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Novel spirooxindole based benzimidazole scaffold: In vitro, nanoformulation and in vivo studies on anticancer and antimetastatic activity of breast adenocarcinoma

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ABSTRACT

The present work provided in vitro anticancer investigation of novel spirooxindole based benzimidazole scaffold SP1 and its nanoformulation with in vivo evaluation of anticancer and antimetastatic activity as potential drug for breast adenocarcinoma. The synthesized compound SP1 exhibited potent growth inhibitory efficacy against four types of human cancer (breast, prostate, colon and lung) cell lines with IC50 = 2.4, 3.4, 7.24 and 7.81 μM and selectivity index 5.79, 4.08, 1.93 and 1.78 respectively. Flow cytometric analysis illustrated that SP1 exhibited high apoptotic effect on all tested cancer cell lines (38.22–52.3 %). The mode of action of this promising compound was declared by its ability to upregulate the gene expression of p21 (2.29–4.50 fold) and NF-κB (1.26–1.44 fold) in the treated cancer cells. Also, it enhanced the protein expression of apoptotic marker p53 and moderate binding affinity for MDM2 (Kd=7.94 μM). Notwithstanding these promising impressive findings, its selectivity against cancer cell lines and safety on normal cells were improved by nanoformulation. Therefore, SP1 was formulated as ultra-flexible niosomal nanovesicles (transethoniosomes). The ultra-deformability is attributable to the synergism between ethanol and edge activators in improving the flexibility of the nanovesicular membrane. F8 exhibited high deformability index (DI) of (23.48 ± 1.4). It was found that % SP1 released from the optimized transethoniosomal formula (F8) after 12 h (Q12h) was 84.17 ± 1.29 % and its entrapment efficiency (%EE) was 76.48 ± 1.44 %. Based upon the very encouraging and promising in vitro results, an in vivo study was carried out in female Balb/c mice weighing (15–25 g). SP1 did halt the proliferation of breast cancer cells as well as suppressed the metastasis in other organs like liver, lung and heart.

1. Introduction

Cancer is a major disease affecting population in all regions in the world leads to morbidity and mortality [1]. The p53 protein plays an important and essential role in controlling important and related biological processes (including glycolysis, cell cycle, apoptosis, autophagy and cell differentiation) [2]. The activity of p53 – often referred to as the tumor suppressor - is controlled at various stages: transcription, translation and stability. Much recent cancer research has focused on the p53-MDM2 loop [3]. p21 (p21WAF1 or p21Cip1) is a cyclin-dependent kinase inhibitor (CKI) represents a major target of p53 activity and thus associated with linking DNA damage to cell cycle arrest [4]. Since NF-κB
activation generally inhibits p53, their crosstalk seems to be antagonistic [5,6]. Mechanistically, NF-κB can induce MDM2, thereby leading to p53 degradation through a ubiquitin-dependent pathway [7]. Herein, we set our design rationale to tailor new MDM2 inhibitors endowed with NF-κB inhibitory potential for maximizing their p53 induction capacity.

In addition, NF-κB plays a critical role in regulating the survival, activation and differentiation of innate immune cells and inflammatory T cells [8]. Consequently, deregulated NF-κB activation contributes to the pathogenic processes of various inflammatory diseases. Ki-67 is a nuclear protein and is expressed mainly in S, G1, G2 as well as M phase and absent in G0 phase [9]. Immunohistochemical analysis for Ki-67 is a popular mean for evaluating the effectiveness of tested compound in treating cancer cells. During metastasis, cancer cells break away from the original (primary) tumor, travel through the blood or lymph system, and form a new tumor in other organs or tissues of the body [10]. To design a drug that has a dual effect against cancer as well as metastasis is challenging.

Spirooxindoles present unique and attractive structures that fit in the binding domain of MDM2, thereby blocking the interaction and leads to the activation and translocation of p53 to the nucleus [11-24]. The nuclear translocation activates p53-targeted genes that are involved in the regulation of different signaling pathways. Although some inhibitors have included spirooxindole-containing compounds, there is a need to improve the efficacy and bioavailability of MDM2 inhibitors for pharmacological use in the treatment of cancer. Barakat et al, recently have been reported some examples that shown high efficacy towards the p53-MDM2 protein-protein interactions inhibitors (Fig. 1) [20-23].

