CHAPTER EIGHT
EFFECTS OF ISOLATED COMPOUNDS ON NBLE CD44+ CD24- CELLS
8.1 INTRODUCTION

Recent studies have demonstrated the presence of stem cells in various human tissues including the breast (Rodríguez Salas et al., 2010). Additionally, several cancers, such as, leukemia, glioma, pancreas, colon and breast carcinoma and others have been shown to harbor a rare population of cells termed ‘cancer stem cells’ (CSCs) that appear to be critical for tumor growth and maintenance (Tan et al., 2006). The hierarchical organization of cancers and the phenotypic and functional resemblance between normal and CSCs has led to the speculation that cancers arise in normal, tissue specific stem/progenitor cells (Shackleton, 2010, Phesse and Clarke, 2009, Rodríguez Salas et al., 2010, Pardal et al., 2003). Recent reports suggest that a small sub-population of human mammary epithelial cells (HMECs) was able to form three-dimensional (3D) spheroids called “mammospheres” when grown in suspension in vitro (Dontu et al., 2003). Mammospheres are largely comprised of primitive mammary stem and progenitor cells of varying proliferation potential, and capable of undergoing both self-renewal and multilineage differentiation in vitro (Dontu et al., 2003). Moreover, transplantation of mammospheres into cleared mammary fat pad generated complete mammary structures in vivo, further confirming the presence of long-term self-renewing stem/progenitor population within mammospheres (Liao et al., 2007). Thus, unlike HMECs grown in adherent conditions that promote differentiation (Muschler et al., 1999), growth in suspension appears to enrich for mammary stem and progenitor cells.

A recent study led to the generation of immortalized NBLE cells by overexpression of SV40ER and hTERT into stem cell enriched mammosphere-derived single cells from normal primary breast tissue (Paranjape et al., 2011). These cells showed retention of stem cell properties as with the presence of 32% CD44+/CD24- population, ability to form mammospheres at high frequency (32%), increased stemness related genes, reduced differentiation markers and retain the ability to differentiate in vitro. The NBLEs contain heterogeneous clonal populations with varying stemness properties. These NBLE cells even in the absence of oncogenic Ras are tumorigenic and interestingly form adenocarcinomas in nude mice. The CD44+/CD24- fraction within NBLE possess stemness property and are tumorigenic but not the 'Rest' fraction. The gene profile of cancer stem cell fraction of NBLE resembles that of primary breast cancer-derived cancer stem cells. The NBLE cells also showed heterogeneous karyotype and have few unique cancer stem cells specific changes.
The cancer stem cell fraction of NBLE showed the acquisition of epithelial to mesenchymal phenotype, DNA damage response and deregulated self-renewal pathways (Notch, Wnt and hedgehog). Thus, NBLEs provide a novel breast cancer model system to understand the molecular mechanisms involved in the transformation of primitive breast cells with stem-like properties and provide a potent experimental tool to study the effects of drugs against breast CSCs (Paranjape et al., 2011). The CD44$^{\text{high}}$/CD24$^{\text{low}}$ population of NBLE cells were sorted by FACS sorting and cultured in DMEM-F12 with growth factors to derive a cell line called NBLE CD44+/CD24- (Paranjape et al., 2011).

We have found that the compounds TC-A, TC-B, TC-C and TC-D isolated from the plant Tinospora cordifilia inhibits side population, CD44$^{\text{high}}$/CD24$^{\text{low}}$ population and cancer sphere formation in breast cancer cells. Moreover, the detailed study revealed that these compounds selectively induce cytotoxicity against SP and CD44$^{\text{high}}$/CD24$^{\text{low}}$ cells. These findings corroborated that compounds TC-A, TC-B, TC-C and TC-D were able to inhibit breast cancer stem cells. Our next question was whether TC-A, TC-B, TC-C and TC-D have any effect against NBLE CD44+/CD24- cells which is a cancer stem cell enriched cell line. First, we addressed this question by determining the cytotoxic and growth inhibitory activities of TC-A, TC-B, TC-C and TC-D against NBLE CD44+/CD24- cells. Since the NBLE cells harbor 30-40% CD44$^{\text{high}}$/CD24$^{\text{low}}$ cells and are enriched for cancer stem cells, the ability of the compounds to inhibit CD44$^{\text{high}}$/CD24$^{\text{low}}$ population of NBLE CD44+/CD24- cells were investigated. Moreover, the current study also investigated the ability of the compounds TC-A, TC-B, TC-C and TC-D to induce specific cell death against NBLE CD44+/CD24- cells.

