Screening of Amodiaquine for its in vitro Anti-cancer Activity on Breast Cancer Cell Lines- a Case Study for Drug Reprofiling

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Abstract

Background: Cancer is one of the foremost contributors to global disease burden and constantly requires new therapeutic options. The development of new drugs has failed to keep up with its incidence. Hence, drug reprofiling strategies are emerging as novel therapeutic options. The study aimed to evaluate the anti-cancer activity of amodiaquine (anti-malarial drug) using a combination of murine and human breast cancer cell lines

Methods: Amodiaquine was authenticated by ultra-violet spectrophotometry, high- performance liquid chromatography and \textsuperscript{1}D nuclear magnetic resonance. \textit{In vitro} cytotoxicity of amodiaquine was evaluated against three breast cancer cell lines. MDA-MB-453, 4T1 and MDA-MB-231 cells were incubated with the drug at different concentrations (0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 µM) for 72 h, after which cell viability testing was conducted using the cell counting kit-8 assay. Negative control in which no drug was added to the cells was also evaluated. The flow cytometry analysis of MDA-MB-231 cells when treated with amodiaquine was also evaluated by a flow cytometer using annexin V/propidium iodide staining assay.

Results: Cell viability studies showed that the IC\textsubscript{50} values of amodiaquine on MDA-MB-453, 4T1, and MDA-MB-231 cells were 6.48 ± 1.12, 10.50 ± 1.17, and 19.23 ± 1.16 µM, respectively. The flow cytometry analysis of MDA-MB-231 cancer cells treated with amodiaquine showed cancer cell death by necrosis.

Conclusion: This study has shown that amodiaquine may be potentially reprofiled as an anti-cancer agent in managing androgen receptor-positive / HER-2 positive and triple-negative breast cancer types. An additional probable mechanism of action of anti-cancer activity of amodiaquine was found to be necrosis.

Keywords: Amodiaquine, drug reprofiling, breast cancer, necrosis
1.0 INTRODUCTION

According to the Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) statistics, an estimated 19.3 million new cancer cases occurred worldwide in just 2020, with almost 10.0 million cancer deaths [1]. In addition, the World Cancer Research Fund report has stated that breast cancer is the most prevalent malignancy in women worldwide, and it is the second most common type of cancer. There were over 2 million new cases of breast cancer disease in 2018 [2, 3]. Cancer, being one of the leading contributors to global disease burden, constantly requires new therapeutic options. However, the development of new anticancer drugs has failed to keep up with the increasing incidence of cancer. Hence, drug reprofiling strategies are emerging [4]. Drug reprofiling is a science-driven process of discovering new therapeutic uses of already approved drugs, different from their initial pharmacological indications [5].

Drug reprofiling has many advantages over traditional de novo drug discovery approaches. First, it is often more cost-effective [6]. Second, there is a reduction in development time [7]. Third, reprofiled drugs are good, quick alternatives for rare life threatening diseases. For example, in the recent coronavirus disease 2019 (COVID-19) pandemic, where remdesivir is being reprofiled for its management [8, 9]. Anti-malarial drugs have a long history of clinical use and tolerable safety profile [10]. Amodiaquine is a mannich base 4-aminoquinoline anti-malarial drug, with an analogous mode of action to chloroquine [11]. It has been shown to inhibit autophagy and stabilize p53 through ribosome biogenesis stress, and as such, it has been reprofiled in the anti-cancer activity of some murine and human cancer cells [12, 13]. Moreover, it has been shown to be non-cytotoxic to healthy cells like Vero cells [14,15].

Metastasis is the primary factor causing cancer morbidity and mortality [16]. MDA-MB-453 cell line is a metastatic cell line whose proliferation is stimulated by androgens [17]. Also, triple-negative breast cancer cells of murine and human origin are greatly important in cancer metastasis [18]. Therefore, this study aimed to evaluate amodiaquine's anti-cancer activity on MDA-MB-453 breast cancer cell lines and triple-negative breast cancer cell lines of both murine (4T1 cells) and human (MDA-MB-231) origin.

