Chapter-4

Part-2

Studies with Resveratrol and its analog
Results

4.1. C1 is more potent than Res in breast cancer cell lines.

Res and its analog C1 differ with each other structurally as C1 is a hybrid molecule of Res and Combrestatin. The structural difference, mass and molecular weight of Res and C1 are indicated below (Fig. 4.1.1). Thus it was essential to see whether the structural differences have any implication on the activity of the analog as well. To compare the potency of Res and C1, IC_{50} of Res and C1 were determined using MTT assay in three breast cancer cell lines MCF-7, MDA-MB-468 and T47D. In MCF-7 cells, Res and C1 showed IC_{50} of 35 and 15 µM respectively (Fig. 4.1.2. A, B). The analog C1 showed almost half fold less IC_{50} compared to Res. To validate the result in other breast cancer cells, we determined IC_{50} in MDA-MB-468 and T47D as well. In MDA-MB-468, Res and C1 showed IC_{50} of 36 and 19 µM (Fig. 4.1.2. C, D). T47D was found to be more sensitive to both Res and C1 compared to other two cell lines showing IC_{50} of 19.8 and 8 µM for Res and C1 respectively (Fig. 4.1.2. E, F). Despite of being more sensitive to Res and C1, T47D still followed similar trends in the IC_{50} like the other two cell lines. Thus the analog C1 was found to be more potent than Res in all the three cell lines as the IC_{50} values of the analog C1 was two times less than that of Res indicating greater potency.
Figure. 4.1.1. Structural difference between Res and C1. The chemical formula, exact mass and molecular weight of Res and C1.

MCF-7

A

B
Table 4.1. IC_{50} values of Res and C1.

<table>
<thead>
<tr>
<th>IC_{50} values (µM)</th>
<th>MCF-7</th>
<th>MDA-MB-468</th>
<th>T47D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Res</td>
<td>35</td>
<td>36</td>
<td>19.8</td>
</tr>
<tr>
<td>C1</td>
<td>15</td>
<td>19</td>
<td>8</td>
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</tbody>
</table>

Figure. 4.1.2. The analog of Res was more potent than Res in breast cancer cells. The IC_{50} values of Res and AS-9 were determined by MTT assay in MCF-7 (A) & (B), MDA-
MB-468 (C) & (D) and T47D (E) and (F) on exposing cells with different doses of Res and C1 for 72 h. Error bars represent mean ± SD of 2-3 independent experiments of duplicate/ triplicate samples. Table. 4.1 summarizes the IC_{50} values of Res and C1 in MCF-7, MDA-MB-468 and T47D cell lines (C= control, Res= resveratrol, C1= compound 1).

