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Synthesis, Molecular Docking, And Cytotoxic Activity Of *N*-Ethyl-*N*-(Ethylcarbamoyl)Benzamide Derivatives Against MCF-7 Cell Line.

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ABSTRACT

The urea derivatives has play important role as anticancer agents because of their good inhibitory activity against receptor tyrosine kinases. Benzoylurea derivatives could produced from acylation of *N,N'*-diethylurea by substituted-benzoyl chlorides. The aim of this study to synthesize new benzoylurea derivatives, namely *N*-ethyl-*N*-(ethylcarbamoyl)benzamides (**a-c**) and to examine their cytotoxic activity against human breast cancer cells (MCF-7). The structures of the compounds were analyzed by FT-IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectroscopies. The molecules were docked into ATP-binding site of EGFR (PDB. 1XKK) and displayed high binding affinity. All synthesized compounds were subjected to cytotoxic test by MTT assay. As the result, the spectroscopic data confirmed that the compound **a** is 3,5-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide**,** compound **b** is 2,4-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide**,** and compound **c** is 4-nitro-*N*-ethyl-*N*-(ethylcarbamoyl) benzamide. The compound **b** revealed more potent cytotoxic activity than 5-fluorouracil as reference drug, their IC_{50} value are 3.41 μM and 8.15 μM respectively. The compound **b** showed also higher binding affinity than 5-fluorouracil. It can be concluded that such benzoylation of *N,N'*-diethylurea produced the *N*-ethyl-*N*-(ethylcarbamoyl)benzamides which shows potency as antitumor agent.

Keywords: *N*-ethyl-*N*-(ethylcarbamoyl)benzamide, synthesis, cytotoxicity, MCF-7

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INTRODUCTION

Various urea derivatives have been reported to have a variety of biological activities, such as anti-inflammatory [1], antibacterial [1-4], antifungal [3,4], anti-HIV [5], anticonvulsant and analgesic [6], antiparkinson [7], and anticancer activities [8-10]. The urea derivatives such as nitrosoureas, hydroxyurea, benzoylureas, thioureas, and diarylsulphonyl ureas, which have anticancer activity, work through different mechanisms. Benzoylurea and aryl chloroethylurea derivatives work by inhibiting microtubuli [11-13], while some arylurea and other diarylurea reported to inhibit a variety of receptor tyrosine kinases. The urea derivatives has play important role as anticancer agents due to it have good inhibitory activity against receptor tyrosine kinases, protein tyrosine kinases and NADH oxidase [14]. A serie of dianilinopyrimidineureas are reportedly worked as inhibitors of receptor tyrosine kinase VEGFR2 [15], imidazo[1,2-a]pyrazine diarylureas are inhibitors of receptor tyrosine kinase EphB4 [16], some aryl-heteroarylureas based on 4-aminoquinoline could inhibit the receptor tyrosine kinase IGF-1R [17,18], the 4-anilinoquinazoline derivatives bearing diarylurea and tertiary amino moiety can serve as anticancer agents and receptor tyrosine kinase EGFR inhibitors [19].

The epidermal growth factor receptor (EGFR) is receptor tyrosine kinase that is over-expressed in a wide variety of solid human cancer. The role of EGFR has been most thoroughly studied in breast cancer although EGFR over-expression is also seen in ovarian cancer, lung cancer, and in hormone refractory prostate cancer. Compounds that inhibit the kinase activity of EGFR after binding are of potential interest as new therapeutic antitumor agents [18].

The urea functionality forms two crucial hydrogen bonds, one with the backbone aspartate and the other with the glutamate side chain [19]. While the urea moiety appears crucial for the ligand binding, many of the undesirable properties, such as poor water solubility, may be the result of the diarylurea functionality. Therefore, in our effort we start to improve the physicochemical properties of the diarylurea by inserting only one aromatic ring, as a benzoyl group, and two ethyl groups into the amino of urea while attempting to retain antitumor activity.

Generally, diarylurea derivative is not directly synthesized from the starting material urea but synthesized by the reaction of an intermediate aryl isocyanate and aromatic amine [20-22]. In this study we attempted the synthesis of benzoylurea derivatives directly from reaction of *N,N'*-diethylurea with substituted-benzoyl chlorides.

