

## Research Paper

# 7-(O)-Carboxymethyl daidzein conjugated to N-t-Boc-hexylenediamine

A novel compound capable of inducing cell death in epithelial ovarian cancer stem cells

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**Abbreviations:** EOC, epithelial ovarian cancer; OCSC, ovarian cancer stem cell; mOCC, mature ovarian cancer cell; N-t-boc-Daidzein, 5-{2-[3-(4-Hydroxy-phenyl)-4-oxo-4H-chromen-7-yloxy]-acetylamino}-pentyl-carbamic acid tert-butyl ester; CD44<sup>+</sup>, CD44-positive; CD44<sup>-</sup>, CD44-negative; XIAP, X-linked inhibitor of apoptosis protein; p-p70S6K, p-p70 S6 kinase

**Key words:** ovarian cancer stem cells, apoptosis, caspases, XIAP, mitochondrial depolarization

One of the major difficulties in the treatment of epithelial ovarian cancer (EOC) is the high rate of recurrent disease. This is thought to be due to the survival of a population of chemo-resistant cells within the tumor, the ovarian cancer stem cells (OCSCs), that are able to regenerate the tumor following chemotherapy. Therefore, the identification of a compound that can target the OCSCs is one of the main steps in improving overall survival of ovarian cancer patients. The objective of this study was to determine the effect of N-t-boc-Daidzein, a novel daidzein derivative, on OCSCs. The efficacy of this compound was evaluated in OCSC and mature ovarian cancer cell (mOCC) lines isolated from malignant ovarian cancer ascites. Cells were treated with increasing concentrations of N-t-boc-Daidzein (0.003–10  $\mu$ M) and cell growth was monitored by “real time in vitro micro-imaging” using the InCuCyte system. Cell viability was measured using the CellTiter 96 assay. Apoptosis was determined by caspase-Glo 3/7, 8 and 9 assays. The components of the apoptotic cascade were characterized by western blot analysis. N-t-boc-Daidzein was able to significantly inhibit cell growth and decrease cell viability of OCSC as well as mOCC cells in a dose and time dependent manner. This effect was due to the induction of apoptosis, which is characterized by caspase activation, XIAP and AKT degradation, and mitochondrial depolarization. This study describes a novel compound that can target the OCSCs. These findings may provide vital aide in improving overall survival in patients with EOC.

## Introduction

Epithelial ovarian cancer (EOC) is the fourth-leading cause of cancer-related deaths in women and the leading cause of gynecologic cancer death.<sup>1</sup> This high mortality rate is largely due to difficulties with diagnosis in early stages of the disease and a high rate of recurrence.<sup>2,3</sup> The troubles with early detection of the disease are caused by an absence of signs and symptoms in the initial stages of the disease and the lack of a widely available screening test. Additionally, although eighty percent of cancers initially respond to chemotherapy, the majority ultimately recurs with less than fifteen percent remaining in remission.<sup>4</sup> This is thought to be due to a small subset of cells within the tumor, the ovarian cancer stem cells (OCSCs), that are resistant to conventional chemotherapy treatments.<sup>5</sup>

Current chemotherapy agents are aimed at targeting rapidly dividing cells;<sup>6</sup> however, since the OCSCs are slow-dividing, they are not effectively killed by these compounds.<sup>5</sup> A number of previous studies have shown that there is indeed a population of cells within EOC tumors that are CD44-positive (CD44<sup>+</sup>),<sup>7</sup> chemo-resistant, and are able to regenerate a heterogeneous tumor. In contrast, the CD44-negative (CD44<sup>-</sup>) cell population (referred to here as the mature ovarian cancer cells or mOCCs) are chemo-sensitive, and non-tumorigenic.<sup>5</sup> Thus, the CD44<sup>+</sup> cells are thought to be the OCSCs, whose survival during chemotherapy is a large factor in the high recurrence rate of ovarian cancer. Therefore, developing new therapies that can target the OCSCs is extremely important for the treatment of patients with ovarian cancer.

Daidzein is an isoflavone found in soy products that has been shown to affect cell cycle progression and apoptosis in human cancer cells.<sup>8</sup> 5-{2-[3-(4-Hydroxy-phenyl)-4-oxo-4H-chromen-7-yloxy]-acetylamino}-pentyl-carbamic acid tert-butyl ester (N-t-boc-Daidzein) is a synthetic derivative of Daidzein that preliminary studies have demonstrated can inhibit cell growth in ovarian and colon cancer cells in vitro.<sup>9</sup> The long chain on the daidzein molecule imparts lipophilic character facilitating

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Submitted: 04/20/09; Revised: 06/09/09; Accepted: 06/17/09

Previously published online as a *Cancer Biology & Therapy* E-publication: <http://www.landesbioscience.com/journals/cbt/article/9285>