2.0 METHODOLOGY

2.1 Authentication of Amodiaquine Dihydrate Dihydrochloride

The ultraviolet (UV) spectrophotometry of 2.5 µL of 0.1 mg/mL of amodiaquine dihydrate dihydrochloride (98% purity, BOC Sciences, NY, USA) in 0.1% trifluoroacetic acid (99% purity, Sigma-Aldrich, MO, USA) solution was analysed using UV spectrophotometer (Nanodrop ND-1000 spectrophotometer, NC, USA). The UV spectrophotometer was scanned from 220 nm - 750 nm [19].

The high-performance liquid chromatography (HPLC) of 30 µg/mL of amodiaquine dihydrate dihydrochloride prepared in water was analyzed as described by Luo et al. [20] with some modifications. The mobile phase consisted of 0.1 % trifluoroacetic acid prepared in water and acetonitrile. The mobile phase was delivered into the HPLC (Agilent Eclipse 1100 series, Column- Eclipse XDB - C18 (4.6 mm x 250 mm), CA, USA) at ambient temperature; at a flow rate of 1mL/min, the detection for the amodiaquine sample was done at 350 nm using UV detector. The injection volume was 10 µL and the run time was for 35 min [20].

The amodiaquine dihydrate dihydrochloride was also characterized by proton 1D Nuclear Magnetic Resonance (Varian Mercury 400 MHz NMR, CA, USA) in deuterium oxide [21].

2.2 Cell Culture

Cell lines and culture conditions: Human (MDA-MB-453 cells) HER-2 positive/androgen receptor-positive breast cancer cell lines, triple-negative cell lines of murine (4T1 cells), and human origin (MDA-MB-231 cells) were used for this research. All these cell lines were obtained from the Center for Controlled Chemical Delivery, the University of Utah.

MDA-MB-453 cells were cultured in Dulbecco's modified eagle medium (DMEM) IX (Gibco®-Thermo Fisher Scientific, USA), supplemented with 10% fetal bovine serum heat-inactivated and 1% penicillin-streptomycin while 4T1 and MDA-MB-231 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum heat-inactivated and 1% penicillin-streptomycin. These cells were maintained at 37°C, in a humidified atmosphere with 5% CO₂.
2.3  *In vitro* Cytotoxicity of Amodiaquine Dihydrate dihydrochloride on MDA-MB-453, 4T1 and MDA-MB-231 Cells

The *in vitro* cytotoxicity of amodiaquine dihydrate dihydrochloride was assessed, as described by Tominaga et al., [22], with some modifications. Cells were seeded in triplicates in three 96-well microplates (100 µL / well) at a density of 10,000, 4,000, and 5,000 cells per well for MDA-MB-453, 4T1 and MDA-MB-231 cells respectively in supplemented DMEM or RPMI 1640 medium. After a period of incubation (48 h for MDA-MB-453; 24 h for 4T1 and MDA-MB-231 cells) at 37 °C with 5 % carbon dioxide, the cells were treated with 100 µL of known concentrations of amodiaquine dihydrate dihydrochloride (0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 µM) in supplemented DMEM or acidified RPMI 1640 (pH 5.5) medium. After 72 h, the treatment was removed, and the cells were washed with 100 µL of phosphate-buffered saline for each well.

Afterward, cell viability was quantified using the CCK-8 (Lot no: EQ646; Dojindo Laboratories, Japan). A volume of 100 - 150 µL of CCK-8 reagent diluted 15 fold in media was added to the each well in the three plates, and they were incubated (Periods of incubation were 10 minutes, 1 h 40 min, and 34 h 30 min for MDA-MB-453, 4T1 and MDA-MB-231 cells respectively). The absorbance was measured using a plate reader (Tecan Infinite M200 PRO, Austria) at 450 nm, the absorbance of the control group (negative control) in which no drug was added was also measured as well as the absorbance of blank in which there were no cells or drug [22].