Thus, an improved format for delivering substituted spirooxindoles is desired. Within this approach, the representation of benzimidazole scaffold in various anticancer agents [25-29], and more specifically targeting NF-κB sparked our interest [30-36]. Therefore, it would lead to a path for the development of novel target-specific and highly effective benzimidazole-based anticancer agents (Fig. 1). Accordingly, in continuation to our research [19-23], we utilized spiro[3H-indole-3,2′-pyrrolidin]-2(1H)-one as a promising chemically stable core for installing benzimidazole motif to the main skeleton spiro ring via a carbonyl spacer. This scaffold hopping approach was also supported by the observation that some efficient MDM2 inhibitors are based on benzimidazole ring.

Nanotechnology is a promising approach with several medical uses. Nanomedicine refers to the application of nanotechnology to the fabrication of drug delivery systems. Indeed, nano-based drug delivery systems improve bioactive component efficiency by increasing solubilization and hence bioavailability [37]. Niosomes are nanovesicles made of non-ionic surfactants that provide controlled drug delivery. Niosomes have numerous advantages over liposomes, including enhanced stability, thermoresponsiveness, and increased permeation. Non-traditional classes of niosomes, such as proniosomes, disomes, and multifaceted niosomes, have been developed and characterized. Ethioniosomes are novel elastic nano-sized niosomes containing ethanol.

Fig. 1. Reported spirooxindoles and benzimidazoles with anti-cancer activity and our designed compound SP1.
and a small amount of cholesterol. Ethoniosomes have the potential to be excellent drug delivery systems for both water-soluble and insoluble pharmaceuticals. Because ethoniosomes include cholesterol, a bilayer membrane stabilizer, they are more physically stable than spanlastics [38,39]. The addition of EAs to ethoniosomes may increase vesicle deformability by disrupting the lipid bilayer and therefore boosting flexibility [39]. As a result, because of its ultradisperse nature, a carrier composed of both ethanol and EAs would enhance the drug’s penetration through different membranes. This work described a promising nano-sized niosomal system with elastic properties (trans-ethoniosomes) as a prospective drug delivery technology based on the concept of applying ethanol as a hydrating solution to surfactant/lipid materials and EAs for increasing vesicular membrane deformability.

In this text, we discussed in vitro, nanoformulation and in vivo anticancer and antitumor activity of novel spirooxindole-based benzimidazole scaffold of formula SP1 as a potential anticancer drug for breast adenocarcinoma. The nanoformulation described a novel nano-sized niosomal system with elastic properties (trans-ethoniosomes) as a viable drug delivery system based on the concept of applying ethanol as a hydrating solution to surfactant/lipid materials and EAs for increasing vesicular membrane deformability.

2. Results and discussion

2.1. Chemistry

The lead compound candidate was synthesized in a large-scale quantity for cytotoxicity in vitro, nano-formulation and animal model study according to scheme 1. The requisite dipolarophile required for 32CA reaction was synthesized and amended in the supplementary material. Next, by applying the one-pot three components reaction approach afforded the constructed final lead compound which was the reaction refluxed in MeOH for 2-3 h. The reaction proceeded via [3 + 2] cycloaddition reaction (ortho/endo-path) [40] which furnished the final drug in regioselective and stereoselective manner as shown in scheme 1. The chemical structure of the final drug was elucidated by a set of spectrophotometric tools including 1H NMR, 13C NMR; MS and IR. The absolute configuration for the final drug was assigned based on the single crystal x-ray diffraction analysis. The crystallographic data of SP1 was collected by using Cu Kα radiation at 120 K. The structure of SP1 was solved and refined in the chiral orthorhombic space group P212121. The Flack parameter was refined to 0 (formally –0.007(3)) indicating presence of only one enantiomer shown in the Scheme 1.

2.2. Biological evaluation

2.2.1. NCI screening

Initially, the synthesized spirooxindole based benzimidazole scaffold SP1 was screened for panel of cancer cell lines at NCI (DTP- development therapeutic program) at 10 µM single dose. The GI50 (percentage of growth inhibition) reported in the Table S1(SI). As summarized in the table, the tested compound possessed promising antiproliferative activities against most of NCI-60 cell lines.