8.2 MATERIALS AND METHODS

8.2.1 Drugs and chemicals

DMEM-F12, Trypsin-EDTA, HBSS, mitoxantrone, MTT, propidium iodide, hEGF, hydrocortisone, insulin and heparin were purchased Sigma Chemical Co. (St. Louis, MO, USA). CD44-PE was procured from BD Biosciences (USA). B27 and CD24-Alexa Fluor 610 were purchased from molecular probes, Invitrogen (USA).

8.2.2 Cell culture

The NBLE CD44+/CD24- cells were cultured in DMEM-F12 with growth factors (hEGF, hydrocortisone, insulin, heparin and B27) in a humidified 5% CO$_2$ incubator at 37°C Cells
were cultured in healthy condition and exponentially growing cells were taken for the experiments.

8.2.3 Cytotoxicity assay

NBLE CD44+/CD24- cells were treated with TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide and mitoxantrone (Each compounds were treated with four different concentration viz., 0.1 μg/ml, 1 μg/ml, 10 μg/ml and 100 μg/ml, respectively) in triplicates for 48 hours. DMSO was used as vehicle control. At the end of treatment, MTT assay was performed as described earlier (Section, 3.2.4.2). IC\textsubscript{50} (Concentration of the drug required to reduce the percentage cell viability to 50 percentage) values for TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide and mitoxantrone were determined by nonlinear regression (curve fit) analysis by plotting log (inhibitors) vs. Normalized response using software, Graph Pad Prism 5. Statistically significant difference from IC\textsubscript{50} values for the control cells exposed to vehicle control (DMSO) (p<0.001) were determined by one way ANOVA using Dunnett's Multiple Comparison Test.

8.2.4 CD44/CD24 analysis

NBLE CD44+/CD24- cells in log phase were cultured with and without compounds TC-A, TC-B, TC-C, TC-D (20 μg/ml each), salinomycin (2 μM), parthenolide (2 μM) and mitoxantrone (0.2 μM) for 48 hours. DMSO (0.5%v/v) was used as a vehicle control. At the end of the treatment period, cells were trypsinized and single cell suspension in HBSS (1x10\textsuperscript{6} cells/ml) after a recovery period (60 minutes) was stained with 5μl of CD44 conjugated with PE and 5μl of CD24 conjugated to Alexa Fluor 610 in ice for 30 minutes in the dark. At the end of incubation, cells were washed twice with ice-cold HBSS and resuspended in 500μl of HBSS. Cells were analyzed on a flow cytometer (FACSCanto II, Beckmen Dickinson, USA). CD44-PE was excited with a 488 nm laser and emission was detected at 585/42 nm. Similarly, CD24-Alexa Fluor 610 was excited with a 633 nm laser and emission was detected at 660/20 nm. Fluorescence compensation was done to eliminate spectral overlap. CD44-PE and CD24-Alexa Fluor 610 were plotted on y-axis and x-axis respectively. CD44\textsuperscript{high} CD24\textsuperscript{low} population was gated as blue color. Data were analyzed by the software Summit v 4. 3 and statistical analysis was carried out by one way ANOVA using Dunnett's Multiple Comparison Test.
8.2.5 Propidium iodide assay

Propidium iodide (PI) assay was used to identify the number of dead cells. Briefly, NBLE CD44+/CD24-cells were cultured with and without TC-A, TC-B, TC-C, TC-D (20 μg/ml each), salinomycin (2 μM), parthenolide (2 μM) and mitoxantrone (0.2 μM) for 48 hours. DMSO was used as a vehicle control. At the end of the treatment period, cells were trypsinized and single cell suspension in HBSS was stained with PI at 2 μg/ml for 5 minutes. Cells were immediately analyzed on a flow cytometer to detect PI-positive dead cells (FACSCanto II, Beckmen Dickinson, USA). PI was excited with a 488 nm laser and emission was detected at 585/52 nm. Data were analyzed by Summit v 4.3 and statistical analysis was carried out by one way ANOVA using Dunnett's Multiple Comparison Test.