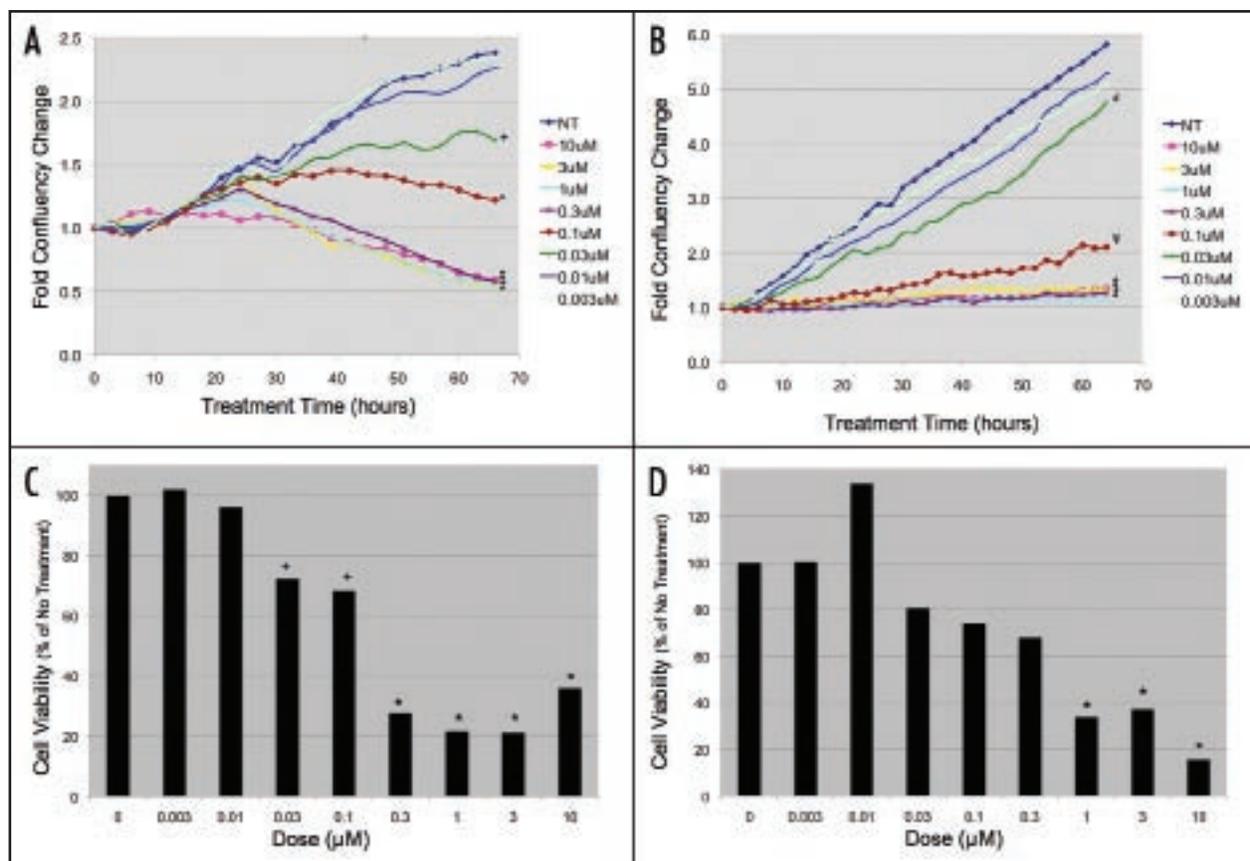


Figure 1. In vitro effect of N-t-boc-Daidzein on EOC cells. (A and B) EOC cells were treated with increasing concentrations of N-t-boc-Daidzein (0–10  $\mu$ M) for an average of 65 h. Confluency was measured by real time in vitro micro-imaging using the InCuCyte system and fold confluency change over time plotted for (A) OCSC1 and (B) TARA R127. \* $p < 0.001$ ,  $^{\#}p < 0.01$ ,  $^{\ast}p < 0.025$ ,  $^{\#}p < 0.05$ , when compared to control. (C and D) Percent viable cells after 65 h of treatment, (C) OCSC1 and (D) TARA R127. \* $p < 0.001$ ,  $^{\ast}p < 0.025$ . Similar results were observed for the other cell lines tested. Experiments were performed in triplicate.

endocytosis and the N-t-Boc group at the end of the side chain provides metabolic stability, which may increase its efficacy in vivo.<sup>9</sup> The objective of this study was to evaluate the effect of N-t-boc-Daidzein on EOC cells, especially the OCSCs.

We report here the molecular characterization of this novel compound that has the ability to induce apoptosis in OCSCs in vitro.

## Results

**N-t-boc-Daidzein significantly inhibits growth of EOC cells in vitro.** Our first objective was to characterize the in vitro response of EOC cells to N-t-boc-Daidzein treatment. Two CD44<sup>+</sup> OCSC cultures (OCSC1, OCSC2) and their corresponding CD44<sup>-</sup> mOCCs (TARA R182, TARA R127) were incubated in the presence of increasing concentrations of N-t-boc-Daidzein (0.003–10  $\mu$ M) for an average of 72 ( $\pm 11$ ) h, and cell growth was monitored by “real time in vitro micro-imaging” using the InCuCyte system (Essen Instruments, Ann Arbor, MI). The InCuCyte system allows for the hourly monitoring of cell growth by determining the confluency of the cells and displaying the morphologic changes associated with treatment. Figure 1A and B show the growth curves of representative EOC cell lines. For the OCSCs (Fig. 1A), the