Acylation of *N,N'*-diethylurea by benzoyl chloride capable of producing *N,N'*- dibenzoylurea-*N,N'*-diethylurea as the reaction proceeds at both the secondary amino group of the urea [23] although theoretically one of the two amino groups are also able to undergo mono substitution [24]. This study described the synthesis of a serie of benzoylurea derivatives, namely N-ethyl-N-(ethylcarbamoyl)benzamides, which bear urea backbone N-CO-N, based on the Schotten-Baumann reaction using an organic solvent and base catalyst. The advantage of this method includes reactivity of ring-substituted benzoyl chlorides towards the secondary amine, a less reactive nucleophile, and simplicity of the reaction which only takes place in one step.

As a part of preliminary investigation on ligand-protein interaction which plays a key role in rational drug design, the new compounds were subjected to molecular docking study into EGFR protein. Docking result will enable us to predict and identify most promising ligands as protein inhibitors which potentially generate antitumor activity. The *in vitro* cytotoxic activity of the new compounds were evaluated against human breast carcinoma cell lines (MCF-7). Based on the result, the most important one is the possibility to make various derivatives that makes it as a basis for further research to produce as many as possible benzoylurea ligands which will undergo biological trials against cancer cells.

MATERIALS AND METHODS

The chemicals and reagents obtained from commercial supplier were used as received. Melting points were determined by Fischer Johns apparatus and were uncorrected. Purity of the compounds was checked by TLC using silica gel $60F_{254}$ coated aluminum plates and the spots were detected by exposure to UV lamp at 254 nm. IR spectra were recorded in KBr on Jasco FT-IR 5300 spectrometer, ^{1}H-NMR and ^{13}C-NMR spectra were

recorded on JEOL ECS-400 (400 MHz) spectrometer in $CDCl_3$ and TMS as an internal standard. Mass spectra (Electron Ionization) were recorded on GC-MS Agilent 6890N. The chemical names given for the synthesized compounds are according to the IUPAC nomenclature.

General procedure for synthesis of a-c

N,N'-diethylurea (0.0125 mol) dissolved in THF (20 ml) was mixed with triethylamine (4 ml) in a 200 ml conical flask. The mixture was added drop wise by solution of substituted-benzoyl chloride (0.025mol) in THF at 0–5 °C ice bath while stirring for 30 minutes. Then the temperature was raised to 70 °C and the mixture was refluxed for 3 hours. Reflux was continued at room temperature for next 20 hours and the mixture was concentrated by evaporating the solvent. The product was washed with cold water and saturated sodium bicarbonate respectively, and the solid residue was crystallized from ethanol–water (1:1) to obtain the benzoylurea derivatives.

(a) 3,5-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide

White crystal, yield 84%, mp: 94 ^{0}C. IR (KBr) ν, cm^{-1}: 3291 (N–H); 3073 (=C–H), 2876 (C–H); 1668 (C=O), 1567 (C=C), 858 (C_{ar}–H). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.13 (t, *J*= 7.6, 3H, -C<u>H</u>$_3$), 1.22 (t, *J*= 6. 8, 3H, C<u>H</u>$_3$), 3.39 (q, *J*= 5.6, 4H, C<u>H</u>$_2$), 3.71 (q, *J*= 7, 4H, C<u>H</u>$_2$), 7.30 (s, 2H, =C<u>H</u>), 7.47 (s, 1H, =C<u>H</u>), 8.79 (s, 1H, N<u>H</u>). ^{13}C NMR (400 MHz, $CDCl_3$, δ ppm): 14.86, 15.17, 35.58, 43.25, 124.26, 130.34, 135.76, 139.17, 153.82, 171.76. MS(EI) m/z: 287 (M+), 289 (M+2), 173 (base peak), 216, 200, 145, 109.

