each. The first group was used as control groups 2, 3, and 4 were injected by tamoxifen citrate and groups 5, 6, and 7 by compound **2**. Tamoxifen citrate and compounds **2** doses ($C1=1/10$, $C2=1/50$, $C3=1/100$ of LD_{50} of compound **2** (75mg/kg) were not lethal to mice. Both molecules were dissolved in water and daily intraperitoneally injected to mice. Food, water intake and body weights of mice were daily registered. All mice were killed by cervical decapitation after 15 days of treatment. Heparinized tubes were used to collect the blood, and after centrifugation for 10 min at $2200 \times g$, plasma samples were collected and stored at -80 °C for biochemical analysis.

2.7. Clinical signs feed intake and body weight measurement

The general health appearance of each mouse was daily monitored using the following score system: 1 (no sign of discomfort); 2 (slightly ruffled fur, active); 3 (ruffled fur and back circumflexed, active); 4 (ruffled fur, back circumflexed, inactive, buprenorphine was administered); 5 (ruffled fur, inactive, hunched posture, and closed or sunken eyes, with or without ocular secretions). Mice were weighed every three days and expressed as average for each group throughout the experiments.

2.8. Biochemical assays

2.8.1. Hemogram

Also called CBC (Complete Blood Count), it is an automated way to obtain quickly a set of quantitative data about red blood cell (erythrocytes) parameters, white blood cell (leukocytes) count and platelets, including leukocyte formula. Blood was collected in tubes with heparin, and CBC was determined just after animal sacrifice, this service is assumed by the hematology service of Habib Bourguiba Hospital.

2.8.2. Biochemical markers in plasma

Plasma lipid parameters including total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL) were determined using commercial kits (#20111, #20131 and #20113) purchased from Biomaghreb (Ariana, Tunisia). The low-density lipoprotein cholesterol (LDL) was determined according to Friedewald equation [27]:

$$LDL = \frac{TC - (TG - HDL)}{HDL}$$

2.9. Statistical analyses

Data are presented as mean values with their standard errors (SEM). Data were subjected to ANOVA using the GLM procedure of SAS (SAS Institute, 2003). The means were compared with LSD test. A probability of $P < 0.05$ was required for statistical significance. Treatments with different letters are significantly different ($p < 0.05$).

3. Results and Discussion

In a previous published work, several diaryl-butene analogous and derivatives of tamoxifen were tested against pathogenic Gram-positive and Gram-negative bacteria. The idea came from the fact that other research teams have found that tamoxifen citrate has an inhibitory power as bactericidal against the pathogenic germs. Several diaryl-butene have shown some

very interesting activities. However, compound **2** have not been tested. To urge this query and keen interest about the question that whether compound **2** has a potential for tumor and bacterial inhibition? and to compare its activity versus tamoxifen citrate.

We evaluated cytotoxicity of compound **2** compared to tamoxifen citrate on MCF7 breast cancer cells. As the compound **2** is an analogue of tamoxifen, those cells seem to be a good choice given their invasive appearance. The results appearing in the figure 2 shown that both of the two molecules have an antiproliferative activity against MCF7 cells, however, we noted significant effects differences that favor the compound **2**. Indeed, cell's death caused by this compound compared to tamoxifen citrate is equivalent to twice at the concentration of 10 μ M. Our results showed that in the case of MCF7 cells, based on the LD₅₀ value, compound **2** is five times more toxic than the tamoxifen citrate with respective LD₅₀s of 5 μ M and 25 μ M.

Inhibition test was applied with two types of *E. coli* as gram-negative, and *Staphylococcus aureus*, *Listeria monocytogenes* as gram-positive bacterial strains. The antibiotic levofloxacin was used as a control. The MIC and MBC for the different molecules are shown in the table 1.

Compound **2** and tamoxifen citrate show a better potential against gram-positive than against gram-negative. The inhibition profiles obtained for tamoxifen citrate and compound **2** are different, a difference that manifested by a better inhibitory activity for compound **2** than for tamoxifen citrate, whether for the Gram-positive or Gram-negative (Figure 3). Compound **2** and tamoxifen citrate have a ratio MBC/MIC less than or equal to 4, sign of bacteriostatic and not bactericidal activity. MBC of compound **2** for *Staphylococcus aureus* is 6.25 μ g/mL, this value corresponds to the MIC of tamoxifen citrate. These results point out that compound **2** is much better and more active than tamoxifen citrate, and also has the potential to be used against gram-positive strains especially against *Staphylococcus aureus*.