4.2. Res and C1 induces cell death through apoptosis in breast cancer cells.

Since C1 was found to be more potent than Res in terms of their IC_{50} values, it was essential to identify the mode of cell death induced by them. We used MCF-7 and MDA-MB-468 for our further studies. Res. 10, 20 and 30 µM were the dose selected for Res and 1, 5 and 10 µM for C1, keeping in mind their IC_{50} values. Since we wanted to see the effect of the compounds on different cell death pathways, it was essential to keep the dose of drugs lower than that of their IC_{50} values. To know whether apoptosis was responsible for Res and C1 mediated cytotoxicity, apoptotic markers were studied. Percentage apoptotic cells on exposure to Res and C1 were determined by Annexin-V FITC/ Propidium iodide (PI) staining. In MCF-7 cells, there was significant increase in the percentage of apoptotic cells compared to control in both Res and C1 exposed cells in a dose dependent manner (Fig. 4.2.1. A). The PARP (cleaved) levels in MCF-7 also showed significant increase on exposure to Res and C1 compared to control. Moreover the analog induced apoptosis in the breast cancer cells at a much lower dose compared to Res (Fig. 4.2.1. B, C). MDA-MB-468 cells also showed similar results. There was significant increase in the percentage of apoptotic cells on exposure to Res and C1 in MDA-MB-468 cells as well (Fig. 4.2.2. A). Even PARP (cleaved) levels were significantly enhanced in Res and C1 treated cells compared to control (Fig. 4.2.2. B, C). In both the cell lines, C1 induces apoptosis at a much lower dose (1, 5 and 10 µM) compared to Res (10, 20 and 30 µM) indicating the higher potency of C1.
**Figure. 4.2.1. Res and C1 induces apoptosis in MCF-7 cells.** (A) Percentage apoptotic cell on exposure to Res (10, 20, 30 μM) and C1 (1, 5, 10 μM) for 24 h as determined by Annexin-V FITC/ PI staining and analysed by Flow cytometer. Error bars represent mean ± SD of 3 independent experiments of duplicate/ triplicate samples (**p < 0.01). (B) and
(C) Upper panel: Western blot analysis for PARP (cleaved) levels on exposure to Res (10, 20, 30 µM) and C1 (1, 5, 10 µM). Lower panel: Densitometric analysis of PARP (cleaved) levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Error bars represent mean ± SD of 3 independent experiments (C= control, Res= resveratrol, C1= compound 1).
Figure. 4.2.2. Res and C1 induces apoptosis in MDA-MB-468 cells. (A) Percentage apoptotic cell on exposure to Res (10, 20, 30 µM) and C1 (1, 5, 10 µM) for 24 h as determined by Annexin-V FITC/ PI staining and analysed by Flow cytometer. Error bars represent mean ± SD of 3 independent experiments of duplicate/ triplicate samples (**, p< 0.01). (B) and (C) Upper panel: Western blot analysis for the PARP (cleaved) levels on exposure to Res (10, 20, 30 µM) and C1 (1, 5, 10 µM). Lower panel: Densitometric analysis of PARP (cleaved) levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (**, p< 0.01; ***, p< 0.001). Error bars represent mean ± SD of 3 independent experiments (C= control, Res= resveratrol, C1= compound 1).

4.3. Res induces autophagy in breast cancer cell lines but analog inhibits autophagy.

To study the effect of Res and C1 on the other cell death pathway, autophagy we analysed autophagy markers like LC3 and Beclin-1. During the autophagic vacuole formation, a cytosolic form of LC3 (LC3-I) is associated with phosphatidylethanolamine to form LC3-phosphatidylethanolamine complex (LC3-II). This LC3-II is then recruited in the membrane of autophagic vacuoles (1). The detection of LC3 in autophagic vacuoles has thus become a reliable assay for monitoring autophagy. Beclin-1 also has a central role in formation of the autophagy complex (2). Therefore increase in the autophagy associates with concomitant increase in the levels of LC3-II and Beclin-1 proteins and decrease in the autophagy will cause decrease in the levels of these proteins. Res treatment causes a dose dependent increase in the LC3-II and Beclin-1 levels in MCF-7. LC3-II expression in MCF-7 cells increased to ~ 2 fold at 10 µM and went on to increase to ~3.5 fold at 30 µM of Res exposure. Beclin-1 levels also increased significantly (Fig. 4.3.1.A). The analog, C1 however showed significant decrease (~ 2 fold) in the both LC3-II and Beclin-1 levels (Fig. 4.3.1. B). The formation of autophagic vacuoles was further confirmed by the distribution of GFP-LC3 dots in transfected breast cancer cells. We transfected both the
breast cancer cell lines with GFP-LC3 followed by the exposure to the maximum doses of both Res and C1, which is 30 and 10 µM respectively. Fig. 4.3.1. C depict significant increase in the distribution of the GFP-LC3 dots in the Res treated cells while decrease in GFP-LC3 dots were observed in the C1 treated cells.

To further validate our results we analysed the autophagic markers in MDA-MB-468 cells as well. Even MDA-MB-468 cells showed significant increase in the LC3-II and Beclin-1 levels in the Res exposed cells compared to control in dose dependent manner (Fig. 4.3.2. A). C1 showed significant decrease in LC3-II and Beclin-1 levels compared to untreated cells (Fig. 4.3.2. B). The GFP-LC3 dots in the transfected MDA-MB-468 further confirmed the results. The GFP-LC3 distribution increased significantly in Res treated cells but there was decrease in GFP-LC3 dots in C1 treated cells compared to control (Fig. 4.3.2. C).