2.2.2. MTT assay

Furthermore, the newly spirooxindole based benzimidazole SP1 was screened for its cytotoxic effect on normal fibroblasts (Wi-38) in the term of IC_{50} which was equivalent to 13.9 µM, compared to 3.86 µM for standard chemotherapeutic drug (Doxorubicin, Dox) (Table 1). Then the screened compound was evaluated for its anticancer activity on four human cancer types, which are among the top 10 cancer types in the term of the estimated cases and deaths worldwide, namely breast, prostate, colon and lung cancers (MDA-MB 231, PC3, HCT-116 and A549, respectively). SP1 had lower IC_{50} values (2.4, 3.4, 7.24 and 7.81 µM, respectively) than Dox as shown in Table 1. Accordingly, the calculated SI values of SP1 against MDA-MB 231, PC3, HCT-116 and A549 were 5.79, 4.08, 1.93 and 1.78, respectively, compared to < 1 in the case of Dox. This powerful anticancer effect is supported with severe morphological alterations (cell shrinking and loss of normal spindle shape) of the treated cancer cells in comparison with untreated cancer cells (Fig. 2).

All values are presented as mean ± SEM.

2.2.3. Apoptosis studies

2.2.3.1. Flow cytometric analysis of apoptosis. Dot plots, as shown in Fig. 3, illustrate four quadrants including QC1, QC2, QC3, and QC4 which represent annexin V-stained early apoptotic cells, annexin V/PI-dual stained late apoptotic cells, healthy unstained cells, and PI-stained necrotic cells, respectively. Fig. 3 and Table 2 clarify that SP1 treated cancer cells demonstrated higher percentage of annexin/PI-stained late apoptotic population cells than untreated cells. As shown in Fig. 3 and Table 2, SP1 induced apoptosis-dependent death (38.22–52.3 %) in the treated breast, prostate, colon and lung cancer cell lines. This effect may attribute to the potency of SP1 up-regulating p21 expression by 2.29–3.91 folds as well as down-regulating the expression of the level of cyclin D-mediated cell cycle progression and NF-kB in the treated cancer cells relative to the untreated cells (Fig. 4). SP1 exhibited higher suppressive effect on cyclin D expression (1.9–8.93 folds) than that of NF-kB (1.26–1.44 fold).

2.2.3.2. Immunohistochemical analysis of p53 overexpression. Fig. 5 A, B shows the immunohistochemistry for tumor suppressor protein (p53) of four test human cancer cell line after treatment for 72 h with SP1. SP1 elevated protein level of p53 to 51.51, 57.53, 49.83 and 34.31 %, in the treated MDA-MB 231, PC-3, HCT-116 and A549 cells, respectively. This was illustrated by increasing brown color stained cells in comparison with purple color stained untreated control cells (Fig. 5A). Despite, the above-mentioned promising results of SP1, it is necessary to improve its selectivity against cancer cells and safety on normal cells, making its nanoformulation valuable.

2.2.3.3. MDM2 binding analysis by microscale thermophoresis assay. Compound SP1 exhibited promising safety profiles and anticancer activities as detected by MTT assay, and induced apoptosis via p53 mechanism. Accordingly, the compound SP1 then tested for MDM2 binding analysis by Microscale thermophoresis assay (MST) which is an emerging sensitive technique that is utilized to assess and quantify biomolecular interactions between various binding partners. The tested compound was incubated with a fluorescently labeled MDM2 at increasing concentration (0.763 nM to 25 µM; the highest concentration was limited by solubility). MST binding curves (Fig. S6, SI) showed that spirooxindole based benzimidazole SP1 showed moderate binding affinity (K_{D}7.94 µM).

2.2.3.4. Molecular docking of the studied compound SP1. To better understand the binding mode of SP1 to the MDM2 pocket we performed modeling studies. For this, we used the native p53-MDM2 interaction (PDB ID 1YCR) and we removed the p53 peptide. Next, we modeled the SP1 oxindole part onto W23 and energy minimized the molecule in the presence of the receptor (Fig. 6). SP1 fits well into the MDM2 binding pocket (Fig. 6) and forms a lot of close contacts with the receptor amino acids. Alignment of SP1 with the p53 peptide reveals that the oxindole indeed mimicks W23 including the key hydrogen bond to the backbone L54. The benzimidazole moiety mimics the L26, and the bicyclic octa-hydroindole substructure nicely mimics F19. Additionally, the phenyl group of SP1 is forming non-canonical interactions with the surface of MDM2 (H96, K94) which are not presented by the native p53 interaction. Overall, this model is able to explain the biological activity of the spirocyclic SP1. The representative compound SP1 exhibited potent binding affinity with the MDM2 which leads to the inhibition of the
Scheme 1. Synthesis and plausible mechanism for the desired spirooxindole derivative SP1.
interactions of the tumor suppressor protein p53 with its negative regulators MDM2.