control group follows a time-dependent growth curve characterized by a small increase in growth in the first 10 h, a logarithmic growth over the next 35 h, and the beginning of a plateau after 45 h. During the first 15 h, no differences were found in growth between the treated and non-treated groups. After this time, the effect of the treatment was clearly delineated according to dose and time. After 18 h of N-t-boc-Daidzein treatment, we began to observe a decrease in cell growth for cells treated with the highest dose of N-t-boc-Daidzein (10  $\mu$ M), as represented by changes in the growth curves. These changes further increased following 30 h of treatment. Significantly decreased cell growth was also observed with treatment doses of 0.3, 1 and 3  $\mu$ M, although the changes in growth curves with these doses were observed at later times (24–30 h of treatment). Lower doses such as 0.1 and 0.03  $\mu$ M affected cell growth curves even later, after 32 h of treatment, and the effect on cell growth was significantly less than with the higher doses. N-t-boc-Daidzein treatment at doses of 0.01 and 0.003  $\mu$ M were found to have no effect on cell growth.

Similar results were observed during the treatment of mOCCs with N-t-boc-Daidzein (Fig. 1B), with doses of 0.3  $\mu$ M and higher significantly inhibiting cell growth, doses of 0.1 and 0.03  $\mu$ M causing some, but less of an effect, and doses of 0.01 and 0.003

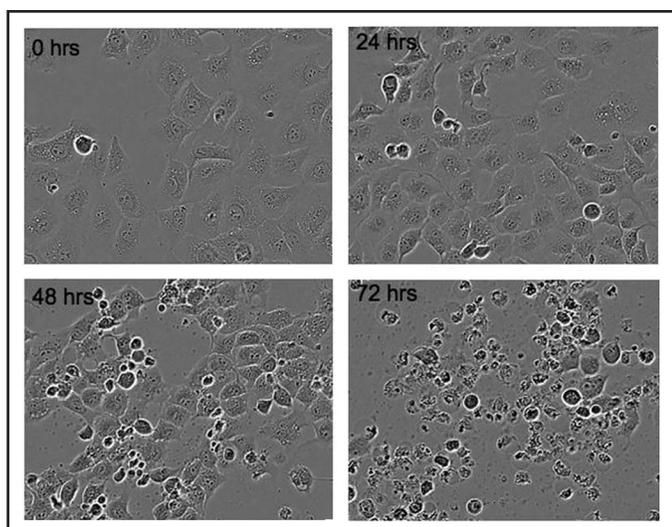


Figure 2. Induction of apoptosis in OCSCs with N-t-boc-Daidzein. OCSCs were treated with 10  $\mu\text{M}$  N-t-boc-Daidzein and their morphologic changes were followed using real time in vitro micro-imaging. Results shown are for OCSC1. Similar results were observed with the other cell lines tested.

$\mu\text{M}$  having no significant effect on growth. Cell growth of the mOCCs was inhibited by treatment at earlier time points than in the OCSCs, with changes in the mOCC growth curves after 10 h of treatment for all effective concentrations of N-t-boc-Daidzein (0.03–10  $\mu\text{M}$ ).

In order to determine whether the changes in the cell growth curves observed following treatment with N-t-boc-Daidzein were due to a decrease in cell viability, we performed the conventional cell viability assay on the cultures at the end of the treatment (Fig. 1C and D) using the CellTiter 96 assay. These results correlated with those observed using the IncuCyte system, with no changes in cell viability for the OCSCs treated with doses 0.003 and 0.01  $\mu\text{M}$  when compared to the control, small decreases in cell viability ( $p < 0.025$ ) with doses 0.03 and 0.1  $\mu\text{M}$ , and significantly decreased cell viability ( $p < 0.001$ ) for doses of 0.3  $\mu\text{M}$  or greater. The  $\text{GI}_{50}$  for OCSCs treated with N-t-boc-Daidzein was approximately 0.2  $\mu\text{M}$ . Cell viability results for the mOCCs also correlated with the data obtained from the Incucyte system with the  $\text{GI}_{50}$  for mOCCs at approximately 0.6  $\mu\text{M}$ .

**Decrease in cell growth with N-t-boc-Daidzein treatment is associated with apoptosis.** Our next objective was to determine the morphologic changes associated with the decrease in cell growth and viability observed with N-t-boc-Daidzein treatment. As shown in Figure 2 and movies 1–4 (Suppl. material), OCSCs and mOCCs treated with N-t-boc-Daidzein (10  $\mu\text{M}$ ) undergo morphologic changes characteristic of apoptosis, including cell shrinkage, chromatin condensation, membrane blebbing, formation of apoptotic bodies, and lastly, DNA fragmentation.<sup>10</sup>

In order to determine whether these morphological changes were indeed induced by apoptosis, we evaluated the activation levels of the caspases, the main mediators of apoptotic cell death. OCSCs were treated with 10  $\mu\text{M}$  N-t-boc-Daidzein for different time points (0–48 h) and the activity of initiator caspases-8 and -9

and the effector caspase-3 was measured using the Caspase-Glo 8, 9 and 3/7 Assays, respectively. As shown in Figure 3A, a significant increase in caspase activation was observed in a time-dependent manner for all three caspases tested. Activity of caspases-3 and -8 doubled after 24 h, while caspase-9 activity doubled after 48 h, suggesting that N-t-boc-Daidzein is able to induce apoptosis in OCSCs.

