Antineoplastic Effect of Sulfanilamide Hybridized with Ciprofloxacin "In Vitro Study"

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ABSTRACT

Many efforts have focused on the therapeutic approach of cancer for a long time by studying cytotoxic activities of different compounds including synthetic, semi synthetic and plant constituents. This work involved synthesis of 1-cyclopropyl-4-fluoro-3-oxo-2-(4- sulfamoylphenylamino) ethyl piperazin-1-y1-1,4-dihydroquinoline-3-carboxylic acid compound named as (CSA) prepared by hybridization of sulfanilamide with ciprofloxacin was applied against HCT116 colorectal adenocarcinoma cell line. This study was planned to evaluate cytotoxicity of CSA by thiazolyl blue tetrazolium bromide (MTT) cytotoxicity test by using HCT116 cell line aiming to introduce an alternative therapeutic compound as compared with standard chemotherapeutic drugs such as doxorubicin (DOX) and 5-Fluorouracil (5-FU), by the assessment of half maximal inhibition concentration (IC50). Results showed that CSA compound was effectively and significantly inhibit HCT116 cells proliferation (p<0.0001) at different concentrations involved 1, 10, 100, 500 and 1000 μg/ml with IC50 of 182.4 μg/ml. Values of DOX and 5-FU IC50 were 58.94μg/ml and 36.73μg/ml respectively. We concluded that CSA compound may have anticancer effect against HCT116 colorectal adenocarcinoma cell line, and future in vivo study to confirm this result is strongly recommended.

Key words: HCT116 colorectal carcinoma, Sulfanilamide, Ciprofloxacin, Cell culture.

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INTRODUCTION

Cancer is a disease involving uncoordinated abnormal neoplastic cell proliferation with the high potency to spread or invade other parts of the body. Cancer involves uncontrolled cell growth, invasion, and sometimes different grade of metastasis [1-2] Colorectal cancer (CRC) is usually markedly varies according to the mortality and incidence around the world and regarded as the third most commonly diagnosed neoplastic disease in the population of males and the second in females, with the incidence of nearly 1.8 million new emerging cases and mortality of 861,000 deaths recorded during 2018 according to the database of World Health Organization GLOBOCAN. Rates of CRC between genders are substantially higher in males than in females [3]. In the United States, both the incidence and mortality have been slowly but steadily decreasing. Annually, approximately 145,600 new cases of large bowel cancer are diagnosed, of which 101,420 are colon and the remainder are rectal cancers [4]. Conventional chemotherapy that used for cancer treatment is seriously limited by emerging of multidrug resistance (MDR) exhibited by tumor cells. The failure of the curative treatment often occurs as a result of intrinsic or acquired drug resistance of the neoplastic cells to chemotherapeutic agents. The resistance of tumors occurs not only to a singular cytotoxic drug, but as a cross-resistance to various ranges of drugs with different structures and cellular targets resulting in multiple drug resistance (MDR). Once MDR emerged, the long-term use of drugs become without benefit, resistance is further stimulated and toxic effects appear. Multidrug resistance (MDR) severely limits the effectiveness of chemotherapy in a variety of malignancies and is responsible for the health retardation and overall cancer chemotherapy poor efficacy [5-7]. Multidrug resistance is usually correlated and frequently associated with amphiphatic natural products including hydrophobic moieties in their structure such as the taxanes (docetaxel and paclitaxel), vinca alkaloids (vinorelbine, vinblastine and vincristine), anthracyclines (doxorubicin, daunorubicin, and epirubicin), epipodophyllotoxins (topside and teniposide), ant metabolites (fluorouracil, Methotrexate, cytosar, 6-mercaptopurine, 5-azacytosine, and gemcitabine) topotecan, daclimycin, and mitomycin C [8-11]. A living cell can harbor multiple drug resistances by the mechanism of active efflux of a wide range of anticancer drugs through their cellular membrane by the action of MDR proteins. MDR drugs were not specifically developed to inhibit MDR; instead, they had other pharmacological properties with low affinity for MDR transporters. They involved compounds of variable structure and function, such as cyclosporine and verapamil, and may cause side effects. Another fact that the new drugs were more inhibitor-specific, and were designed to minimize such side effects e.g., dexniguldipine, R-verapamil, etc. Recently, pharmaceutical industry introduced new compounds obtained from drug development programs characterized by a high affinity to MDR transporters and is efficient at level of Nano molar concentrations. Some of these compounds e.g., M-5(209) giving a future hope for cancer therapy and are currently under clinical trials [5-12]. Fluoroquinolones are synthetic compounds act as antibacterial agents that may stabilize the ternary complex structure of prokaryotic topoisomerase II enzymes (Topo IV and gyrase), resulting in enormous DNA fragmentation and bacterial killing. Despite the structural folds similarity between prokaryotic and eukaryotic topoisomerase, fluoroquinolones display a remarkable selectivity for prokaryotic topoisomerase II, with relevant safety records in humans. There are many specific agents that take human topoisomerase as a target (doxorubicin, topside and mitoxantrone) are frequently associated with subsequent events of secondary
malignancies and significant toxicities, while fluoroquinolones are not known to reveal such clinical adverse effects. Despite fluoroquinolones action of topoisomerase-independent anti-proliferative effects against various human cancer cells, those that reveal significant action against eukaryotic topoisomerase show similar DNA damaging properties as other topoisomerase poisons. Empirical models also showed fluoroquinolones unique immunomodulatory activities of suppressing super-inducing interleukin-2 and pro-inflammatory cytokines [13–15]. In view of the mechanistic similarities and sequence homologies exhibited by the prokaryotic and eukaryotic type II topoisomerase, targeted efforts to specifically and selectively shift from an antibacterial to antineoplastic activity was made by synthesizing novel newly designed classes of quinolones [16]. There are rare data on the effects of fluoroquinolones antibiotics on HCT116 colorectal carcinoma cell proliferation and apoptosis. We therefore investigated the cytotoxic effect of ciprofloxacin hybridized with cyclosporine.