The cell viability was then calculated using the formula below:

\[
\text{% cell viability} = \frac{\text{Absorbance of treated} - \text{Absorbance of control}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100
\]

2.4  Flow Cytometry Analysis of Amodiaquine Dihydrate Dihydrochloride on MDA-MB-231 Cells

The flow cytometry analysis of MDA-MB-231 cells when treated with amodiaquine was evaluated by a flow cytometer (Cytex DxP 8 Analyzer, Cytex Biosciences, CA, USA) using annexin V/ propidium iodide staining assay as described by Kwan et al., [23] with some modifications. Cells were seeded (in duplicate) in a 6-well microplate (1000 µL/ well) at a density of 100,000 cells per well in supplemented RPMI 1640 medium. After 24 h incubation at 37 °C with 5 % carbon dioxide, the cells were treated with 1000 µL of 307 µM of amodiaquine dihydrate dihydrochloride in supplemented RPMI 1640 medium (pH 5.5). The control group (in duplicate) was treated using media without drug.

The cells were further incubated for 72 h. The cells were washed, detached with trypsin and centrifuged at 3000 rpm for 3 min in four Eppendorf tubes, and the supernatant was discarded. A concentration of 90 µg/mL of 0.0125 % annexin V was prepared in PBS and 400 µL each of the prepared solution was transferred into the four Eppendorf tubes containing the cell pellets and used to reconstitute the cells. Afterward, 2 µL of propidium iodide (50 µg /mL) was also transferred to each Eppendorf tube and then kept in the dark for 18 min at ambient temperature. The stained cells were then analyzed using a flow cytometer. The number of unlabelled (viable cells) cells, cells bound to Annexin V-FITC only (early apoptotic cells), those that have both bound to Annexin V-FITC and been labelled with propidium iodide (late apoptotic/ secondary necrotic cells), and those that have been stained with propidium iodide (necrotic cells), were evaluated [23].

2.5 Statistical Analysis

Quantitative data were managed in GraphPad Prism 8.30 (GraphPad Software, Inc., CA, USA). The data were expressed as mean ± standard error of the mean (SEM) as well as mean ± standard deviation (SD).

3.0  RESULTS

3.1  UV spectrophotometry of amodiaquine dihydrate dihydrochloride

The UV spectrophotometry of amodiaquine dihydrate dihydrochloride showed the wavelength of its maximum absorption (λ<sub>max</sub>) to be 343 nm (Fig 1). This value is similar to the λ<sub>max</sub> value of 342 nm stated in USP pharmaco-poeia [24] and according to Scholar in 2007 [25].

3.2  HPLC of Amodiaquine Dihydrate Dihydrochloride

The HPLC analysis of amodiaquine salt showed a single peak with the retention time being 13 min (Fig 2).

3.3  NMR of Amodiaquine Dihydrate Dihydrochloride in Deuterium Oxide

Analysis of the NMR (Fig 3) of amodiaquine dihydrate dihydrochloride confirmed the structure (Fig 4).
3.4 In vitro Cytotoxicity of Amodiaquine Dihydrate Dihydrochloride on MDA-MB-453, 4T1 and MDA-MB-231 Cells

Concisely, the graph of cell viability of MDA-MB-453, 4T1 and MDA-MB-231 cells vs concentration of amodiaquine dihydrate dihydrochloride (Fig 5) showed that the higher the concentration of amodiaquine, the lower the cell viability of MDA MB-453, 4T1 and MDA MB-231 cells. The in vitro cytotoxicity studies of amodiaquine showed the half maximal inhibitory concentration (IC$_{50}$) values of amodiaquine on MDA MB-453, 4T1 and MDA MB-231 cells (Table 1).

Table 1. Half maximal inhibitory concentration (IC$_{50}$) values of amodiaquine dihydrate dihydrochloride on MDA-MB-453, 4T1 and MDA-MB-231 cells.

<table>
<thead>
<tr>
<th>Breast Cancer Cells</th>
<th>IC$_{50}$ values (µM)</th>
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<tbody>
<tr>
<td>MDA MB-453</td>
<td>6.48 ± 1.12</td>
</tr>
<tr>
<td>4T1</td>
<td>10.50 ± 1.17</td>
</tr>
<tr>
<td>MDA MB-231</td>
<td>19.23 ± 1.16</td>
</tr>
</tbody>
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IC$_{50}$ values were derived from data fitted with dose-response curve using Graphpad Prism software. Data are expressed as means ± SEM (n=3).
The flow cytometry analysis of MDA-MB-231 cells treated with amodiaquine for 72 h has shown that most of the cells were found in the necrotic region (Figures 6 and 7).