**Early changes in the apoptotic pathway induced by N-t-boc-Daidzein treatment.** There are two main pathways that lead to caspase activation and the initiation of apoptosis. One is the intrinsic, or mitochondrial, pathway that involves disruption of the mitochondrial membrane and caspase-9 activation. The second is the extrinsic pathway, which is activated by binding of ligands to transmembrane death receptors on the surface of the cells, leading to caspase-8 activation and initiation of the apoptotic cascade. These two pathways are interconnected and can activate each other. Both the intrinsic and extrinsic apoptosis pathways are controlled by a delicate balance between pro and anti-apoptotic proteins.<sup>11,12</sup> Our next objective was to characterize the activation status of some of these key regulatory proteins in OCSCs following N-t-boc-Daidzein treatment.

The earliest changes observed after N-t-boc-Daidzein treatment were caspase-2 activation and XIAP degradation. As shown in Figure 3B, following 8 h of N-t-boc-Daidzein treatment we observed a decrease in the inactive form of caspase-2 (p48), representing caspase-2 activation, and appearance of the activated caspase-2 fragment at 15 h (p20). Additionally, after 8 h of treatment we observed a decrease in the anti-apoptotic protein, X-linked inhibitor of apoptosis protein (XIAP) and later observed the appearance of its p30 cleavage product, thought to be formed when XIAP is cleaved by caspases.

**Late involvement of the intrinsic apoptosis pathway in N-t-boc-Daidzein-induced apoptosis.** The increase in caspase-9 activity that we observed suggested involvement of the mitochondrial pathway in N-t-boc-Daidzein-induced apoptosis. To further study mitochondrial pathway involvement, we looked at several proteins in the Bcl<sub>2</sub> family of proteins, which are the main regulators of mitochondrial stability and the intrinsic apoptosis pathway. These proteins can be divided into three groups: (1) pro-survival proteins, such as Bcl<sub>2</sub>, (2) pro-apoptotic proteins, including Bid, and (3) the Bax subfamily. In normal, healthy cells, the pro-survival proteins outweigh the pro-apoptotic proteins, resulting in cell survival. However, when a cell is given a stress signal, the activated pro-apoptotic proteins bind and neutralize the pro-survival molecules, allowing pro-apoptotic family members like Bak and Bax to oligomerize and create an open channel in the mitochondrial membrane, resulting in mitochondrial depolarization.<sup>13</sup> These events may lead to the activation of caspase-9, which we observed after 48 h of N-t-boc-Daidzein treatment.

Bid functions as a link between the extrinsic apoptosis pathway, involving caspase-8 activation, and mitochondrial integrity. Indeed, it has been shown that Bid is cleaved and activated by caspase-8, and the truncated form translocates to the mitochondria initiating the process of mitochondrial depolarization.<sup>14,15</sup> After 15 h of N-t-boc-Daidzein treatment, Bid was activated, as shown by a decrease in the amount of full-length, inactive Bid (p22) and