8.3 RESULTS

8.3.1 TC-A, TC-B, TC-C and TC-D inhibits cell proliferation

In order to evaluate the cytotoxic and growth inhibitory effects of TC-A, TC-B, TC-C and TC-D, NBLE CD44+/CD24- cells were treated with various concentrations of the compounds TC-A, TC-B, TC-C and TC-D for 48 hours. Standard inhibitors of cancer stem cells (salinomycin, parthenolide) and anticancer drug (mitoxantrone) were used as positive controls. Results indicated that compounds isolated from Tinospora cordifolia TC-A, TC-B, TC-C and TC-D imparts cytotoxicity and dose dependent inhibition of proliferation against NBLE CD44+/CD24- cells (Fig. 8.1). The IC_{50} values of TC-A, TC-B, TC-C and TC-D in NBLE CD44+/CD24- cells were found to be 5.83 ± 0.31 μg/ml, 1.26 ± 0.11 μg/ml, 2.48 ± 0.12 μg/ml and 21.33 ± 1.85 μg/ml, respectively (Table 8.1). Thus, among the four compounds investigated for potential cytotoxicity and inhibition of cell proliferation, TC-B demonstrated the most potent activity. The chronological order of cytotoxicity against NBLE CD44+/CD24- cells were TC-B>TC-A>TC-C>TC-D. Results also showed that salinomycin, parthenolide and mitoxantrone possess dose dependent inhibition on proliferation of NBLE CD44+/CD24-cells (Fig. 8.2). The IC_{50} values of salinomycin, parthenolide and mitoxantrone were 0.41 ± 0.02 μM, 2.22 ± 0.14 μM and 4.34 ± 0.48 μM, respectively (Table 8.1). Salinomycin was found to have potent cytotoxic effects in NBLE CD44+/CD24- cells as compared to parthenolide. However, mitoxantrone was found to have less cytotoxic effects as compared to salinomycin and parthenolide. The IC_{50} values of TC-A, TC-B, TC-C and TC-D was quite comparable with salinomycin and parthenolide. These results corroborated that
compounds TC-A, TC-B, TC-C and TC-D possess potent cytotoxicity against cancer stem cells (NBLE CD44+/CD24-). The data represent ± SD of three independent experiments (n=3).

8.3.2 TC-A, TC-B, TC-C and TC-D inhibits CD44\textsuperscript{high} CD24\textsuperscript{low} population

Next, we have investigated the effects of compounds in CD44\textsuperscript{high} CD24\textsuperscript{low} population of NBLE cells by flow cytometry. It was found that NBLE CD44+/CD24- cells harbor 36.76 ± 2.15% of CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype. In order to understand the effects of purified compounds against CD44\textsuperscript{high}/CD24\textsuperscript{low} population, NBLE CD44+/CD24- cells were treated with and without the isolated compounds (TC-A, TC-B, TC-C and TC-D), salinomycin and parthenolide as described in materials and methods. Results indicated that CD44\textsuperscript{high} CD24\textsuperscript{low} population in untreated and vehicle control treated NBLE CD44+/CD24- cells were 36.76 ± 2.15% and 35.77 ± 2.85%, respectively. Upon treatment with TC-A, TC-B, TC-C and TC-D, CD44\textsuperscript{high} CD24\textsuperscript{low} population was found to be 0.54 ± 0.24% 0.29 ± 0.13% 1.50 ± 0.43% and 7.27 ± 0.45%, respectively. Similarly, in salinomycin and parthenolide treated groups, CD44\textsuperscript{high} CD24\textsuperscript{low} population were 1.36 ± 0.17% and 8.54 ± 0.47% respectively (Fig. 8.3). These results revealed that TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibits CD44\textsuperscript{high} CD24\textsuperscript{low} population of NBLE CD44+/CD24- cells. The compounds TC-B and TC-A found have most potent activity against CD44\textsuperscript{high} CD24\textsuperscript{low} population. Standard drug salinomycin found to have potent activity against CD44\textsuperscript{high} CD24\textsuperscript{low} population as compared to parthenolide (Fig. 8.4). All values are mean ± SD of three independent experiments, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test.