3.6 Direct Necrotic Analysis Using Propidium Iodide

Direct necrotic analysis from the data obtained in figure 8, has shown that the percentage of direct necrotic cells from the treated group was 47.15 ± 0.78 % while that of the untreated was 7.21 ± 0.32 % (Figures 8 and 9).

4.0 DISCUSSION

The qualitative assessment of amodiaquine dihydrate dihydrochloride obtained was necessary to ascertain its authenticity. The UV spectrophotometry analysis showed that the wavelength of maximum absorption ($\lambda_{\text{max}}$) obtained was 343 nm (Fig 1). The $\lambda_{\text{max}}$ is a qualitative parameter that provides some information on the electronic structure of molecules, and it is characteristic of the particular substance in question. The $\lambda_{\text{max}}$ value obtained in this study is very close to the wavelength of maximum absorbance of amodiaquine dihydrate dihydrochloride (342 nm) as stated in the USP monograph [24] and according to Scholar in 2007 [25]. The HPLC analysis of
amodiaquine salt showed that the salt is a pure sample showing a single peak at 350 nm at 13 min (Fig 2), indicating that the salt is unadulterated. This unadulterated salt was however confirmed by NMR (Fig 3). On analysis, the integral areas and chemical shifts have proven the structure of amodiaquine dihydrate dihydrochloride, as the addition of the major integral areas of the spectrum was 20 (Fig 3). This showed the total number of hydrogens attached to the carbon atoms in the molecule (Fig 4). NMR is used to characterize molecules by identifying carbon-hydrogen frameworks within the molecules [26]. The remaining eight hydrogens are obtained from those attached in the secondary amine, hydroxyl group, two molecules of water, and hydrochloride as deuterium oxide tends to exchange hydrogen atoms attached to nitrogen and oxygen, so it cannot be read directly from the spectrum [26]. The total number of hydrogens in amodiaquine dihydrate dihydrochloride was therefore 28.

The in vitro cytotoxicity study of amodiaquine on MDA-MB-453 cells showed that amodiaquine was cytotoxic to MDA-MB-453 breast cancer cell lines (Fig 5) with an IC₅₀ of 6.48 µM. (Table 1). MDA-MB-453 breast cancer cell lines are androgen receptor-positive cells that also express human epidermal growth factor receptor 2 (HER-2). The cell line exhibits a characteristic apocrine carcinoma steroid receptor profile: ER-α-negative, PR-negative, and AR-positive [27]. The IC₅₀ of doxorubicin which is one of the most commonly used chemotherapeutics, was 0.69 µM on MDA-MB-453 cells in a previous research work [28]. This shows the superior potency of doxorubicin over amodiaquine against MDA-MB-453 cells.

A previous research study has shown that 100 µM of chloroquine (an analogue of amodiaquine) has been shown to slightly decrease HER-2 levels in MDA-MB-453 cells, which means that chloroquine could be cytotoxic to these cells since HER2 signaling helps to mediate cell survival or proliferation in these breast cancer cells [29]. Increased proliferation in response to androgens is also a key feature of MDA-MB-453 cell lines. However, they can be blocked by anti-androgens, such as flutamide [27]. Therefore, if amodiaquine can elicit an anti-cancer effect on MDA-MB-453 cells which are androgen receptor-positive, it is probable that amodiaquine can be further reprofiled in the treatment of diseases that are associated with androgen over-expression e.g., acne, polycystic ovary syndrome, hirsutism, alopecia, acanthosis nigricans, hypersexuality to mention but a few [30].

The in vitro cytotoxicity of amodiaquine on murine triple-negative breast cancer 4T1 cell lines has shown that amodiaquine dihydrate dihydrochloride was cytotoxic (Fig 5) with an IC₅₀ value of 10.50 µM (Table 1) in acidified media of pH 5.5. This pH mimics the acidified condition of tumor microenvironment and so the behavior of
these virulent, metastatic cells in acidic media when treated with amodiaquine was investigated [31]. The negative control group was also subjected to the same conditions as the treated groups. This shows that amodiaquine is substantial enough to elicit an anti-cancer effect on 4T1 cells, derived from spontaneous tumors arising in a BALB/c mouse. They have been found to be extremely aggressive, and they have a high metastatic potential [32]. They closely mimic stage IV human breast cancer in their growth and they have immunogenic properties. Hence, they are good models for preclinical applications of anti-cancer study [32]. 4T1 cells can spontaneously metastasize from the primary tumour in the mammary gland to multiple distant sites, including lymph nodes, blood, bone, lung, liver, and brain [33, 34].