MATERIALS AND METHODS

Chemical synthesis of the target compound was according to the following steps [17]

Synthesis of 2-chloro-N-(4-sulfamoylphenyl) acetamide, compound (A)

This compound synthesized by the reaction of Sulfanilamide (A) zur Pharma international ltd, Canada) with chloroacetyl chloride (Alpha chemical, India) according to Saeedi et. al. [18]: Sulfanilamide (2g, 11.6 mol), was dissolved in Dimethyl form amide (DMF) (Market Research Engine, USA): Benzene (China steel chemical corp., China) in vol/vol ratio of 1:3 mixture (total of 40 ml), then TEA (Bioteaque GmbH & Co. KG, Germany) (1.6 ml, 11.6 mmol) was added. The reaction mixture was stirred at room temperature overnight. The solvent was then evaporated under vacuum; the residue was triturated with acetone and crystallized from methanol.

Synthesis of 1-cyclopropyl-6-fluoro-4-oxo-7-((piperazin-1-yl)-1, 4-dihydroquinoline-3-carboxylate, compound (B)

Ciprofloxacin Himedia, India etherification was achieved using the method of Jaehde et. al. (19): (2 g, 6 mmol) of ciprofloxacin was suspended in absolute methanol (BDH, UK) (50 ml), then cooled down to -15°C, thionyl chloride (Gujarat, India) (0.45 ml, 6 mmol) was then added in drop wise manner (at -15°C). Then the reaction mixture was kept at 40°C for 3 hrs followed by refluxing for 35 hrs until the HCl gas was ceased, then kept at room temperature overnight. The solvent was then dried by evaporation under vacuum; the residue was dissolved again in methanol and evaporated. Thionyl chloride was then removed by repeating this process several times. The residue was collected and crystallized from methanol-chloroform (CLN GmbH, Germany).