(b) 2,4-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide

White crystal, yield 91%, mp: 79 ^{0}C. IR (KBr) ν, cm^{-1}: 3414(N–H); 3085 (=C–H), 2942 (C–H); 1714 (C=O), 1567 (C=C), 824 (C_{ar}–H). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.06 (t, *J*= 7.0, 3H, C<u>H</u>$_3$), 1.22 (t, *J*= 7.2, 3H, C<u>H</u>$_3$), 3.38 (m, 2H, C<u>H</u>$_2$), 3.81 (m, 2H, C<u>H</u>$_2$), 7.24 (d, *J*= 8, 1H, =C<u>H</u>), 7.32 (d, *J*= 8.8, 1H, =C<u>H</u>), 7.45 (s, 1H, =C<u>H</u>), 8.99 (s, 1H, N<u>H</u>). ^{13}C NMR (400 MHz, $CDCl_3$, δ ppm): 14.82, 14.90, 35.58, 41.57, 127.69, 129.99, 130.64, 134.73, 136.21, 139.17, 153.82, 170.83. MS(EI) m/z: 287 (M+), 289 (M+2), 173 (base peak), 216, 200, 145, 109.

(c) 4-nitro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide

White yellowish crystal, yield 49%, mp: 101 ^{0}C. IR (KBr) ν, cm^{-1}: 3313(N–H); 3077 (=C–H), 2937 (C–H); 1696 (C=O), 1521 (C=C), 854 (C_{ar}–H). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.09 (t, *J*=7.2, 3H, C<u>H</u>$_3$), 1.21 (t, *J*= 7.2, 3H, C<u>H</u>$_3$), 3.37 (m, 2H, C<u>H</u>$_2$), 3.64 (q, *J*= 6.4, 2H, C<u>H</u>$_2$), 7.56 (d, *J*= 9.2, 2H, =C<u>H</u>), 8.31 (d, *J*= 8.4, 2H, =C<u>H</u>), 8.81 (s, 1H, N<u>H</u>). ^{13}C NMR (400 MHz, $CDCl_3$, δ ppm): 15.14, 14.85, 35.62, 42.01, 124.27, 126.80, 142.44, 148.56, 153.73, 172.58. MS(EI) m/z: 265 (M^+), 150 (base peak), 193, 178, 123, 107.

Molecular Docking Simulation

Chemical structures of the compounds have been drawn using ChemBioDraw Ultra 12.0 (Cambridge Soft), their 3D geometries were subjected to energy minimization using MMFF94, and the energy presented as E_{total}. The molecular docking were performed by Molegro Virtual Docker (MVD) version 6.0 (CLC Bio) into crystal structure of the EGFR (PDB. 1XKK). The tested compound were placed into ATP-binding site of EGFR (cavity-1) by align method to the reference ligand GW572016 [25]. The binding affinity between ligand and protein (docking score) was predicted using MolDock Score. The highest-scoring pose (lowest energy) should represent the best-found binding mode. Evaluation of the interaction was based on their MolDock Score which is the sum of ligand-protein interaction energy, including hydrogen bonds between ligands and protein, and internal energy of ligand. The validation of docking was carried out by re-docking the GW572016 into cavity-1 of 1XKK. Docked into 1XKK, hydroxyurea and 5-Fluorouracil, the reference compounds, have docking scores -50.35 and -62.88 respectively.

A high binding affinity indicates stronger binding. The best docked structures have to follow these criteria: i) they have the lowest binding energy (MolDock Score); ii) geometrically, they must occupy the same cavity in the receptor similar to GW572016. This can be observed visually by comparing the structure of docked molecule with crystal structure of GW572016 inside the binding site. The ligand-protein complexes of the top-scoring poses were used for further visual inspection.

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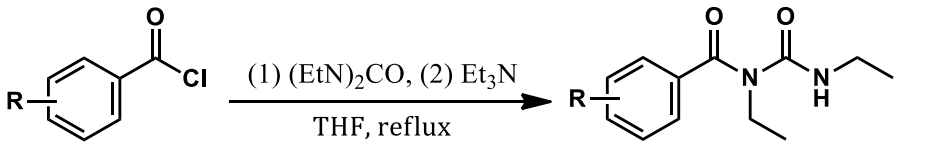
Cytotoxic assay

Cytotoxic activity was tested against MCF-7 cell line by MTT assay and expressed in IC_{50}, a concentration of the compounds inducing 50% inhibition of cell growth of treated cells compared to the growth of control cells. Hydroxyurea (HU) and 5-fluorouracil (5-FU) were used as the reference drugs [26]. Cancer cell lines were seeded at a density of $8x10^3$ cells/well in 96-well plates. After 24 hours, exponentially growing cells were exposed to the test compounds in DMSO at final concentration ranging from 100 to 600 µg/ml. After 24 hours incubated in a 5 % CO_2 incubator at 37 ^{0}C, cell survival was determined by the addition of MTT solution (100 µl of 0.5 mg/ml MTT in PBS). Once formazan was formed, 100µL 10 % SDS in 0.1 N HCl was added and plates were incubated in the dark at 37 ^{0}C overnight. The absorbance was observed at 595 nm on ELISA-reader and survival ratio of living cells were expressed in percentages with respect to untreated cells. Each experiment was performed at least three times.