223 Following 15 days of intraperitoneal injection to the mice with tamoxifen citrate and
224 compound **2**, a hematologic study was conducted. The findings obtained shows a disturbance
225 in the blood count for different groups injected with tamoxifen citrate and with compound **2**,
226 compared to the control groups without being injected as depicted in Figure 4. In the scientific
227 literature tamoxifen citrate, as part of these secondary effects, is described as disruptive of
228 blood parameters. In this context, our work has shown a decline in red blood cell count
229 (Figure4A), and variance statistical study on different doses used for each molecule
230 ($LD_{50}/100, LD_{50}/10$) showed no significant differences. However, variance becomes
231 significant by comparing each molecule to the control group. Although the two molecules to
232 be tested reduce the number of red blood cells, a phenomenon already described for tamoxifen
233 and its active derivatives like hydroxytamoxifen known as factors causing erythrocyte
234 hemolysis [28,29]. Compound **2** shows that having a similar effect of reducing the number of
235 these cells, but with a less acute form (Figure 4A). The mean corpuscular volume (MCV)
236 reflecting the size of red blood cells also showed a fall, a decline that seems not to be
237 statistically significant compared to the control group (Figure 4B). However, comparing the
238 effect of the two molecules on the MVC value, the rates of significance become very strong,
239 with a relative decline less important than compound **2**. One of the most relevant and
240 interesting things that was revealed by this study is the difference in effect of the molecules
241 tested on platelets. In contrast to tamoxifen citrate which decreases dramatically the number
242 of platelets in the blood, compound **2** has an opposite effect by a slight increase in platelet
243 count relative to the control (Figure 4D). Detrimental effect of Tamoxifen on blood platelets
244 is already well known [30]. The reason might be explained by the fact that tamoxifen causes
245 mini injury into blood vessel, which explains the appearance of hematomas in some organs
246 such as the liver. Due to their role in cicatrization these thrombosis injuries can occur
247 anywhere in the body (lungs, heart and even the brain). Thrombosis risk increase may lead to

dangerous consequences, like sudden death. Our results also show a significant drop in the number of leukocytes for tamoxifen citrate (Figure 4C). This important downfall for tamoxifen citrate compared to compound **2**, can induce a mess and declining reliability of the immune system, making the body vulnerable for attack by pathogens [31].

In summary comparison between tamoxifen citrate and compound **2** as well as their effects on hematological parameters demonstrates that these two molecules cause anemia, but the severity of the decline is greater for tamoxifen (Figure4A). Compound **2**, unlike tamoxifen citrate, does not cause leukopenia and thrombocytopenia (Figure 4C, and 4D). In nut shell, compound **2** found to be far less toxic and better than tamoxifen citrate.

The analysis of blood biochemical parameters following tamoxifen citrate and compound **2** injections shows majorly changes in triglycerides, High-Density Lipoprotein Cholesterol (HDL) and other cholesterol profiles. Statistical analysis of the dose effect for tamoxifen are not significant, therefore all doses are equivalent (Figure 5). The same thing was observed for the different doses of compound **2** used, except for triglycerides and Low-Density Lipoprotein Cholesterol (LDL) where it seems that the dose is related to the effect of the product (Figure 5).

Outcome of the study suggested that the two molecules alter in a clear manner the serum concentration of cholesterol and HDL (Figure 5B, and 5C). However, compound **2** alters the serum concentration of triglycerides less than tamoxifen citrate and does not register a significant difference with the control (Figure 5A). Although this change, the statistical scale seems to be not significant for the two molecules for LDL (Figure 5D). Cholesterol decrease in the blood during tamoxifen citrate treatment is something that is well known [32]. However, the problem for the triglyceride levels in the blood following administration of tamoxifen citrate is controversial. The majority of studies carried out leads to the fact that

blood triglycerides level increases following administration of tamoxifen citrate. This increase is due to inhibition of hepatic enzymes involved in lipid metabolism which increases the rate of cell and serum triglycerides [33]. Some studies, like our results, found that tamoxifen citrate causes a drop in plasma lipids cholesterol, triglycerides, LDL and HDL [34-37].

4. Conclusion

The results emerged from the present study confirmed the antitumor and antimicrobial effect of tamoxifen citrate and its hematological side effects. Compound **2** seems to be more effective than tamoxifen citrate for antitumor and antimicrobial treatment while having less hematological side effects and less disruption of the blood biochemical parameters. These findings encourage us to push further studies for compound **2** and test it for other therapeutic usage for which tamoxifen was effective.

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400

Figure legends

Figure 1. Preparation of compound **2** and structures of the compounds object of the study

Figure 2. Inhibition of MCF7 cells in presence of tamoxifen citrate or compound **2**.

Figure 3. Growth inhibition of *Listeria monocytogenes* ATCC 7644 (A) and *Escherichia coli* K12 ATCC 23716 (B) in presence of tamoxifen citrate (square) or compound **2** (triangle).

Non-linear regression equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}$.

Figure 4. Effect of tamoxifen citrate and compound **2** at (A) Erythrocytes enumeration; (B) VCM; (C) Leucocytes enumeration and (D) platelets.

Figure 5. Effect of tamoxifen citrate and compound **2** on (A) TG; (B) Cholesterol; (C) HDL and (D) LDL profile in blood.

Table 1: Determination of MIC and MBC of Levofloxacin, tamoxifen citrate and compound 2

Bacterial strains	Compound concentration (µg/mL)					
	Levofloxacin		Tamoxifen citrate		Compound 2	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>Listeria monocytogenes</i> ATCC 7644	6.25	>100	12.5	25	6.25	12.5
<i>Staphylococcus aureus</i> ATCC 6538	12.5	25	12.5	25	6.25	12.5
<i>Escherichia coli</i> ATCC 10536	<0.7	<0.7	> 100	>100	100	>100
<i>Escherichia coli</i> K12 ATCC 23716	<0.7	<0.7	> 100	>100	25	50

1,1-Bis[4-(3-dimethylaminopropoxy)phenyl]
-2-phenyl-but-1-ene

1,1-Bis[4-(3-dimethylaminopropoxy)phenyl]
-2-phenyl-but-1-ene dihydrochloride

Tamoxifen citrate

Figure 1

423

424 **Figure 2**

A

B

425 **Figure 3**

Figure4

Figure 5