Synthesis of methyl 1-cyclopropyl-6-fluoro-4-oxo-7-4-2-oxo-2-(4-sulfamoylphenylamino) ethyl (piperazin-1-yl)-1, 4-dihydroquinoline-3-carboxylate, compound (C)

This compound synthesized by the reaction of compound IC with compound IID, according to method of Saeedi et. al. [17–18]: A mixture of both compound- B (2g, 5.8 mmol), and compound- A (1.44 g, 5.8 mmol), was then dissolved in DMF (25 ml), then TEA (0.81 ml, 5.8 mmol), was added. The mixture was then stirred at room temperature overnight. The solvent was then evaporated and residue was triturated with acetone and crystallized from methanol.

![Scheme (1): Chemical synthesis of 1-cyclopropyl-6-fluoro-4-oxo-7-4-2-oxo-2-(4-sulfamoylphenylamino) ethyl (piperazin-1-yl)-1, 4-dihydroquinoline-3-carboxylic acid, compound and its intermediates](image-url)
Synthesis of 1-cyclopropyl-6-fluoro-4-oxo-7-(4-(sulfamoylphenylamino)ethyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid, compound D (as named CSA)

Ester hydrolysis was performed according to Bodansky et al. (20); Compound C (0.8 g, 1.43 mmol), dissolved in minimum volume of ethanol 99%: THF (Donacchem GmbH, Austria) (3:1) mixture and the solution were cooled to 18°C. Then NaOH (BDH, UK) (2N, 0.86 ml, 1.73 mmol) was added drop wise, with continuous stirring over a period of 30 min., stirring was continued at 18°C for additional three hours. The reaction mixture was acidified with HCl (2N, 0.86 ml, 1.73 mmol), then excess of cold water was added. The precipitated compound was filtered, dried and crystallized from methanol: chloroform (9:1), to give compound D, as 1-cyclopropyl-6-fluoro-4-oxo-7-(4-(sulfamoylphenylamino)ethyl)piperazin-1-yl)-1,4 dihydroquinoline-3-carboxylic acid. The target compound (D) through its intermediates was synthesized according to the scheme (1) and figure (1).

Figure (1): The newly synthesized compound D (CSA), as 1-cyclopropyl-6-fluoro-4-oxo-7-(4-(2-oxo-2-(4-sulfamoylphenylamino)ethyl)piperazin-1-yl)-1,4 dihydroquinoline-3-carboxylic acids

**Anticancer assay of CSA, DOX and 5-FU**

The anticancer activity of CSA compound, DOX (Pfizer Pty Ltd, Australia) and 5-FU (Sigma, USA) against HCT116 colorectal adenocarcinoma cell line(Kindly provided by dr Hamid Naji/ College of medicine/ Babylon university) was evaluated by MTT assay (21). Cells were seeded onto 96 well plates with a concentration of 10³ cells/ml. Different concentrations of microtitered CSA compound (1, 10,100,500 and 1000) µg/ml, microtitered DOX and 5-FU were added to culture wells at a final volume of 100 µl in each well. The maximum concentration of Dimethyl sulfoxide (DMSO) (SANTA CRUZ, USA) not exceeds 0.1% as a final concentration was used as negative control. After 24hr incubation at 37°C in 5% CO2, the MTT test was carried out as described above (in cytotoxicity assay). MTT reagent (bio WORLD, USA) (20 µl) was then added to each well. After 3 to 4 hrs of incubation at 37°C, the formazan was solubilized by addition of 100µl of 1:1 volume of DMSO: isopropanol, and the absorbance was read at 490 nm with a reference wavelength of 630 nm by an ELISA reader Data were calculated as described previously; the percentage of inhibition was calculated using the formula: \( \text{I}=1-(A-A_0)\times100; \) Where A= absorbance of untreated samples, A= absorbance of the treated test/standard. While the 50% of maximum inhibition (=Y in the formula of IC50 calculation) was calculated according to the following equation: 50% of maximum inhibition=M ax % of growth Inhibition - % of growth Inhibition - 50% x (Max % of growth Inhibition - min % of growth Inhibition)