2.3. Nano formulation study

2.3.1. The 2³ factorial design of SP1-loaded ethoniosomes

The optimization technique can estimate the best amounts of many factors needed to generate high-quality formulations [41]. The effects of several independent variables on the parameters of SP1-loaded ethoniosomal formulations are investigated in Table SI. The optimization technique was used to maximize the observed responses; EE percent and Q₁₂h to choose the optimum SP1-loaded ethoniosomal formula. Table 3 shows the results of the factorial design of SP1-loaded ethoniosomes. The signal-to-noise ratio is denoted by the adequate precision. In the current model, the adequate precision values were greater than 4 (the intended value) for both responses, indicating that this model could successfully navigate the design space. The acquired data from both responses (EE percent and Q₁₂h) fit the linear model well, with R² of 0.9892 and 0.9960, respectively. The high R² values of both EE percent and Q₁₂h explored the statistical validity of the calculated equations. The predicted R² denotes the predictability of both responses in the current model. There is a reasonable harmony between the predicted and adjusted R² which is attributed to the small difference between their values (lower than 0.20) [42].

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Wi-38</th>
<th>MDA-MB 231</th>
<th>PC3</th>
<th>HCT-116</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀(µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP1</td>
<td>13.9±1.1</td>
<td>2.4±0.2</td>
<td>3.4±0.3</td>
<td>7.2±0.3</td>
<td>7.8±0.3</td>
</tr>
<tr>
<td>Dox</td>
<td>3.86±0.2</td>
<td>5.82±0.4</td>
<td>8.80±0.3</td>
<td>13.1±2.1</td>
<td>11.5±0.8</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB 231</th>
<th>PC3</th>
<th>HCT-116</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.835±0.1</td>
<td>3.6±0.19</td>
<td>0.835±0.3</td>
<td>2.395±0.9</td>
</tr>
<tr>
<td>untreated</td>
<td></td>
<td>0.045±0.9</td>
<td></td>
<td>0.125±0.9</td>
</tr>
<tr>
<td>SP1</td>
<td>51.65±0.69</td>
<td>52.3±0.71</td>
<td>38.22±0.3</td>
<td>45.44±0.4</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM. Different letters are significantly different in the same column at P<0.05.

Fig. 2. Morphological alteration of SP1-treated MDA-MB 231, PC-3, HCT-116 and A549 cells in comparison with the untreated control cells (Magnification, 100 X).

Fig. 3. Flow charts of annexin-PI analysis of SP1-treated cancer cells lines.
Furthermore, diagnostic curves for EE percent and $Q_{12h}$ were generated to validate the credibility and goodness of fit of the existing model, as shown in Figs. 7 and 8, respectively. The differences between the real and predicted responses were described as the residuals. Fig. 7a and 8a show a normal distribution of residuals and a linear pattern in their normal probability plots, indicating that the collected data do not require transformation. Fig. 7b and 8b indicate that there is no constant error where the values of both responses are close to the zero-axis. Fig. 7c and 8c show that there are no lurking variables influencing the tested responses. Fig. 7d and 8d show the close relationship between the real and predicted responses ($Y_1$ and $Y_2$) of SP1-loaded ethoniosomes in the current model. The ANOVA analysis (Table 4) shows the significance of the effect of numerous independent factors on both EE percent and $Q_{12h}$.

2.3.2. The EE percent of SP1-loaded ethoniosomes

Table 4 investigates that the EE percent of SP1-loaded ethoniosomes ranged from $55.43 \pm 1.61$ to $92.66 \pm 1.33 \%$. Fig. 9 demonstrates the influence of the studied variables on the EE% of SP1-loaded ethoniosomes. ANOVA, Table 4 explores that the EE percent is significantly influenced by the quantity and type of non-ionic surfactant as well as the quantity of EA.

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**Fig. 4.** Relative change in the gene expression of p21, cyclin-D and NF-κB in SP1-treated four cancer cell lines.

**Fig. 5.** p53 immunostaining for the untreated and SP1-treated MDA-MB 231, PC-3, HCT-116 and A549 cells. (A) Microscopic image for p53 of the untreated and SP1-treated cancer cells showing p53− purple colored negative immunostained cells (yellow arrow) and p53+ brown colored positive immunostained cells (red arrow) (magnification × 200). (B) Representative percentages of positive immunostaining cancer cells. Different letters are significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The results of the optimization technique for Table 3 sticks) Highlighting the binding hotspot F19W23L26 (green sticks). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 3**
The results of the optimization technique for SP1-loaded ethoniosomes.