RESULTS AND DISCUSSION

Chemistry

The synthesis procedure used (Scheme 1) was capable to produce the designed compound. The structural change of *N,N'*-diethylurea to *N*-ethyl-*N*-(ethylcarbamoyl) benzamide was characterized by the conversion of one NH moeity of the diethylurea to N-C=O(ar). There are two symmetric NH moeity in *N,N'*-diethylurea, but the new compounds bear only one benzoyl group attached on their N atom. The main feature for the formation of the compound is the absence of hydrogen on one N atom of *N,N'*-diethylurea and the presence of a benzoyl group. This is proved by ^{1}H-NMR spectrum showing an additional H peak in the benzene region ranging from δ= 7.3 ppm to 8.8 ppm. The C=O of benzoyl is proved by ^{13}C-NMR chemical shift at 170-172 ppm, supported by the IR spectrum showing absorption band at 1668-1714 cm^{-1}.



compound **a**: R = 3,5-dichloro
compound **b**: R = 2,4-dichloro
compound **c**: R = 4-nitro

Scheme 1. The synthesis of compounds a-c

The synthesized derivatives **a-c** were structurally determined by spectral data of FT-IR, ^{1}H NMR, ^{13}C NMR, and GCMS analysis. The structure of the *N*-ethyl-*N*-(ethylcarbamoyl) benzamides was elucidated as discussed below. Structure of 3,5-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide (compound **a**) was shown in Figure 1.

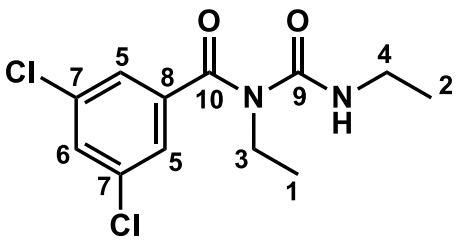


Figure 1: structure of 3,5-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide (compound a)

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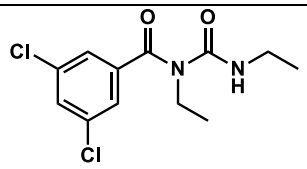
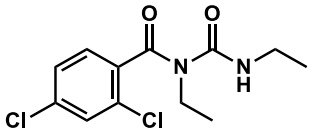
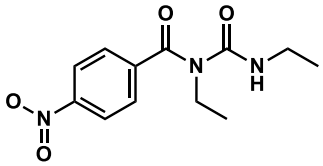
The ^{1}H NMR spectrum of 3,5-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide (compound **a**) exhibited peak at δ= 7.47-8.79 ppm (singlet, *meta* coupling) corresponds to 3 ArH , aromatic proton of benzene ring. The proton of amine (NH) shows broad singlet at δ = 8.79 ppm. Multiplet at δ= 3.39-3.71 ppm corresponds to methylene group (CH_2 (2H)) due to coupling with amine and methyl protons and triplet at δ= 1.13-1.22 was corresponds to methyl group (CH_3 (3H)) with coupling constant *J*= 7.2 Hz, due to adjacent methylene protons. Additional support to elucidate the structure (Fig. 1) was obtained from ^{13}C NMR spectrum. The appearance of peak at δ= 14.86 and 15.17 ppm were for CH_3(C1) and CH_3(C2) respectively, peak at 35.58 ppm for CH_2(C3), peak at 43.25 ppm for CH_2(C4). The aromatic carbon was found to appear at δ between 124.26-139.17 ppm respectively. Peak at 153.82 ppm was corresponds to quarternary carbon linked by two nitrogen (N-CO-NH, C9) and peak at 171.76 ppm was corresponds to quarternary carbon linked by nitrogen and aromatic ring (N-CO-C_{ar}, C10). Further the mass spectrum of compound **a** was recorded as additional evidence for the proposed structure. It was exhibited three isotopic peaks in the molecular ion region (M+, M+2, M+4) with peak heights in the ratio of 9:6:1 due to two chlorine atoms. From all these spectral data the structure of compound **a** was confirmed. Similarly the structures of the other derivatives were determined (Table 1).