**MCF-7**

![MCF-7 Graph](image1)

![MCF-7 Graph](image2)
Figure. 4.3.1. Res induces autophagy but C1 inhibits autophagy in MCF-7 cells. (A) Upper panel: Western blot analysis for the of LC-3, Beclin-1 levels on exposure to Res (10, 20, 30 µM) and (B) C1 (1, 5, 10 µM) for 24 h. Lower panel: Densitometric analysis of LC3-II levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (**, *p < 0.01). Error bars represent mean ± SD of 2-3 independent experiments. (C) GFP-LC3 transfected cells were treated with 30 and 10 µM of Res and C1 respectively for 24 h time period and observed under fluorescence microscope (C= control, Res= resveratrol, C1= compound 1). Scale bar represents 50 µm.

MDA-MB-468
Figure. 4.3.2. Res induces autophagy but C1 inhibits autophagy in MDA-MB-468 cells. (A) Upper panel: Western blot analysis for the LC-3, Beclin-1 levels on exposure to Res (10, 20, 30 µM) and (B) C1 (1, 5, 10 µM) for 24 h. Lower panel: Densitometric analysis of LC3-II levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (*, p< 0.05; **, p< 0.01). Error bars represent mean ± SD of 2-3 independent experiments (C) GFP-LC3 transfected cells were treated with 30 and 10 µM of Res and C1 respectively for 24 h time period and observed under fluorescence microscope (C= control, Res= resveratrol, C1= compound 1). Scale bar represents 50 µm.


Res and C1 both induced apoptotic cell death in breast cancer cells. Res induced dose dependent autophagy while C1 inhibited autophagy in both the cell lines. So to establish the interconnection between autophagy and apoptosis induction by Res and its analog, we used autophagy inhibitor, 3-methyladenine (3-MA). Autophagy was inhibited by pre-treating the breast cancer cells with 10 mM of 3-MA for 2 h, followed by Res (30 µM) and C1 (10 µM) for 24 h. In MCF-7 cells, the percentage of apoptotic cells on exposure to Res increased ~ 6 fold but 3-MA significantly decreased the Res induced percentage of apoptotic cells similar to control. C1 also induced significant percentage of apoptotic cells (~ 7 fold) which on pre-treatment with 3-MA significantly increased further up to ~ 20 fold
(Fig. 4.4.1.A). We further analysed the effect of 3-MA on the LC3-II and PARP levels of MCF-7 cells. 3-MA significantly downregulated the LC3-II levels. The PARP levels induced by Res reduced to significant levels, when MCF-7 cell were pre-treated with 3-MA (Fig. 4.4.1.B). However the PARP levels induced by C1 further increased significantly in the presence of 3-MA (Fig. 4.4.1.C).

MCF-7

A
Figure. 4.4.1. Res and C1 induced apoptosis involves differential role of autophagy in MCF-7 cells. (A) Percentage apoptotic cell on pre-treatment with 3-MA or DMSO for 2 h followed by exposure to Res (30 µM) and C1 (10 µM) for 24 h determined by Annexin-V FITC and PI staining and analysed by Flow cytometer. Error bars represent mean ± SD of 3 independent experiments of duplicate/ triplicate samples (*, $p<0.05$). (B) Upper
panel: Western blot analysis for the LC3, PARP (cleaved) levels on pre-treatment with 3-MA or DMSO for 2 h followed by exposure to Res (30 µM) and (C) C1 (10 µM) for 24 h. Lower panel: Densitometric analysis of LC3 II and PARP (cleaved) levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (*, p< 0.05; **, p< 0.01). Error bars represent mean ± SD of 3 independent experiments (C= control, 3-MA= 3-Methyladenine, Res= resveratrol, C1= compound 1).

Pre-treatment with 3-MA significantly reversed the apoptosis induction by Res as determined by Annexin-V/PI staining (Fig. 4.4.2.A) and PARP levels in MDA-MB-468 cell also (Fig. 4.4.2. B). But in case of C1, the combination of C1 and 3-MA significantly increased the percentage of apoptotic cells (Fig. 4.4.2.A) and PARP levels (Fig. 4.4.2.C). The LC3-II levels however were decreased in the presence of 3-MA in both Res and C1 treated cells (Fig. 4.4.B, C) Thus autophagy plays different role in apoptosis induction by Res and C1 in both the breast cancer cells.