The IC50 of doxorubicin was 1.49 µM on 4T1 cells in a previous work [35]. This shows that doxorubicin is more potent than amodiaquine against 4T1 cells. Furthermore, another study on the in vitro cytotoxicity of hydroxychloroquine which is structurally similar to amodiaquine on 4T1 cells showed that the IC50 was 70 µM [36]. This current cell viability study showed that amodiaquine (IC50 value–10.50 µM) is more potent when compared with hydroxychloroquine. A previous work of clonogenic cell viability activity of 4T1 cells has also shown that amodiaquine was more potent when compared with chloroquine [37].

Amodiaquine was also found to be cytotoxic to MDA-MB-231 cell lines (Fig 5). The half-maximal inhibitory concentration (IC50) of amodiaquine on MDA-MB-231 cell lines was 19.23 µM (Table 1) in acidified media of pH 5.5. This pH mimics the acidified condition of tumor microenvironment [31]. The negative control group was also subjected to the same conditions as the treated groups. A previous study on the in vitro cytotoxicity of amodiaquine on MDA-MB-231 cells showed that the IC50 value obtained was 840 µg/mL [13]. This current cell viability study showed that amodiaquine is more potent against these MDA-MB-231 cells where the IC50 value obtained was 8.94µg/mL (19.23 µM) (Table 1). Another study in 2019 showed that the IC50 value obtained for the in vitro cytotoxicity of amodiaquine on MDA-MB-231 cells was 10.50 µM [38]. The variations in these IC50 values could be due to differences in the state of the cells used, the cell densities, the incubation, and treatment times and conditions for each study [39].

The IC50 of doxorubicin on MDA-MB-231 cell lines was 3.16 µM in previous work [28]. This shows that doxorubicin is more potent than amodiaquine against these cell lines. However, amodiaquine is substantial enough to elicit an anti-cancer effect on MDA-MB-231 cells, which are highly aggressive, invasive, and poorly differentiated cells with high metastatic potential.

Triple-negative breast cancer has worse prognostic outcomes in many patients when compared with hormonal breast cancer, and there are limited treatment options [40].

On comparing the in vitro cytotoxicity of amodiaquine on 4T1 cells which are murine triple-negative breast cancer cells (IC50 values −10.50 µM) and MDA-MB-231 cell lines which are human triple-negative breast cancer cells (IC50 values −19.23 µM), it can be inferred that the murine cells were more sensitive to amodiaquine when compared with the human cells as amodiaquine was more potent against the murine cells.

Flow cytometry counts and analyzes viable, early apoptotic, late apoptotic/secondary necrotic cells, and necrotic cells [23]. The lower left quadrant (Fig 6a, b) represents viable cells and the flow cytometry analysis of amodiaquine on triple-negative MDA-MB-231 human breast cancer cells has shown that there was a reduction in the number of live cells in the quadrant which is the Annexin V- FITC - / propidium iodide - (annexin V- / PI-) quadrant to 30.20 % (Fig 6b, 7) when compared with the percentage of these cells in the control which was 81.20 % (Fig 6a, 7). At this point, it is expected that the cancer cells are healthy and intact and there is no altered morphologic features like plasma membrane blebbing, plasma membrane asymmetry and attachment, chromatin condensation, nuclear fragmentation, and internucleosomal cleavage of DNA [41, 42]. Thus, having only a few viable cells in the treated group (Fig 6b) showed an obvious cell death being observed and that amodiaquine has a cytotoxic effect against these cells.