<table>
<thead>
<tr>
<th>Studied responses</th>
<th>$R^2$</th>
<th>Adjusted $R^2$</th>
<th>Predicted $R^2$</th>
<th>Adequate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE percent (Y1)</td>
<td>0.9892</td>
<td>0.9810</td>
<td>0.9567</td>
<td>32.619</td>
</tr>
<tr>
<td>Q_{12h} (Y2)</td>
<td>0.9960</td>
<td>0.9930</td>
<td>0.9841</td>
<td>53.276</td>
</tr>
</tbody>
</table>

In Abbreviations: $R^2$, r-squared or the coefficient of determination; EE percent, entrapment efficiency percent; $Q_{12h}$ % SP1 released after 12 h.

Concerning the amount of the non-ionic surfactant (X1), it is obvious that higher amounts of both Span 60 and Span 80 has a significant positive influence on the EE percent of SP1-loaded ethoniosomes ($p < 0.001$). That may be elucidated by enhancing the stiffness of the lipid bilayer while minimizing drug leakage [43].

Besides, the type of non-ionic surfactant (X2) showed a significant effect on the EE percent. The EE% from ethoniosomal preparations fabricated using Span 60 was significantly greater than that of Span 80 ($p < 0.001$). That might be attributed to the formation of less leaky Span 60-based vesicles due to Span 60’s saturated alkyl chain and its high phase-transition temperature ($53^\circ C$). Span 80-based vesicles, on the other hand, are leakier due to the non-saturation of Span 80’s alkyl chain of and its low phase-transition temperature ($12^\circ C$) [44] that impede the creation of a tight membrane [45,46].

With respect to the amount of EA, transeoniosomes have a significantly ($p < 0.001$) higher EE% than the corresponding ethoniosomes. That may be explained on the basis of the localization of amphiphilic molecules (EAs) at the polar-nonpolar interface which result in increasing the packing density of the vesicular membrane and hence improving the entrapment efficiency [47]. These findings are in agreement with other researchers such as Garg et al. [48] and Shaji et al. [39].

2.3.3. The in vitro release of SP1-loaded ethoniosomes

Fig. 10 investigates that the $Q_{12h}$ of the prepared SP1-loaded ethoniosomes ranged from 52.43 ± 1.14 to 94.43 ± 1.58 %. The in vitro release of SP1 from different ethoniosomal formulations was clearly more sustained than that of free SP1, which exhibited 92.73 ± 2.11 % drug released after 4 h. These outcomes indicated that ethoniosomal preparations act as reservoirs for SP1 and could effectively sustain its in vitro release.

Fig. 11 investigates the impact of the selected variables on $Q_{12h}$ of SP1 (Y2). The amount of the non-ionic surfactant (X1) has a significant detrimental impact effect on the $Q_{12h}$ of SP1-loaded ethoniosomes ($p < 0.001$) [49]. That could be explained on the basis of increasing the rigidity of the lipid bilayer that leads to decreasing the drug efflux from the ethoniosomal vesicles. These findings were also shared by Surya et al. [50].

Additionally, the non-ionic surfactant type (X2) had a significant impact ($p < 0.01$) on $Q_{12h}$ % SP1 released from Span 80-based ethoniosomal formulations was significantly greater than those containing Span 60. This could be interpreted in terms of the higher phase transition temperature and the saturated alkyl chain, which lead to the development of more rigid and less permeable bilayers than Span 80 [51].

Respecting the EA amount, it is noticeable that transeoniosomes have a significantly higher $Q_{12h}$ ($p < 0.001$) than the corresponding ethoniosomes. That might be interpreted on the basis of the combined effect of both ethanol and edge activator effect [39,48] that result in the formation of more deformable vesicles [52].

The kinetic analysis investigated that the release profile of both SP1-loaded ethoniosomes and SP1 dispersion followed the Baker & Lonsdal model, Table 5.

2.3.4. The optimization of SP1-loaded ethoniosomes

The SP1-loaded ethoniosomes were optimized using Design-Expert software via numerical analysis on the premise of the highest values of EE percent and $Q_{12h}$ [54]. The choice of the optimized ethoniosomal formulation was performed by the concurrent optimization process of various response variables which was followed by choosing the formula with the greatest desirability [55,56]. It was found that the transeoniosomal formula (F8) had the greatest desirability value; hence it was chosen as the optimized ethoniosomal formula.