8.3.3 TC-A, TC-B, TC-C and TC-D imparts cell death in NBLE CD44+/CD24- cells

Previous results indicated that compounds TC-A, TC-B, TC-C and TC-D possess dose dependent inhibition of cell proliferation against NBLE CD44+/CD24- cells. Next, we had the question that whether these compounds induce any specific cell death in NBLE CD44+/CD24- cells. This question was addressed by performing a direct cell death assay based on propidium iodide assay using flow cytometry. Briefly, NBLE CD44+/CD24- cells treated with and without the isolated compounds (TC-A, TC-B, TC-C, TC-D), salinomycin, parthenolide and mitoxantrone for 48 hours. Results showed that percentage number dead cells in untreated
control cells were 4.84 ± 0.43%. DMSO did not have any significant cell death (5.78 ± 0.81%). Upon treatment with TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide and mitoxantrone, percentage number dead cells were 79.59 ± 5.11%, 79.32 ± 3.33%, 44.73 ± 1.25%, 13.86 ± 2.49%, 71.44 ± 2.39%, 53.25 ± 3.51% and 23.88 ± 2.50%, respectively (Fig. 8.5). These results revealed that compound TC-A, TC-B, TC-C and TC-D induces a significant cell death against NBLE CD44+/CD24- cells. The effects of the compounds were comparable with standard cancer stem cell inhibitors such as salinomycin and parthenolide. The compounds TC-B and TC-A possess most potent activity as compared to other compounds. However, mitoxantrone found to have less cell death against NBLE CD44+/CD24- cells as compared to the isolated compounds, salinomycin and parthenolide. All values are mean ± SD of three independent experiments ***p<0.001, **p<0.01 (for compound TC-D) determined by one way ANOVA by using Dunnett's Multiple Comparison Test.

8.4 DISCUSSION

NBLE cells harbored a sub-population of CD44+/CD24- cells that resembled the breast cancer stem cells and generated adenocarcinomas in immunocompromised mice. The CD44+/CD24- fraction of NBLEs showed, mammosphere formation, EMT phenotype and exhibited activated self-renewal pathways. Thus, the plasticity exhibited by the NBLE cells with respect to differentiation, self-renewal and tumorigenicity makes them a unique model system to study the pharmacological effects of drugs which targets cancer stem cells (Paranjape et al., 2011).

The CD44+/CD24- fraction of NBLE cells were isolated by FACS and cultured in DMEM-F12 with growth factors to derive a cancer stem cell line, NBLE CD44+/CD24-. Thus, the derived cell line is a valuable tool to study the cancer stem cell specific effects of drugs. Our data suggests that compounds purified from the plant *Tinospora cordifolia* imparted dose dependent cytotoxic and growth inhibitory effects against NBLE CD44+/CD24- cells. The cytotoxic effects of compounds TC-A, TC-B, TC-C, and TC-D were comparable with standard inhibitors of cancer stem cells. Mitoxantrone, a chemotherapeutic drug was found to have less sensitivity against NBLE CD44+/CD24- cells. Our previous studies revealed that mitoxantrone is resistant towards SP and CD44<sup>high</sup> CD24<sup>low</sup> population of human breast cancer cells. These results correlate with the resistance properties of
mitoxantrone against cancer stem cells. More interestingly, the CD44/CD24 analysis by flow cytometry revealed that compounds TC-A, TC-B, TC-C, and TC-D significantly inhibited CD44\textsuperscript{high} CD24\textsuperscript{low} population of NBLE CD44+/CD24- cells. TC-B was found to have most potent activity against CD44\textsuperscript{high} CD24\textsuperscript{low} population. The induction of specific cell death against cancer stem cells is considered as the major strategy to eliminate the cancer stem cell population. Flow cytometry based cell death assay showed that the purified compounds impart significant cell death against NBLE CD44+/CD24- cells. The cancer stem cell inhibitory effects of the compounds isolated from the plant may be due to the blockade of self-renewal pathways such as Wnt signaling, Notch signaling, hedgehog pathway, Bmi-1, PTEN etc. It may be also due to the down regulation of stemness genes such as Oct-4, Nanog, Lef-1, ABCG-2 etc. Recent report suggests that epithelial mesenchymal transition (EMT) lead to the cancer stem cells characteristics. Therefore, these compounds may also down regulate EMT genes like Snail, Twist, N-cadherin, vimentin etc.
Cytotoxicity of compounds isolated from *Tinospora cordifolia* viz. TC-A, TC-B, TC-C and TC-D in human cancer stem cell line (NBLE CD44+CD24+). The cells were treated with specified concentrations of TC-A, TC-B, TC-C and TC-D for 48 hours and the percentage cell viability was determined by MTT assay. Percentage cell survival in untreated control cells was calculated as 100%. TC-A, TC-B, TC-C and TC-D significantly inhibited proliferation of NBLE CD44+CD24- cells. Each point represents the mean ± SD of three independent experiments performed in triplicate (n=3).