The early apoptotic cells in the annexin V+ / PI- (lower-right) quadrant showed that the percentage of these cells in the control group were insignificant (Fig 6a, 7). The treated group for the early apoptotic cells also showed very insignificant number of cells (Fig 6b, 7). It can therefore be inferred that amodiaquine at a concentration of 307 µM did not induce early apoptosis on MDA-MB-231 cells. This might be due to the concentration that
was used, as a high chemotherapeutic dose of drug may not usually elicit cancer by apoptosis [43]. This might also be due to the long-term treatment of 72 h; early apoptosis might have occurred after 24 h or 48 h. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment, and so one of the earliest features of apoptosis is loss of plasma membrane asymmetry [42]. Annexin V is a 35–36 kDa Calcium ion (Ca²⁺) dependent phospholipid-binding protein with high affinity for PS. Annexin V can usually be conjugated to fluorochromes like fluorescein isothiocyanate (FITC) while retaining its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis by binding to the exposed apoptotic cell surface [42]. Therefore, annexin V+ / PI-quadrant analysis showed that early apoptosis did not occur when MDA-MB-231 cells were exposed to 307 μM of amodiaquine. The late apoptotic/secondary necrotic cells in the annexin V+ / PI+ (upper right) quadrant showed that the percentage of the cells found there was 28.25 % (Fig 6b, 7) after treatment with amodiaquine, while the untreated control was 6.53 % (Fig 6a, 7). PS translocation that causes loss of membrane asymmetry in early apoptosis precedes the loss of membrane integrity, which accompanies the later stages of cell death resulting from either apoptotic or necrotic processes. Thus, late apoptosis or secondary necrosis will occur because of loss of plasma membrane integrity since PI, a DNA-binding dye molecule can only enter cells with ruptured membranes [42]. During late apoptosis / secondary necrosis, annexin can also enter the ruptured membrane and bind to the PS that is left inside the cells, thereby creating a false positive result [44]. Hence apoptosis or necrosis cannot be categorically confirmed from this quadrant.

The necrotic cells in the annexin V- / PI+ (upper left) quadrant showed that the percentage of the cells was 41.70 % (Fig 6b, 7) after treatment with amodiaquine, while the control was 7.18 % (Fig 6a, 7). Figure 7 shows a statistically significant difference between the percentage of untreated and treated cells in this quadrant. This could mean that one of the mechanisms by which amodiaquine elicits its anti-cancer activity is by induction of necrosis. Propidium iodide cannot penetrate viable cells because they possess intact membranes. However, the membranes of dead and damaged cells are permeable to propidium iodide [45, 46]. Therefore, the percentage of annexin V-/ PI + cells analyzed will contain both dead (necrotic cells) and damaged cells. The exact percentage of necrotic cells present was then analyzed by gating the cells in the annexin V-/ PI + quadrant using direct necrotic analysis by propidium iodide.

The direct necrotic analysis showed that the percentage of direct necrotic cells from the treated group was 47.15 % (Fig 8b, 9), while that of the untreated was 7.21 % (Fig 8a, 9). This gives a truer picture of the necrotic activity of propidium iodide because of the exclusion of damaged cells or cell debris that may be present in the annexin V- / PI + cells previously analyzed. The necrotic effect observed with this high dose of amodiaquine (307 μM) supports previous findings showing that one of the cell death pathways of cancer cells is necrosis [43, 47] and also that necrotic effect is usually caused by a high concentration of anti-cancer drugs [47, 48].

This study has shown that amodiaquine may be potentially reprofiled as an anti-cancer agent in managing various breast cancer diseases like androgen receptor-positive / HER-2 positive breast cancer and triple-negative breast cancer. In addition to amodiaquine's well-established autophagy inhibitory activity, necrosis was found to be a probable mechanism of action for the anti-cancer activity of amodiaquine on MDA-MB-231 cells.

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Conflict of Interest
Authors declare that there is no conflict of interest.

Authors' Contributions
KSS conceived and designed the study, contributed to data collection, data analysis tools, analysis of data and manuscript writing. CPA contributed to study design, immensely contributed to thorough editing of the manuscript draft and in overall study supervision. MOA...
gave a critical review of the manuscript as an expert in Pharmaceutical Chemistry and served as an advisor for the research work. **BOS** contributed immensely to the ingenious editing of this manuscript, also as the main supervisor of this research; he provided guidance as well as advisory and supervisory roles throughout the period of this research.

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