2.4. Characterisation of the optimized SP1-loaded transeoniosomes

2.4.1. Morphological description of the optimized SP1-loaded transeoniosomes by SEM

Fig. 12 investigates the SEM micrograph of the SP1-loaded transeoniosomes (F8) as spherical and distinct nanovesicles. The spherical outline of SP1-loaded transeoniosomal vesicles within the aqueous colloidal nanodispersions. A large zeta potential of F8 (~35.60 mV)
Fig. 7. The diagnostic curves for EE percent of SP1-loaded ethoniosomes (a) The graph of normal % probability vs. the internally studentized residuals (b) The graph of internally studentized residuals vs. the predicted values, (c) The graph of internally studentized residuals vs. run number and (d) The graph of predicted versus actual values; Abbreviation. EE%, entrapment efficiency percent.
Fig. 8. The diagnostic curves for $Q_{12h}$ of SP1-loaded ethoniosomes (a) The graph of normal % probability vs. the internally studentized residuals (b) The graph of internally studentized residuals vs. the predicted values, (c) The graph of internally studentized residuals vs. run number and (d) The graph of predicted versus actual values; Abbreviation: $Q_{12h}$ % SP1 released after 12 h.
Fig. 9. The impact of the amount of non-ionic surfactant (a), the type of non-ionic surfactant (b), the amount of EA (c) on EE% of SP1-loaded ethoniosomes. Abbreviation: EE, the entrapment efficiency.
explores the reasonable stabilization of the transethoniosomal dispersion because of the repulsive forces between different ethoniosomal vesicles [60–66].

2.4.3. Measurement of transethoniosomal vesicle deformability

The elasticity of the transethoniosomal vesicles investigates their capability of squeezing through the tiny pores of different biological membranes with no rupture [55]. The DI of the optimized SP1-loaded transethoniosomes (23.48 ± 1.4) was significantly (p < 0.01) higher than that (10.22 ± 0.62) of the corresponding ethoniosomes. That can be interpreted on the basis of enhancing the elasticity of the lipid bilayer [54] of the SP1-loaded ethoniosomes by the addition of EAs.

2.5. Animal model results

SP1 compound did not cause severe toxicity to mice

In order to determine the toxicity of SP1 compound and calculate LD50, a single dose was orally injected to mice. After 14 days of observation, only one mouse was dead in the group treated with 50 mg/kg. The H&E pathological examination revealed that mild to moderate hepatic degeneration, tubular dilation and degeneration in the kidney, cardiomyocytes degeneration, few peribronchial leukocytic cells infiltration and an increase in lymphocytic cell’s depletion in lymphoid follicles was observed in the groups injected with 10 and 25 mg/kg, respectively.

Based on these observations, the antimetastatic activity for SP1 compound was conducted for both 10 and 25 mg/kg doses. After the injection of mammary fat pads with 4 T1 cells, H&E staining showed that lung, heart and liver have tumor infiltration. The treatment of the 4 T1-bearing mouse with 10 or 25 mg/kg showed normal hepatocytes with decreased numbers of perivascular infiltration of tumour cells when compared with their counterpart untreated group injected. Increasing SP1 dose (25 mg/kg) was more efficient than 10 mg/kg (Fig. 14).

The lung sections from group received 4 T1 cells showed congested blood vessels, peribronchial and interstitial aggregation of tumour cells admixed with MNCs. However, the congestion and tumor aggregation were disappeared in a dose-dependent manner (Fig. 15). The same pattern of antimetastatic activity of SP1 compound was seen in the heart sections where hyaline degeneration in cardiomyocytes and interstitial aggregation of tumour cells admixed with MNCs was detected in 4 T1-bearing mice and no tumour cells infiltrating cardiac muscles or coronary fat was detected in mice treated either with 10 or 25 mg/kg (Fig. 16).

The antimetastatic activity of SP1 was confirmed by evaluating the level of Ki-67 proliferative marker. Ki-67 is a nuclear protein and is expressed mainly in S, G1, G2 as well as M phases and absent in G0 phase [67,68]. Immunohistochemical analysis for Ki-67 is a popular mean for evaluating the effectiveness of tested compound in treating cancer cells [9,69]. In this study, the analysis of IHC revealed that less Ki-67 positive nucleus has been detected either in liver, lung or in heart in comparison to the untreated group. The decrease in Ki-67 staining was seen in a dose-dependent manner (Fig. 17A and B).

Further mechanistic study was performed to evaluate the change in angiogenic and metastatic gene expression in groups treated with SP1.
Fig. 11. The impact of the non-ionic surfactant amount (a), the non-ionic surfactant type (b) and the EA amount (c) on $Q_{12\text{h}}$ of SP1-loaded ethoniosomes. Abbreviation: $Q_{12\text{h}}$ % SP1 released after 12 h.
Table 4
The ANOVA study for the optimization of SP1-loaded ethoniosomes.