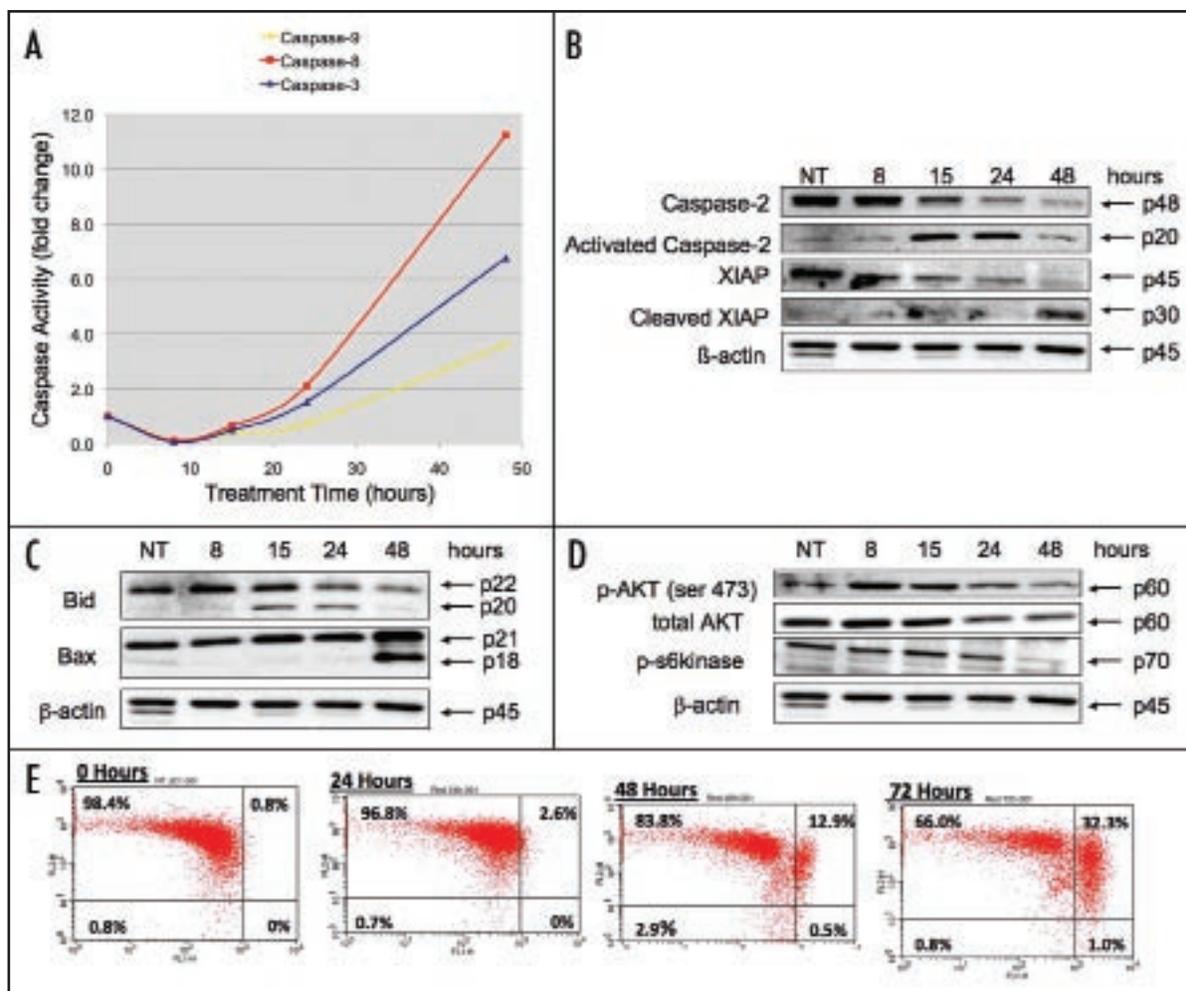


Figure 3. Effect of N-t-boc-Daidzein treatment on components of the apoptotic cascade. OCSCs were treated with 10  $\mu$ M N-t-boc-Daidzein for 8, 15, 24 or 48 h. (A) Activity of caspases-3/7, -8 and -9 was measured using Caspase-Glo assays. (B–D) Effect on pro- and anti-apoptotic proteins, and pro-survival proteins was determined using western blot analyses. (E) Effect on mitochondrial membrane stability was determined using the JC-1 dye. Results shown are for OCSC1. Similar results were observed with the other cell lines tested.

the appearance of its truncated, active form (p20) (Fig. 3C). The other pro-apoptotic Bcl<sub>2</sub> family member, which was activated after N-t-boc-Daidzein treatment was Bax. We detected the cleaved form of Bax (p18) after 48 h of treatment, which is indicative of Bax activation and mitochondrial translocation.

To further confirm the involvement of the mitochondrial pathway, we measured mitochondrial depolarization using JC1 dye, and found that there is a late, slow mitochondrial depolarization, beginning after 48 h of treatment with N-t-boc-Daidzein (Fig. 3E). This time point for mitochondrial depolarization correlated more with Bax activation, which occurred at 48 h, than with Bid activation, which occurred earlier at 15 h. Furthermore, a significant increase in caspase-9 activity was observed after 48 h of treatment. Taken together, our results suggest that N-t-boc-Daidzein primarily initiates apoptosis via the extrinsic pathway and later engages the intrinsic pathway to amplify the cascade.

**Effect of N-t-boc-Daidzein on AKT survival pathway.** As indicated above, apoptosis is the result of changes in the balance

of pro- and anti-apoptotic pathways. Our next objective was to characterize the effect of N-t-boc-Daidzein on one of the main pro-survival pathways, the AKT survival pathway.

AKT promotes cell survival by controlling the activities of caspase-9, Bax, and the mTOR pathway and protecting XIAP from degradation. AKT has been shown to protect ovarian cancer cells against pro-apoptotic signals.<sup>16</sup> Interestingly, we observed a time-dependent decrease in activated, phosphorylated AKT (p-AKT) following N-t-boc-Daidzein treatment. Furthermore, we also observed a decrease in total AKT levels, suggesting that N-t-boc-Daidzein not only affects AKT phosphorylation and activation, but also triggers its degradation. This had downstream effects on other pro-survival pathways regulated by AKT, including the mTOR pathway. We measured the activity of p-p70 S6 kinase (p-p70S6K), one of the main effectors of the mTOR pathway, using western blot analysis and found that the level of p-p70S6K began to decrease after 24 h of treatment, with only a faint band remaining at 48 h (Fig. 3D).