**RESULTS**

Physicochemical Properties or the CSA compound;

Molecular formula; C₂₁H₂₂FN₂O₅S; Molecular weight; 543.57; Appearance: Beige crystals and Melting point 241-243°C

**Spectral Study**

FT-IR (cm⁻¹): 3400 (N-H) stretching, Broad band between 3,300-2,700 (O-H) stretching 3,003 (C-H) of aromatic, 1,699 (C=O) of carboxylic acid, and 1,626 (C=O) of amide. CHN calculated (C₂₁H₂₂FN₂O₅S): C, 55.24; H, 4.82; O, 12.88; found: C, 55.19; H, 4.59; O, 12.57; 1H-NMR (DMSO-d₆) δ (ppm): 9.68 (s,1H,COOH), 8.58 (s,1H, C=C-H), 8.4-7.43 (m,6H,Ar-H), 7.25 (s,1H, N-H=C=O), 5.4 (m,1H, C=H), 3.24 (t,1OH, CH₂=CH=O overlap with CH₃ of piperazine), 2.3 (s,2H,N₂H₂), 1.13 (m,4H,C₄H₉) of cyclopropane.

**Results of Anticancer assay of CSA, DOX and 5-FU**

As shown in figure (2); MTT cytotoxicity assay revealed high significant differences between un treated cells as compared with those treated with different concentrations
of each compound (P<0.0001). Anticancer activity of CSA, DOX and 5-FU were carried out by MTT assay on HCT116 Colorectal adenocarcinoma cell Line. The synthesized CSA composite is selected and applied for anticancer activity. The CSA compound showed significant anticancer activity against HCT116 Cell Line. It has an IC50 value of 182.4 µg/ml (335.56 µmol/ml) which is significant value (p<0.0001), with positive spearman correlation coefficient of 0.6219. Growth inhibition results were shown in Table 1, the curve of IC50 fitting was demonstrated in Fig. (3). The cytotoxic effect of CSA, DOX and 5FU that presented by plotting of concentration log versus growth inhibition % were shown in figures 3, 4 & 5 respectively. The values of growth inhibition % of CSA, DOX and 5FU were also shown in tables 1, 2&3 respectively. These results revealed growth inhibition curve of CSA with significant differences concerning the comparison between treated and non-treated cells. Doxorubicin and 5FU effect on HCT116 cell line monolayer, presented by Mean ± SEM of optical density at 490nm was shown in tables (2 & 3) and figures (4 &5 respectively) with values of growth inhibition %. The conventional anticancer drug DOX showed IC50 value of 58.94 µg/ml (108.45 µmol/ml) with positive spearman correlation coefficient of 0.6929. While 5-FU showed IC50 value of 36.73µg/ml (282.36 µmol/ml) with positive spearman correlation coefficient of 0.7766. The values of concentration log, IC50 and R^2 of all compounds were shown in table (4).

**Figure (2):** A - HCT116 Colorectal adenocarcinoma Cells before treatment with CSA or addition of MTT reagent; B - HCT116 cells after treatment with CSA 182.4 µg/ml and addition of MTT reagent; arrows (1-green) refers to dead cells and 2-black refers to remnant live cells.

**Table 1:** Anticancer activity of CSA compound presented by Mean± SEM, IC50 and Growth inhibition % values

<table>
<thead>
<tr>
<th>(Dose(µg/ml))</th>
<th>Mean± SEM</th>
<th>Growth inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000(control)</td>
<td>0.23967±0.013544</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.16500±0.016093</td>
<td>31.1</td>
</tr>
<tr>
<td>10</td>
<td>0.16000±0.009609</td>
<td>33.2</td>
</tr>
<tr>
<td>100</td>
<td>0.15167±0.017901</td>
<td>36.6</td>
</tr>
<tr>
<td>500</td>
<td>0.14233±0.013445</td>
<td>40.5</td>
</tr>
<tr>
<td>1000</td>
<td>0.12467±0.010525</td>
<td>47.9</td>
</tr>
</tbody>
</table>
Figure (3): Anticancer activity of CSA compound presented by plotting of concentration log vs. Growth inhibition % values