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F-value</th>
<th>P-value</th>
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<tr>
<td>Y1</td>
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<tr>
<td></td>
<td>X1</td>
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<tr>
<td></td>
<td>X2</td>
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<td>453.31</td>
<td>180.12</td>
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<tr>
<td></td>
<td>X3</td>
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<td>1</td>
<td>266.34</td>
<td>105.83</td>
<td>0.0005</td>
</tr>
<tr>
<td>Y2</td>
<td>Model</td>
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<td>333.79</td>
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<tr>
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<td>192.86</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>X3</td>
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<td>1</td>
<td>409.27</td>
<td>324.91</td>
<td>&lt;0.0001</td>
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</table>

Notes: *Y1: EE percent (%), Y2: Q12h (%), the amount of non-ionic surfactant (X1), the type of non-ionic surfactant (X2), the amount of EA (X3). Abbreviation: SS, the sum of squares; df, the degree of freedom; MS, the mean of squares.

Table 5
The kinetic orders of the in vitro release of SP1 and SP1-loaded ethoniosomes.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi model</th>
<th>Hixson Crowell</th>
<th>Baker &amp; Lonsdal</th>
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<tr>
<td>F1</td>
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<td>0.9784</td>
<td>0.9648</td>
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<tr>
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<tr>
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<td>0.9862</td>
</tr>
<tr>
<td>F4</td>
<td>0.9351</td>
<td>-0.9942</td>
<td>0.9778</td>
<td>0.9868</td>
<td>0.9975</td>
</tr>
<tr>
<td>F5</td>
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</tr>
<tr>
<td>F6</td>
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<td>0.9908</td>
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<td>-0.9953</td>
<td>0.9918</td>
<td>0.9961</td>
<td>0.9964</td>
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<td>0.9974</td>
<td>0.9968</td>
<td>0.9991</td>
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</tbody>
</table>

Fig. 12. SEM analysis of the optimized SP1-loaded transethoniosomal formula (F8).

Fig. 13. Vesicle size distribution graph of the optimized SP1-loaded transethoniosomal formula (F8).
Fig. 14. Microscopic pictures of H&E stained liver sections from groups received (A) $4 \times 10^6$ cells/200 μL showing congested blood vessels (red arrows), aggregation of tumour cells admixed with mononuclear cells (MNCs) was observed in perivascular (black arrows) and in sinusoids (black arrowheads) along with diffuse hydropic degeneration in hepatocytes (yellow arrows). Microscopic pictures of H&E stained liver sections from treated groups (B) 10 or (C) 25 mg/kg) showing normalized hepatocytes with decreased numbers of perivascular infiltration of tumour cells. Low magnification X: 100 with 100 μM scale bar and high magnification X: 400 with 50 μM scale.
Fig. 15. Microscopic pictures of H&E stained lung sections from groups received (A) $4\times10^6$ cells/200 µL showing congested blood vessels (red arrows), peribronchial and interstitial aggregation (black arrows) of tumour cells admixed with MNCs. Microscopic pictures of H&E stained lungs sections from treated groups (B) 10 or (c) 25 mg/kg showing disappeared congestion with decreased numbers of perivascular and interstitial infiltration of tumour cells Increasing dose of treatment 25 mg/kg was more efficient than 10 mg/kg. Low magnification X: 100 with 100 µM scale bar and high magnification X:400 with 50 µM scale.
As shown in Fig. 18 A and B, the fold of change in gene expression for the angiogenic vascular endothelial growth factor (VEGF-A) was significantly decreased in groups treated either with 10 or 25 mg/kg in comparison to untreated group. Angiogenesis is defined as the formation of new blood vessels from existing ones. It is involved in progression of cancer and is considered as a crucial target for cancer treatment [70]. VEGF-A regulates the formation of blood vessels via its receptor VEGFR-1 and -2 [71]. These results confirm the effectiveness of SP1 as anticancer lead compound.

Cell-cell adhesion and its adhesion with the extracellular matrix is regulated by glycoproteins including epithelial-Cadherin (E-Cadherin) [72]. It has been reported that the level of E-Cadherin expression is inversely proportional with tumor prognosis and metastasis [73]. Therefore, in this study, the gene expression level of E-Cadherin has been evaluated. The analysis showed that SP1 treatment led to upregulation in the expression (Fig. 18A).