MDA-MB-468

A

![Graph showing apoptosis population](image-url)
Figure. 4.4.2. Res and C1 induced apoptosis involves differential role of autophagy in MDA-MB-468 cells. (A) Percentage apoptotic cell on pre-treatment with 3-MA or DMSO for 2 h followed by exposure to Res (30 µM) and C1 (10 µM) for 24 h as determined by Annexin-V FITC and PI staining and analysed by Flow cytometer. Error bars represent mean ± SD of 2-3 independent experiments of duplicate/ triplicate samples (*, p< 0.05; **, p< 0.01).
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**, p< 0.01). (B) Upper panel: Western blot analysis for the LC3, PARP (cleaved) levels on pre-treatment with 3-MA or DMSO for 2 h followed by exposure to Res (30 µM) and (C) C1 (10 µM) for 24 h. Lower panel: Densitometric analysis of LC3 II and PARP (cleaved) levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (*, p< 0.05; **, p< 0.01). Error bars represent mean ± SD of 3 independent experiments (C= control, 3-MA= 3-Methyadenine, Res= resveratrol, C1= compound 1).

4.5. Autophagy precedes Res and C1 induced apoptosis.

To further investigate the correlation between autophagy and Res/C1 mediated apoptotic cell death, breast cancer cells were pre-treated with Ac-DEVD-CHO, a caspase-3 inhibitor. Res and C1 treatment in the presence of Ac-DEVD-CHO significantly reduced the percentage of apoptotic cells as determined by Annexin-V/PI staining in MCF-7 cells (Fig. 4.5.1.A). The PARP levels also reduced when pre-treated with caspase inhibitor (Fig. 4.5.1. B, C). To explore the role of autophagy in the presence of caspase inhibitor, LC-3 levels were studied using western blot analysis. The Res induced increase and C1 mediated decrease in LC3-II levels were not altered in the presence of Ac-DEVD-CHO (Fig. 4.5.1. B, C). Hence inhibiting apoptosis by caspase-3 inhibitor suppressed the Res and C1 induced apoptosis but not autophagy. Similar results were obtained for MDA-MB-468 cells as well (Fig. 4.5.2). This suggested that autophagy precedes apoptosis during Res and C1 mediated cancer cell death in both MCF-7 and MDA-MB-468 cells.
MCF-7

A

![Graph showing % of apoptotic population](image)

B

![Graph showing Fold Change in PARP (cleaved) and LC3-II](image)
**Figure. 4.5.1. Autophagy preceeds Res and C1 induced apoptosis in MCF-7 cells.**

(A) Percentage apoptotic cell on pre-treatment with Ac-DEVD-CHO or DMSO for 2 h followed by exposure to Res (30 µM) and C1 (10 µM) for 24 h as determined by Annexin-V FITC and PI staining and analysed by Flow cytometer. Error bars represent mean ± SD of 2-3 independent experiments of duplicate/ triplicate samples (*, p< 0.05; **, p< 0.01).

(B) Upper panel: Western blot analysis for the LC3, PARP (cleaved) levels on pre-treatment with Ac-DEVD-CHO or DMSO for 2 h followed by exposure to Res (30 µM) and (C) C1 (10 µM) for 24 h. Lower panel: Densitometric analysis of PARP (cleaved) LC3-II levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (*, p< 0.05; **, p< 0.01). Error bars represent mean ± SD of 3 independent experiments (C= control, Res= resveratrol, C1= compound 1).
Figure. 4.5.2. Autophagy preceeds Res and C1 induced apoptosis in MDA-MB-468 cells. (A) Percentage apoptotic cell on pre-treatment with Ac-DEVD-CHO or DMSO for 2 h followed by exposure to Res (30 µM) and C1 (10 µM) for 24 h as determined by Annexin-V FITC and PI staining and analysed by Flow cytometer. Error bars represent mean ± SD of 2-3 independent experiments of duplicate/ triplicate samples (*, p< 0.05; **, p< 0.01). (B) Upper panel: Western blot analysis of the LC3, PARP (cleaved) levels on pre-treatment with Ac-DEVD-CHO for 2 h followed by exposure to Res (30 µM) and (C) C1 (10 µM) for 24 h. Lower panel: Densitometric analysis of PARP (cleaved) and LC3-II levels. Quantified bands were normalized with their respective β-actin levels and the fold change was calculated compared to control (*, p< 0.05). Error bars represent mean ± SD of 3 independent experiments (C= control, Res= resveratrol, C1= compound 1).
Discussion