Table 2: Anticancer activity of Doxorubicin compound presented by Mean± SEM , IC50 and Growth inhibition % values

<table>
<thead>
<tr>
<th>Dose(µg/ml)</th>
<th>Mean ± SEM</th>
<th>Growth inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000(control)</td>
<td>0.23967±0.013544</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>0.13833±0.002906</td>
<td>36.3</td>
</tr>
<tr>
<td>10</td>
<td>0.13967±0.022378</td>
<td>62.5</td>
</tr>
<tr>
<td>50</td>
<td>0.12367±0.016169</td>
<td>94.9</td>
</tr>
<tr>
<td>75</td>
<td>0.07967±0.015169</td>
<td>94.6</td>
</tr>
<tr>
<td>100</td>
<td>0.03467±0.027510</td>
<td>92.7</td>
</tr>
</tbody>
</table>

Table 3: Anticancer activity of 5-FU compound presented by Mean± SEM , IC50 and Growth inhibition % values

<table>
<thead>
<tr>
<th>Dose(µg/ml)</th>
<th>Mean ± SEM</th>
<th>Growth inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000(control)</td>
<td>0.88800±0.016503</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>0.62807±0.133718</td>
<td>29.3</td>
</tr>
<tr>
<td>10</td>
<td>0.46240±0.040278</td>
<td>47.9</td>
</tr>
<tr>
<td>100</td>
<td>0.35240±0.012767</td>
<td>46.5</td>
</tr>
<tr>
<td>500</td>
<td>0.33273±0.018985</td>
<td>60.3</td>
</tr>
<tr>
<td>1000</td>
<td>0.32307±0.018370</td>
<td>62.5</td>
</tr>
</tbody>
</table>

*Anova significance of CSA < 0.0001; R Squared of OD response Vs concentration =0.594; DOX Anova significance <0.0001; R Squared of OD Vs concentration =0.766; 5-FU Anova significance < 0.0001; R Squared of OD Vs concentration =0.700; SEM : Standard error of mean; IC50: inhibitory concentration of 50% of cell viability

Table (4): Cytotoxicity of CSA compound presented by IC50 and R² values

<table>
<thead>
<tr>
<th>Compound potency and dose</th>
<th>CSA compound</th>
<th>DOX</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log IC50</td>
<td>2.261</td>
<td>1.770</td>
<td>1.565</td>
</tr>
<tr>
<td>IC50 (µg/ml)</td>
<td>182.4</td>
<td>58.94</td>
<td>36.73</td>
</tr>
<tr>
<td>R²</td>
<td>0.6219</td>
<td>0.6929</td>
<td>0.7766</td>
</tr>
</tbody>
</table>

*DOX=Doxorubicin; 5FU=5-Flourouracil; IC50=Half maximal inhibitory concentration; R²=correlation coefficient of concentration log Vs growth inhibition %.

Figure (4): Anticancer activity of Doxorubicin compound presented by plotting of concentration log vs. Growth inhibition % values
DISCUSSION