Table 1: Physical data of *N*-ethyl-*N*-(ethylcarbamoyl)benzamides

Compound	Structure	Yield (%)	M.P (ºC)
a		84	94
b		91	79
c		49	101

Molecular Docking Results

The compounds **a-c** were subjected to molecular docking study into EGFR protein. The Lipinski's rule which was applied on the molecules (**a-c**) are Log P (the logarithm of octanol/water partition coefficient), molecular weight, MR (molar refractivity), total polar surface area (TPSA), the number of hydrogen bond donors and acceptors. Most "drug- like" molecules have log P ≤ 5, molecular weight ≤ 500, number of hydrogen bond acceptors ≤ 10, and number of hydrogen bond donor's ≤ 5. The Lipinski's rule of five parameters and total polar surface area (TPSA), which has shown to correlate with drug absorption, were obtained using the ChemBioDraw Ultra program (Table 2).

Table 2: Physicochemical properties of synthesized compounds

Compound	MW	Log P	MR	E_{total}*	TPSA	H-bond donor	H-bond acceptor
a	289	3.95	7.26	-67.29	49.41	1	2
b	289	3.95	7.26	-55.69	49.41	1	2
c	265	2.27	6.89	-11.35	101.22	1	2

Compounds containing two chloro groups at position-3,5 (compound **a**) and position-2,4 (compound **b**) showed no difference in the value of Log P and CMR but showed differences in values E_{total}. Log P and MR are only depending on the type of atom or group in the molecule [28]. Most computer programs calculate Log P from two-dimensional (2D) molecular structure. MR only provide information about molecular size, but does not give information about the molecular shape so that it is equal for all isomers of molecules [29]. The E_{total} is intramolecular energy in the most stable conformation [30], which is calculated from molecular geometry optimization method MMFF94. This energy depends on the arrangement of atoms or groups in the stable conformation so characteristic for each molecule. Compound **a** (3,5-dichloro substituted) and compound **b** (2,4-dichloro substituted) which contain the same substituent, but in different position on benzene ring, lead to different total energy of optimized geometry (E_{total}).

2D structures of the ligands (**a-c**) were converted into energy minimized 3D structures and were then used for *in silico* ligand-protein docking. The active crystal structure of EGFR was interacted with pharmacophores of *N*-ethyl-*N*-(ethylcarbamoyl)benzamide derivatives **(a-c)** using molecular docking. The docking results are calculated according to binding energy and RMSD values. Table 3 shows the docking scores of the three compounds, which represents ligand-protein binding energy.

Table 3: Docking score of *N*-ethyl-*N*-(ethylcarbamoyl)benzamide on EGFR tyrosine kinase using MVD software version 6.0

Ligand	Docking score (kcal/mol)	Amino acid	Σ Interaction	
			H-bonds	Steric (van der Waals)
a	-110.91	Ala743, Asp855, Cys775, Leu792, Gln791, Leu844, Lys745, Thr790, Val726	0	17
b	-107.44	Ala743, Gln791, Leu844, Lys745, Thr854, Thr790, Val726	0	16
c	- 99.97	Ala741, Arg776, Gln791, Leu844, Lys745, Thr790, Val726	3	15

The docking of EGFR (1XKK) protein with new ligands **a-c**, exhibited well established interaction with many amino acids in the receptor binding site. Figure. 2 shows the docked images of selected candidate ligands 3,5-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide (**a),** 2,4-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl) benzamide (**b**), and 4-nitro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide (**c)** in the docking field of secondary structure of protein and in electrostatic field of amino acids in the binding site.