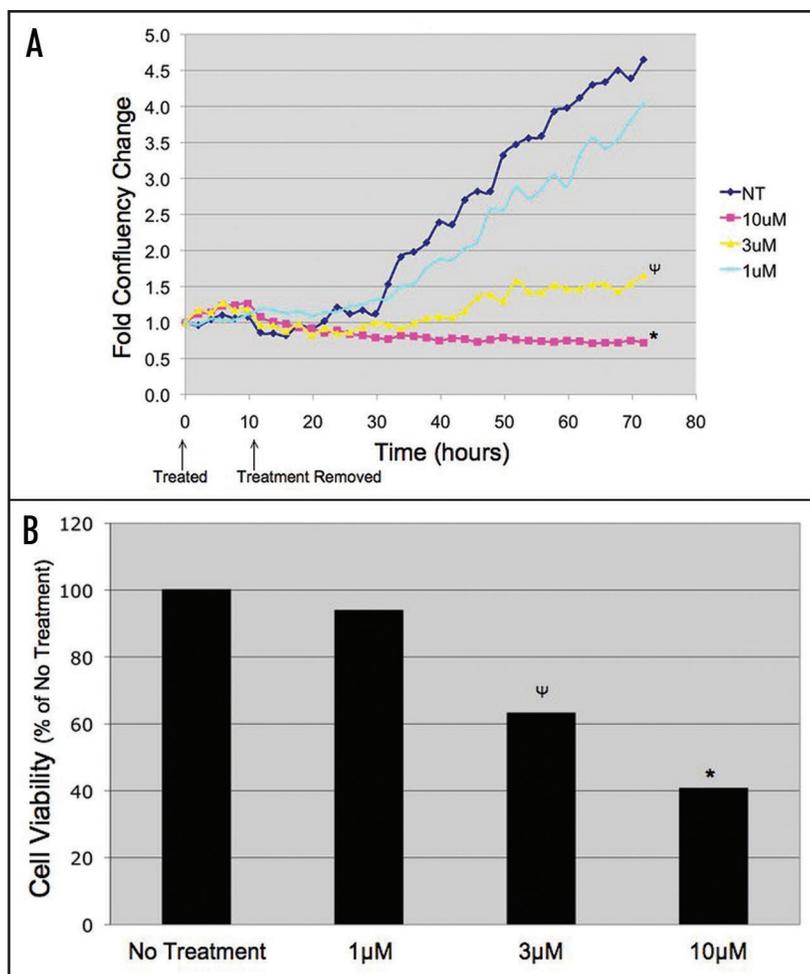


Figure 4. OCSCs failed to recover after a 12 h treatment with N-t-boc-Daidzein. OCSCs were treated with increasing concentrations of N-t-boc-Daidzein (0–10  $\mu$ M) for 12 h, after which media containing the compound was removed and replaced with regular growth media. (A) Fold confluency change over time was measured for the succeeding 60 h using real time in vitro micro-imaging. \* $p < 0.001$ ,  $^{\#}p < 0.01$ . (B) Cell viability was measured at the end of the 60 h recovery period using the CellTiter Assay. \* $p < 0.001$ ,  $^{\#}p < 0.01$ . Experiments were performed in triplicate and the averages were graphed. The results shown are for OCSC2. Similar results were observed with other lines tested.

**EOCs fail to recover from 12 h of N-t-boc-Daidzein treatment.** In order to get a better idea of how effective the drug would be in vivo where it is cleared from the blood, we designed an in vitro wash out system. OCSCs and mOCCs were treated with increasing concentrations (1–10  $\mu$ M) of N-t-boc-Daidzein for 12 h, after which we removed and washed out the compounds, replaced the cultures with regular growth media, and followed the cell confluence using the IncuCyte system. As shown in Figure 4A, OCSCs treated for 12 h with 3  $\mu$ M or greater N-t-boc-Daidzein still exhibited significantly decreased cell growth, even after 60 h of recovery. This gives promising results that the compound will be able to induce cell death prior to its clearance from the blood in vivo. These results were confirmed by cell viability testing after the 60 h recovery period. As shown in Figure 4B, there is a significant decrease in OCSC viability following 12 h of N-t-boc-Daidzein treatment and 60 h of recovery.

## Discussion

We demonstrate in this study that the novel compound N-t-boc-Daidzein is capable of inducing apoptosis in EOC cells, including the OCSCs. The drug exerts its effect through the activation of pro-apoptotic proteins, such as caspases-2, 3, 8 and 9, Bid and Bax, and the degradation of anti-apoptotic proteins XIAP and AKT (Fig. 5). Additionally, the failure of OCSCs to recover following 12 hours of treatment gives promising results for the compound's effectiveness in vivo.

Cancer recurrence is a major obstacle in the treatment of EOC, with over 80% of initially responsive tumors resulting in recurrence. This high rate of recurrence is thought to be due to the OCSCs, which are resistant to conventional treatments.<sup>17</sup> Cancer stem cells are defined as cells within the tumor that have the ability to self-renew and to differentiate into the heterogeneous cell types that make up the bulk of the tumor.<sup>18</sup> The initial response to chemotherapy that is seen in the majority of ovarian cancers is due to death of the bulk of the tumor cells, resulting in a decrease in tumor size, as evidenced by radiography tests and a downward trend in CA-125 levels. However, most of the CSCs survive the treatment and over time, they are able to regenerate the bulk of the tumor resulting in recurrence. Therefore, finding a compound that is capable of killing the OCSCs is extremely important to increasing overall survival and improving rates of tumor remission.