Cytotoxicity of salinomycin, parthenolide and mitoxantrone in NBLE CD44+CD24- cells

Cytotoxicity of salinomycin, parthenolide and mitoxantrone in human cancer stem cell line (NBLE CD44+CD24). The cells were treated with specified concentrations of salinomycin, parthenolide and mitoxantrone for 48 hours and the percentage cell viability was determined by MTT assay. Percentage cell survival in untreated control cells was calculated as 100%. Salinomycin, parthenolide and mitoxantrone significantly inhibited proliferation of NBLE CD44+CD24- cells. Each point represents the mean ± SD of three independent experiments performed in triplicate (n=3).
Table 8.1 IC$_{50}$ values of TC-A, TC-B, TC-C and TC-D in NBLE CD44+CD24- cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bresat cancer stem cell line (NBLE CD44+CD24-)</th>
<th>IC$_{50}$ (μg/mL)</th>
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<tbody>
<tr>
<td>TC-A</td>
<td></td>
<td>5.83 ±0.31</td>
</tr>
<tr>
<td>TC-B</td>
<td></td>
<td>1.26 ±0.11</td>
</tr>
<tr>
<td>TC-C</td>
<td></td>
<td>2.48 ±0.12</td>
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<tr>
<td>TC-D</td>
<td></td>
<td>21.33 ±1.85</td>
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<tr>
<td>Salinomycin*</td>
<td></td>
<td>0.41±0.02</td>
</tr>
<tr>
<td>Parthenolide*</td>
<td></td>
<td>2.22±0.14</td>
</tr>
<tr>
<td>Mitoxantrone*</td>
<td></td>
<td>4.34 ±0.48</td>
</tr>
</tbody>
</table>

IC$_{50}$ values of compounds TC-A, TC-B, TC-C, TC-D, salinomycin, parthenolide and mitoxantrone in human cancer stem cell line (NBLE-CD44+CD24-). IC$_{50}$ (Concentration of the drug required to reduce the percentage cell viability to 50 percentage) were obtained from the graph by non-linear regression analysis as best curve-fit values. Known cancer stem cell inhibitors (salinomycin and parthenolide) and anticancer drug (mitoxantrone) were used as positive controls. *The IC$_{50}$ of doxorubicin is represented as μM. Numerical data are means ± S. D of three independent experiments (n=3). Statistically significant difference from IC$_{50}$ values for the control cells exposed to vehicle control (DMSO) (p < 0.001).
Figure 8.3 CD44/CD24 analysis in NBLE CD44+CD24- cells

NBLE CD44+CD24- Cells were treated with and without purified compounds isolated from *Tinospora cordifolia* (TC-A, TC-B, TC-C, TC-D) and known cancer stem cell inhibitors (parthenolide and salinomycin) for 48 hours. At the end of the treatment, CD44/CD24 assay was performed and cells were analyzed in a flow cytometer to detect CD44<sub>hi</sub> CD24<sub>lo</sub> population. Compounds, TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited CD44<sub>hi</sub> CD24<sub>lo</sub> population. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 8.4 Effects of TC-A, TC-B, TC-C and TC-D in CD44\textsuperscript{high}/CD24\textsubscript{low} population

NBLE CD44\textsuperscript{+}CD24\textsuperscript{-} cells were treated with and without purified compounds isolated from *Tinospora cordifolia* (TC-A, TC-B, TC-C, TC-D) and known cancer stem cell inhibitors (parthenolide and salinomycin) for 48 hours. At the end of the treatment, CD44/CD24 assay was performed and cells were analyzed in a flow cytometer to detect CD44\textsuperscript{high} CD24\textsubscript{low} population. TC-A, TC-B, TC-C, TC-D, parthenolide and salinomycin significantly inhibited CD44\textsuperscript{high}/CD24\textsubscript{low} population of NBLE-CD44\textsuperscript{+}CD24\textsuperscript{-} cells. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
Figure 8.5 TC-A, TC-B, TC-C and TC-D induces cell death NBLE CD44+CD24- cells

NBLE CD44+CD24- cells were treated with and without specified concentrations of TC-A, TC-B, TC-C, TC-D, known cancer stem cell inhibitors (salinomycin and parthenolide) and anticancer drug (mitoxantrone) for 48 hours. At the end of the treatment, percentage number of live cells and dead cell were determined based on propidium iodide assay by flow cytometry. TC-A, TC-B, TC-C and TC-D induces cell death in NBLE CD44+CD24- cells. These figures are representative of three independent experiments. All values are mean ± SD of three independent experiments (n=3, ***p<0.001, **p<0.01 determined by one way ANOVA by using Dunnett's Multiple Comparison Test).
8.5 REFERENCES