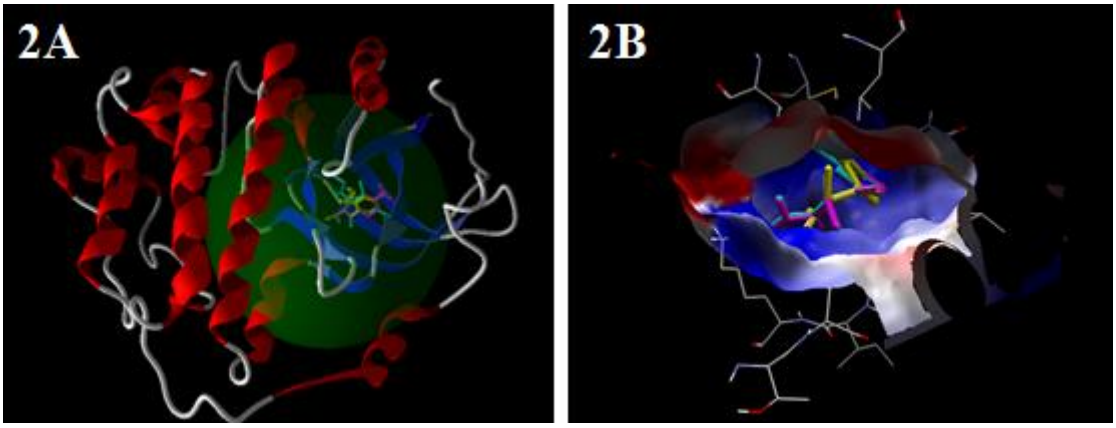


Figure 2: Docked conformation of three molecules derivatives in binding site cavity-1 of EGFR: (2A) molecules in the secondary structure of protein, (2B) molecules in electrostatic field of amino acids in the binding site. Stick in yellow color denoted compound a, light blue color denoted compound b, purple color denoted compound c.

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Figure 3 shows interaction of the compounds with amino acids in binding site of EGFR, including hydrogen bond and steric interactions. 4-nitro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide (**c)** form two hydrogen bonds with Thr790 and one hydrogen bond with Gln791, which are contributed by oxygen of nitro groups as acceptor and nitrogen of nitro groups as donor hydrogen bond.