N-t-boc-Daidzein is a derivative of the isoflavone Daidzein. Isoflavones are plant hormones that regulate normal cell cycle kinetics in plants. Isoflavone derivatives have been shown to be effective in inducing cell death in many forms of cancers, including ovarian cancer.<sup>19</sup> However, many of these compounds have a very short half-life, and therefore, lose their efficacy once tested in humans.<sup>20</sup> The long alkyl chain on the Daidzein molecule in N-t-Boc-Daidzein imparts lipophilic character facilitating endocytosis and the N-t-Boc group at the end of the side chain provides metabolic stability, which we hypothesize will increase its efficacy in vivo.<sup>9</sup>

Our in vitro testing revealed that concentrations of 0.03  $\mu$ M and higher of N-t-boc-Daidzein lead to significant inhibition of OCSC growth, along with a significant decrease in cell viability. Morphologic changes typical of apoptosis, as well as the activation of a number of important caspases, including initiator caspases-2, -8 and -9 and the main effector caspase, caspase-3, proved that N-t-boc-Daidzein treatment is, in fact, activating the apoptotic cascade, resulting in cell death. These results confirmed those found in a preliminary study done by Kohen et al. which showed that N-t-boc-Daidzein was successful in inhibiting the growth of ovarian and colon cancer cells in vitro, as measured by [<sup>3</sup>H]-labeled thymidine incorporation into DNA.<sup>9</sup> They also showed that the action of the drug was inhibited by the addition

of the pan-caspase inhibitor Z-VADFK, supporting our findings that N-t-boc-Daidzein induces a caspase-dependent cell death.<sup>9</sup>

The earliest change in the regulatory proteins of the apoptotic cascade that we observed following *in vitro* treatment of OCSCs with N-t-boc-Daidzein was a decrease in XIAP levels, beginning after 8 hours of treatment. XIAP has been shown to bind directly to and block the activity of caspases-3, 7 and 9, thus inhibiting apoptosis.<sup>21</sup> There is substantial evidence that suggests that XIAP plays a role in chemo-resistance of ovarian cancer cells, with increased ovarian cancer cell death both *in vitro* and *in vivo* following downregulation of XIAP expression using antisense oligonucleotides.<sup>22,23</sup> Therefore, this early decrease in XIAP levels that we observed following N-t-boc-Daidzein treatment may be an important step in the induction of death in OCSCs.

We also observed a decrease in the amount of anti-apoptotic phosphorylated AKT and total AKT protein levels. It has been shown that AKT levels can decrease due to cleavage by caspases, degradation by the proteasome, or translational downregulation as a result of decreasing XIAP levels.<sup>24</sup> Our results show a reduction in both p-AKT and total AKT levels after 15 h of treatment with N-t-boc-Daidzein. This occurs prior to caspase activation, but following the downregulation of XIAP, suggesting that AKT is degraded by XIAP-related mechanisms or by proteasomal degradation. Along with supporting cell growth and survival, AKT has been found to play a role in promoting angiogenesis, increasing telomerase activity, improving cell motility, and altering cell-cell adhesion, all of which are important in tumor metastasis. Hence, N-t-boc-Daidzein's ability to promote the degradation of AKT may have significant implications in reducing tumor growth and spread.

In addition to these important changes observed in the apoptotic pathway in response to N-t-boc-Daidzein treatment, alterations in the mTOR survival pathway were also observed. Levels of p-p70S6K, an important effector kinase in the mTOR pathway, decreased significantly after 24 h of treatment. Previous studies have found that increased nuclear expression of p-p70S6K was associated with malignant ovarian tumors, when compared to benign or borderline tumors.<sup>25</sup> Thus, the ability of N-t-boc-Daidzein to decrease p-p70S6K levels may be important in diminishing tumor aggressiveness and promoting cancer cell death.

In addition to these promising *in vitro* results, preliminary animal studies done by Kohen et al. showed a reduction in tumor size with N-t-boc-Daidzein treatment in an *in vivo* model of ovarian cancer in CD1 nude mice.<sup>9</sup> Furthermore, they found that there was no difference in weight reduction or death of mice

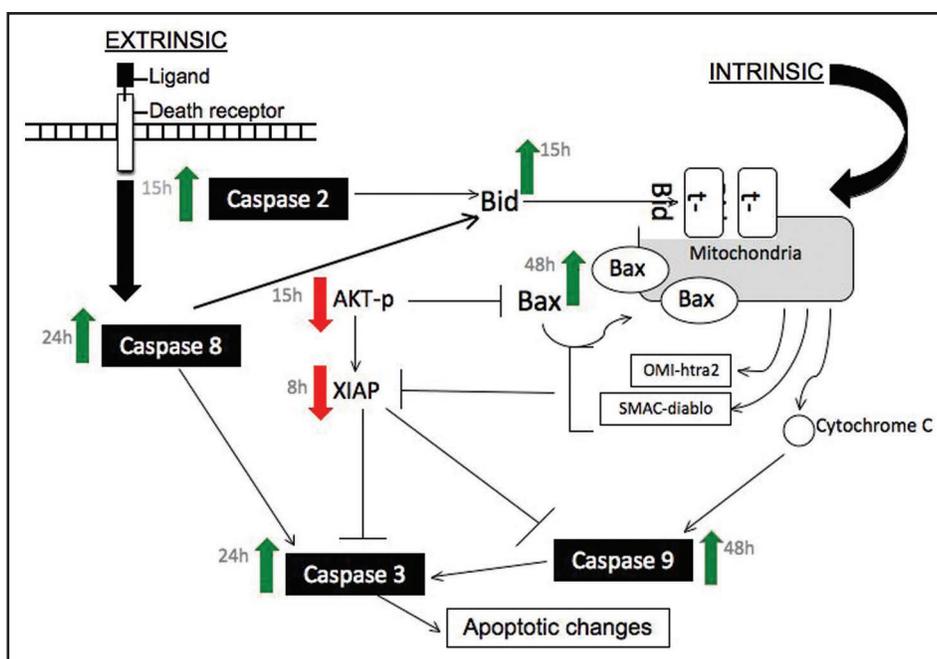


Figure 5. Proposed mechanism for the induction of apoptosis in EOC cells by N-t-boc-Daidzein. N-t-boc-Daidzein induced apoptosis is characterized by activation of caspases-2, 3/7, 8 and 9, downregulation of XIAP, dephosphorylation of Akt and S6 kinase, activation of Bid and Bax, and mitochondrial depolarization.