In addition to that, the metastasis of cancer cells is regulated by the level of metalloproteinases. Metalloproteinases such as MMP9 are Zinc-containing enzymes and they degrade the extracellular matrix components allowing the invasion and metastasis of cancer cells. The level of metalloproteinase MMP9 was evaluated by performing western blotting technique. Fig. 18C revealed that the group treated with 25 mg/kg expressed no MMP9 while the expression was decreased in group treated with 10 mg/kg in comparison to DMSO treated group. The mechanism of action of SP1 in vivo confirms the in vitro activity. SP1 upregulated the expression of p21 (cell cycle inhibitor) and activated p53 (IHC) which both are reported to regulate the expression of Ki-67, VEGF and MMP9 [74,75]. Additionally, the inhibition of NF-κB transcription factor is associated with a reduction in the level of metalloproteinases [76,77] and VEGF while is associated with overexpression of E-Cadherin [78]. Our results present an effective lead compound that has in vitro and in vivo activity.

3. Conclusion

We designed and synthesized successfully a novel lead compound for cancer drug discovery research development. The compound SP1 exhibited potential therapy for several types of cancer cells including, triple negative breast cancer cell line (MDA-MB 231), prostate cancer (PC3), colon cancer (HCT-116) and lung cancer, and A549. The compound SP1 shown safer therapy for human normal lung (wi-38) cells was higher than 81 % after incubation with 5 µM of SP1 which caused wi-38 cell death by over 50 %. Indeed, SP1 pharmaceutically acceptable agent as p21, cyclin-D and nuclear factor-kappa B (NF-xB) genes in the treated cancer cells immunohistochemical detection of apoptosis marker (p53). As antimetastatic drug, SP1 did halt the proliferation of breast cancer

Fig. 16. Microscopic pictures of H&E stained heart sections from groups received (A) 4*10^6 cells/200 µL showing hyaline degeneration in cardiomyocytes (yellow arrows) and interstitial aggregation (black arrows) of tumour cells admixed with MNCs. In addition, tumour cells infiltrate coronary fat was observed. Microscopic pictures of H&E stained heart sections from treated groups showing disappeared hyalinization of cardiomyocytes with no tumour cells infiltrating cardiac muscles in group received either (B) 10 or (C) 25 mg/kg. No infiltrating tumour cells were observed in coronary fat in any of the treated group.
Fig. 17. Antiproliferative activity for SP1 compound. (A) Immunostained liver sections showed decreased numbers of proliferating tumor cells infiltrate portal areas in treated group with 10 mg/kg when compared with untreated group and become very few in treated group with 25 mg/kg. Lung sections revealed decreased numbers of proliferating tumor cells infiltrate interstitial tissue in treated group with 10 mg/kg and much more decreased in treated group with 25 mg/kg when compared with untreated group. Immunostained heart sections showing massive infiltration of tumor cells in coronary fat (*) in untreated group while No tumour cells were seen in infiltrating coronary fat or interstitial tissue in treated groups with 10 or 25 mg/kg. Low magnification X: 100 bar 100 and high magnification X:400 bar 50. IHC counterstained with Mayer’s hematoxylin. (B) Significant reduction in Ki-67 positive nucleus. One-way ANOVA was used in the analysis by Prism 8.0 software. ****: p value was <0.0001 and considered highly significant.

Fig. 18. SP1 treatment attenuated the gene expression of VEGF-A, E-Cadherin and MMP-9. (A) qRT-PCR analysis was normalized to beta-actin and \( \Delta \Delta CT \) was calculated. ****: highly significant difference from the untreated group. (B) representative gel electrophoresis for the product of RT-PCR. The VEGF-A expression was decreased in a dose-dependent manner. (C) down regulation of the metalloprotease MMP9 in group treated either with 10 or 25 mg/kg in comparison to DMSO treated group.
cells as well as suppressed the metastasis in other organs like liver, lung and heart. The present work also deals with developing industry feasible transethoniosomal drug delivery system of SP1 as drug reservoirs that could achieve controlled drug release. SP1-loaded proniosomes exhibited high entrapment efficiency in the range of 76.97% and 83.76%, and could prolong the in vitro drug release rate up to 12 h. The optimized transethoniosomal formula (F8) showed significantly higher cumulative drug release and higher stability than the corresponding ethoniosomes. Thus, the formulation of SP1 compound as transethoniosomal formula (F8) is considered to be a stable and prolonged drug delivery system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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