Cancer cells are subjected programmed cell death in response to cytotoxic chemicals and different kinds of stress (3). There are different types of programmed cell death which a cancer cell encounters (4). These cell death pathways are often closely interlinked with each other. There are several key signalling players which directly or indirectly affect more than one cell death pathway (5). Out of the different cell death pathways, this part of the thesis mainly focusses on apoptosis and autophagy and their interconnection with each other. The role of autophagy in breast cancer is very significant and is different at different stages of tumor development and progression. Initially autophagy is required for the tumor initiation for nutrient recycling. Before the vascularization of tumor takes place, autophagy is necessary for cancer cell survival in hypoxia conditions. Later in a fully transformed breast cancer cells, autophagy functions as a tumor suppressor. (6). While breast tumor susceptibility to autophagy depends upon the tumor genotype and the therapeutic agents used, but it still remains unclear how such new strategies will be beneficial. Thus the issue whether autophagy is aimed at cell survival or at cell death is likely to remain a subject of debate for some time (7). Interestingly, our study shows that autophagy plays a significant role in apoptosis induction by Res and its synthetic analog, C1. C1 is a hybrid analog of Res and Combrestatin.

Res as a natural product has already shown its potential as chemopreventive and chemotherapeutic candidate (8). It has shown to be effective against different types of cancer cells and animal models (9). Res have also shown to induce autophagy and apoptosis synergistically in many cancer cells (10). While in some cases inhibition of autophagy has shown to increase the sensitivity of cancer cells to Res mediated cell death by apoptosis (11, 12). But the fact that Res has limited success at the clinical trials, has led
to researchers to think about alternatives to Res. The lower bioavailability, rapid metabolism and high absorption are the several drawbacks of Res (13). To exploit the beneficial role of Res and at the same time overcome the disadvantages of Res, synthetic analogs are being designed. We have tried to study and compare the effect of Res and its analog C1 on different breast cancer cell line in inducing cell death pathways.

Combrestatin A4 (CA4) is a well known anticancer agent used for inhibition of tubulin polymerization in vitro. It binds to the β-tubulin at the colchicines binding site, promoting the disturbance of the dynamic equilibrium of microtubule formation (14). CA4 also exerts its antitumor effects against the multi-drug resistant (MDR) cancer cells (15). CA4 has also shown to induce autophagy in different cancer cells which when inhibited increases the sensitivity of cells towards CA4 mediated apoptosis (16). However CA4 itself cannot be used as drug because of its poor bioavailability and chemical instability (17).

While doing primary screening of an old repository of compounds, we came across this Res/combrestatin A4 hybrid molecule, C1. Literature survey gave us the initial idea about the involvement of Res and Combrestatin in both apoptosis and autophagy. Hence the possible role of C1 in inducing apoptosis in breast cancer cells has been studied. Also the involvement of autophagy in the cell death mediated by the C1 and Res has been compared. The interconnection between apoptosis and autophagy has also been studied. We found that C1 was two times more potent than Res as determined by the IC$_{50}$ values in three breast cancer cell lines: MCF-7, MDA-MB-468 and T47D (Fig. 4.1.2). Researchers have also shown that structural modification of Res leads towards increased anti-tumor activity in different cancer cell lines indicating the importance of the structural modification in the parent compound (18). It was essential to identify the mode of cell death responsible for the cytotoxic effect of both Res and C1. Both Res and C1 induced significant apoptosis in a dose dependent manner as analysed by the Annexin-V/ PI
staining and PARP levels in both MCF-7 and MDA-MB-468 cells. However C1 was certainly more efficient in inducing apoptosis at much lower doses compared to Res (Fig. 4.2.1 and 4.2.2). This was in accordance with other studies which have shown that both combrestatin and Res analogs are potent in inducing apoptosis in different cancer cell lines including breast cancer (19, 20).