between the treatment and control groups, suggesting that the drug was not toxic to normal cells and was well tolerated *in vivo*.<sup>9</sup> Our next step is to perform additional animal studies to assess the effectiveness of N-t-boc-Daidzein in killing the OCSCs *in vivo* and also to provide additional toxicology studies.

In summary, our results show that N-t-boc-Daidzein is able to successfully induce apoptotic cell death in OCSCs, which are extremely resistant to conventional chemotherapy agents. These results have two major implications. First, despite previous reports, which show that cancer stem cells in general are resistant to apoptosis, our data show that OCSCs can be induced to undergo apoptosis. Second, we show that a naturally occurring compound is potent enough to activate the caspases in OCSCs *in vitro*. The increased stability that the N-t-boc hexylene diamine side chain confers may be vital to improving the effectiveness of this compound on OCSCs *in vivo*. The failure of cells to recover following 12 h of treatment with N-t-boc-Daidzein provides promising evidence for its ability to initiate the apoptotic cascade in OCSCs *in vivo*, prior to its clearance from the blood. As mentioned above, our next objective is to demonstrate if the potency of this compound against OCSCs is reflected *in vivo*. The ability to kill OCSCs *in vivo* is vital to decreasing rates of recurrence and improving overall survival in patients with EOC.

## Materials and Methods

**Cell lines and culture conditions.** Primary EOC cell lines were isolated from malignant ovarian ascites and cultured as previously

described.<sup>26</sup> Cell lines were propagated in RPMI plus 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA).

**Growth curve and cell viability assay.** Cells (4,500 cells/well for OCSCs and 8,000 cells/well for mOCCs) were plated in a 96-well plate. After 24 h, the medium was replaced with OptiMem (Gibco, Invitrogen, Carlsbad, CA) for 4 h followed by treatment. Growth curves were determined by placing cells in the Incucyte system (Essen Instruments, Ann Arbor, MI), which allows an automated and non-invasive method of monitoring live cells in culture. Growth curve is constructed automatically from data points acquired during round-the-clock kinetic imaging. Parallel plates were prepared for cell viability assays where 20  $\mu$ L CellTiter Assay (Promega, Madison, WI) was added to each well and optical density acquired after 1 h at 490 nm. Cell viability was normalized as a percentage of the untreated cells. Each experiment was done in triplicate and the results were averaged.

**Caspase activity assay.** The activity of caspases-3, -8 and -9 was measured using Caspase-Glo<sup>TM</sup> 3/7, 8 and 9 (Promega, Madison, WI), respectively, as previously described<sup>11</sup> using 5  $\mu$ g protein in a 50  $\mu$ L total volume. Fold-increase in activity was calculated based on the activity measured in the untreated cells.

**SDS-PAGE and western blots.** SDS-PAGE and western blots were performed as previously described<sup>11</sup> using the following antibodies: mouse anti-caspase-2 (BD Biosciences, San Jose, CA 1:1,000), rabbit anti-Bid (Cell Signaling, Beverly, MA, 1:1,000), mouse anti-XIAP (BD Biosciences, 1:1,000), rabbit anti-phosphorylated Akt (Cell Signaling, 1:1,000), rabbit anti-Akt (Cell Signaling, 1:1,000), mouse anti-Bax (BD Biosciences, 1:250), anti-phosphorylated p70 S6 Kinase (Millipore, Billerica, MA, 1:2,000), and rabbit anti-actin (Sigma, St. Louis, MO 1:10,000).

**Mitochondrial depolarization assay.** After treatment, cells were trypsinized and stained with JC-1 dye using the Mitocapture<sup>TM</sup> mitochondrial apoptosis detection kit (BioVision Research Products Mountain View, CA) according to the manufacturer's instructions. Data was gathered using the FACS Calibur System and analyzed using CellQuest software (BD Biosciences, Rockville, MD).

**Statistical analysis.** The two-sided t test was used to determine statistical differences between treatment and control groups at the end of treatment.  $p < 0.05$  was considered statistically significant.

#### Acknowledgements

This study was supported in part by grants from NCI/NIH RO1CA127913 and RO1 CA118678; The Janet Burros Memorial Foundation, the Sands Foundation, and the Discovery To Cure Research Program. J.M.G. is the recipient of the Doris Duke Clinical Research Fellowship from the Doris Duke Charitable Foundation.

#### Note

Supplementary materials can be found at:  
[www.landesbioscience.com/supplement/GreenCBT8-18-Sup.mov](http://www.landesbioscience.com/supplement/GreenCBT8-18-Sup.mov)

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