Results of CSA, DOX and SFU IC50 and $R^2$ were shown in tables (1-3). The MTT colorimetric assay provides accurate and reliable quantification of viability. The assay of MTT tetrazolium reduction was the first cell viability assay developed for the evaluation and measurement of any therapeutic agent potency using a 96-well micro titer plate and regarded as valuable and suitable for multivariate screening (24). The activity of the synthesized compound is attributed to the types of linkers between the ciprofloxacin and the sulfonamide derivatives, which affected the orientation of the compound in different sub-pockets of the active site cavity of the target enzymes, and subsequently the binding with the target sites, leading to variable affinities, pharmacological properties and selectivity (17). According to the measurement of CSA anticancer potency, results showed that CSA may have the anticancer potency against with dose dependency somewhat equal to DOX (R Squared of CSA and DOX G1% response Vs concentration log y = 0.6069 and 0.6768 respectively). In fact, these results encourage us to investigate the cytotoxic effects of CSA compound on normal cells both in vitro and in vivo. Chemotherapeutic agents and radiation are the most current cancer treatments rely on that work in the body by killing rapidly dividing cells. The main drawback of conventional chemotherapy is the adverse effects on the body as it lacking deliver selective and specific action to the cancer cells, thus damage extending to the surrounding normal healthy tissues or rapidly dividing healthy cells such as the cells of bone marrow, gastrointestinal tract, hair follicles, causing issues like cardiac, hepatic, pulmonary, gastrointestinal and renal toxicities (25). There are many adverse effects concerning 5-fluorouracil (5-FU) and doxorubicin; Although SFU showing antineoplastic action since interferes with deoxyribonucleic acid (DNA) synthesis by blocking the thymidilate synthesizes (TS), conversion of deoxyuridylic acid to thymidylic acid and incorporation/insertion of its metabolites into RNA and DNA, what made widely used for cancer treatment particularly for colorectal cancer (18,25,26); But it has many adverse effects; topical application is associated with an irritant dermatitis, promotion of vacuolization and decrease of intestinal villi, infiltration of inflammatory cells, other signs of cryptic necrosis, decrease in villus/crypt ratio and loss of cell architecture (27,28). The adverse effects from parenteral administration are more severe and also include bone marrow suppression with clinically significant value, cutaneous reactions and GI toxicity. The two proposed mechanisms by which doxorubicin acts in the cancer cell include (i) intercalation into DNA strands and disruption of topoisomerase II mediated DNA repair (ii) generation of free radicals and subsequent damage to cellular membranes, nucleic acids (DNA) and proteins (29). Adverse reactions are also common after doxorubicin administration and may include fatigue, alopecia, nausea and vomiting, and oral sores. Bone marrow suppression and an increased risk of secondary malignancy diagnoses may occur. Doxorubicin extravasations during intravenous administration can result in severe tissue ulceration and necrosis which worsens over time. Significant cardiac toxicity of doxorubicin was also reported, which limits the long-term use of this drug. Other cardiovascular disturbances such as congestive heart failure may also occur due to the side effect of doxorubicin with other risk factors include a higher cumulative drug dose, extremes of age, combination chemotherapy with other cardio toxic drugs, hypertension, pre-existing left ventricular dysfunction, and previous radiation to the mediastinal region. The 1-year mortality rate is approximately 50%, when congestive heart failure develops after doxorubicin administration (30-31). The sulfonamides constitute interestingly pharmacological agents that possess different biological actions such as anti-carbonic anhydrate, antibacterial, hypoglycemic, diuretic, and antithyroid activity. Recently, a novel numerous newly designed sulfonamide derivatives that possess substantial protease inhibitor effect have ultimately reported. There are some specific metalloprotease inhibitors related to this class, which by their inhibitory action for several matrix metalloprotease (MMPs) show valuable antitumor activity. Some of these newly designed compounds are currently being studied and evaluated in clinical trials (32). Ciprofloxacin was also decreases the proliferation and enhance apoptosis of colorectal carcinoma cells, possibly by blocking the pathway of mitochondrial DNA synthesis (25).

CONCLUSION

We concluded that CSA compound (1-cyclopropyl-6-fluoro-4-oxo-7-(4-(2-oxo-2-(4-sulfamoylphenylamino)ethyl)piperazin-1-yl)-1, 4-dihydroquinoline-3-carboxylic acid) may have antineoplastic activity against colon...
carcinoma cell line HCT116, and we recommend to evaluate CSA compound potency on normal cells and against more than one type of cell lines, then studying apoptosis mechanism by fluorescent assay as well as in vivo antitumorigenic effect of this compound. In our study CSA compound may offer a new medicinal agent with improved potency, good pharmacological actions and hope to be proven as useful therapeutic compound, which may consequently become an object of interest in both industry and academia.

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