For establishing the role of Res and C1 in inducing the other cell death pathway called autophagy, we analysed the different markers for autophagy like LC3 and Beclin-1. Interestingly, we found that Res induced autophagy in dose dependent manner in both MCF-7 and MDA-MB-468 cells. Res and its dimethylated analog, pterostillbene have been reported to induce autophagy in breast cancer cells earlier (21). The analog of resveratrol, C1 however leads to dose dependent decrease in the levels of LC3-II and Beclin-1 in both the cell lines. Exposure of the GFP-LC3 transfected breast cancer cells to Res and C1 further validated the results (Fig. 4.3.1 and 4.3.2). Different classes of compounds like anti-malarial compounds and sodium selenite have shown to inhibit autophagy and this inhibition have different implications on the downstream pathways (22, 23). However there are no reports of autophagy inhibition by Res or its related compounds till now. Thus our findings were interesting and further studies were needed to explore the full picture.

Apoptosis and autophagy involve complex cross talk between them through some common mediators and key signalling molecules (5). Autophagy can increase cancer cell survival by blocking apoptosis in response to certain stress, at the same time it can also cause cell death in some cases. To establish the interconnection between autophagy and apoptosis induction by Res and its analog, we used autophagy inhibitor, 3-methyladenine (3-MA). Pre-treatment with 3-MA significantly reversed the apoptosis induction by Res as determined by the PARP levels and Annexin-V/PI staining in both MCF-7 and MDA-MB-
468 cells (Fig. 4.4.1 and 4.4.2). This suggested that Res mediated autophagy contributed to apoptosis induction in both the breast cancer cells. Res has been shown to induce autophagy mediated apoptosis in colon and ovarian cancer cell lines earlier (24, 25). C1 however showed enhanced apoptosis induction in the presence of 3-MA compared to C1 treatment alone (Fig. 4.4.1 and 4.4.2). This indicates the involvement of autophagy inhibition in the induction of apoptosis by C1. Thus the analog of Res caused apoptosis through autophagy inhibition unlike Res.

To further investigate the correlation between autophagy and Res/C1 mediated apoptotic cell death, breast cancer cells were pre-treated with Ac-DEVD-CHO, a caspase-3 inhibitor. Res and C1 treatment in the presence of Ac-DEVD-CHO significantly reduced the percentage of apoptotic cells as determined by Annexin-V/PI staining and PARP levels in both MCF-7 and MDA-MB-468 cells. The Res induced increase in LC3-II levels and C1 mediated LC3-II decrease was not altered in the presence of Ac-DEVD-CHO. Hence inhibiting apoptosis by caspase-3 inhibitor, suppressed the Res and C1 induced apoptosis but not autophagy (Fig. 4.5.1 and 4.5.2). This suggested that autophagy precedes apoptosis during Res and C1 mediated cancer cell death in MCF-7 and MDA-MB-468 cells.

Our study clearly establishes the importance of the structural difference between Res and its analog. Even though the analog is found to be more potent than Res in terms of inducing apoptosis, the involvement of autophagy is different in both the cases. Res induces apoptotic cell death in breast cancer cells in association with the autophagic pathway as the inhibition of autophagy significantly inhibits the Res mediated cell death by apoptosis. The analog however causes prominent autophagy inhibition, unlike other Res analogs. Autophagy inhibition by C1 is involved with apoptosis induction which increases further in combination with autophagy inhibitor. This mechanism of apoptosis induction by autophagy inhibition was a novel one. The analog certainly proves to be a
potential chemotherapeutic candidate for all those cancer cell lines where autophagy plays a protective role and causes resistance towards different anticancer agents.

Figure 4.6. Graphical representation of the summary of the part-2 of thesis
References