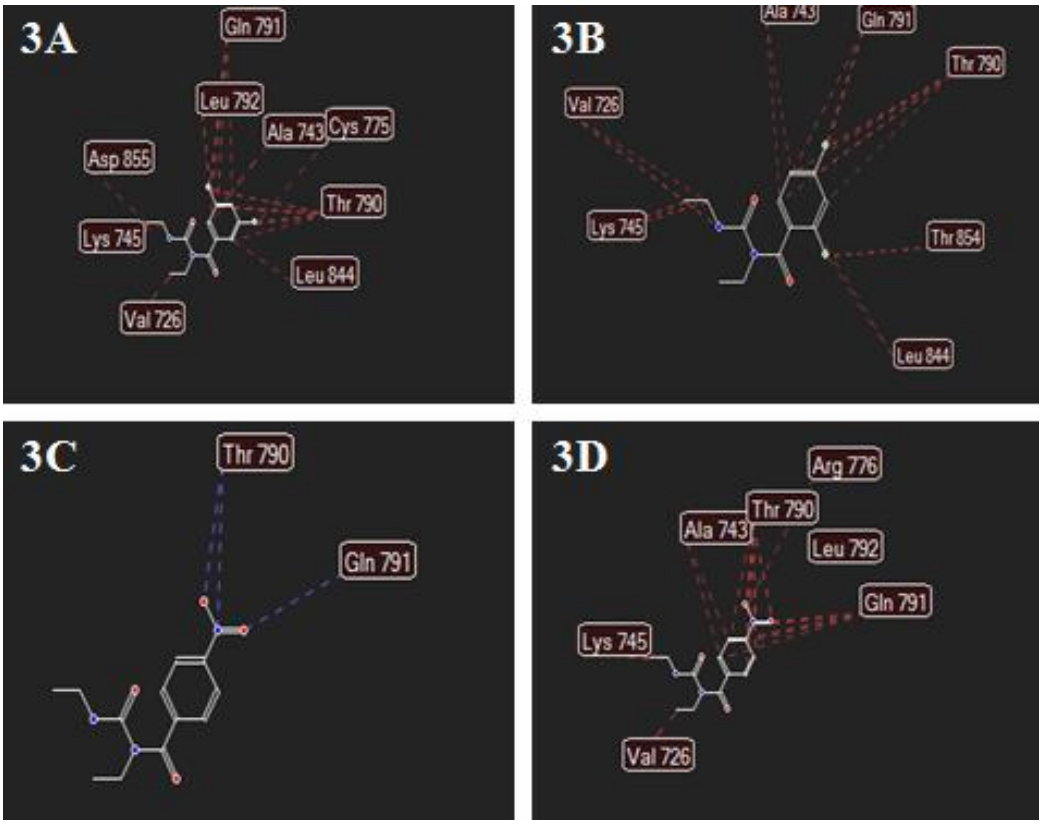

Figure 3: Two dimensional interaction of three molecules derivatives with amino acids inbinding site cavity-1 of EGFR: (3A) steric interactions of compound a, (3B) steric interactions of compound b, (3C) hydrogen bond interactions of compound c, and (3D) steric interactions of compound c. Dashed line in red color denoted steric interaction, blue color denoted hydrogen bond interaction.

In silico studies revealed that all the synthesized molecules showed good binding energy toward the target protein ranging from -99.97 to -110.91 kcal/mol (Table 3). For comparison, hydroxyurea, ie urea derivatives whose structure does not contain an aromatic ring, have a lower binding energy (-50.35 kcal/mol). All molecules (a-**c)** have shown many non hydrogen bond (steric interactions), but only compound **c** shows three hydrogen bonds interaction (Table 3 and Figure 3.). The binding energy is apparently not related to the number of hydrogen bonds but shows the relationship with the number of steric interactions. The compounds show good binding interaction with many amino acids such as Gln791, Thr790, Val726 with 15-17 steric interactions.

Cytotoxic Activity

Cytotoxicity of the derivatives (**a-c**) against MCF-7 cell lines, as shown in Table 4, revealed that all of them have promising anticancer activity if compare with hydroxyurea as reference drug and the IC_{50} values of the compounds diverged according to their structure variations. Compound **a** and **b** which contain the same substituent at different position on benzene ring exhibited different cytotoxic activity. The compound **a** exhibited valuable cytotoxicity against MCF-7 cell lines with IC_{50} values 3.41 µM, compare to 5-fluorouracil (IC_{50} values 8.15 µM). The shift of two chlorines position on the ring from position-3 and 5 to position-2 and 4 as in compound **b** caused an increasing in cytotoxicity, from IC_{50} value 10.95 µM to 3.41 µM, respectively. Compound 4-nitro-*N*-ethyl-*N*-(ethylcarbamoyl) benzamide (**c**), which have a nitro group at position-4 showed

lower activity against MCF-7 (IC_{50}= 9.57 μM). Two chloro substituents at position-3 and 5 have σ value which is almost equal to the nitro at position-4, σ= 0.75 and σ= 0.78 respectively [27]. σ value is Hammet constant for substituent on the benzene ring that describes the influence of substituents on the reactivity of the benzoic acid derivatives. The σ Hammet is one constanta that represents the electronic properties of the substituents.

Table 4: IC_{50} cytotoxic activity against MCF-7 cell line

Compound	IC_{50} (μM)
a. 3,5-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide	10.95
b. 2,4-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide	3.41
c. 4-nitro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide	9.57
Hydroxyurea (urea derivative, anticancer drug)	23.74
5-Fluorouracil (reference drug)	8.15

Data in Table 4 shows that compound **b** has highest activity among all tested compounds, and its activity higher than 5-Fluorouracil, drug which is used in the treatmant of breast cancer. Compare with hydroxyurea, the aromatic rings in the structure of the three benzoylurea derivatives which capable to increase the ligand-protein binding energy (Table 3) could also increase the cytotoxic activity against MCF-7 cells.

CONCLUSION

Three compounds of ring-substituted *N*-ethyl-*N*-(ethylcarbamoyl)benzamides could be produced from acylation of *N,N'*-diethylurea by substutited-benzoyl chlorides in tetrahydrofuran using triethlyamine as catalyst. The compounds showed lower binding energy and higher binding affinity than hydroxyurea and 5-Fluorouracil. All of the compounds showed cytotoxic activity higher than hydroxyurea, but only 2,4-dichloro-*N*-ethyl-*N*-(ethylcarbamoyl)benzamide which generates higher activity than 5-Fluorouracil against human breast cancer cells (MCF-7). The *N*-ethyl-*N*-(ethylcarbamoyl)benzamides have potency as antitumor agent.

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