Effect of Betulinic Acid on Apoptosis in *Caenorhabditis elegans*

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Abstract

Apoptosis is a mechanism of programmed cell death often dysregulated in neurological diseases and cancer. Betulinic acid (BetA), derived from white birch (*Betula pubescens*), induces apoptosis in sixteen cancer cell lines, including drug resistant lines. BetA is hypothesized to induce apoptosis by antagonizing anti-apoptotic Bcl-2 family proteins in a context-dependent manner. I used *ced-1 C. elegans*, which have a genetically sensitive background to apoptosis, to determine BetA's effect on apoptosis. To determine if BetA targets CED-9, I used *ced-1; ced-9(ts)* worms, which have an inactive Bcl-2 homologue CED-9. I used this information to investigate how BetA selectively induces cancer cell death. I quantified apoptotic cells in the germline of *C. elegans* treated with BetA or a DMSO control. My prediction was that BetA would increase the number of apoptotic cells relative to the control in *ced-1* worms, but that it would have no effect in *ced-1; ced-9(ts)* worms. BetA was not found to significantly increase apoptosis in the *ced-1* or the *ced-1; ced-9(ts)* background. These results indicate that BetA had no effect on apoptosis in *C. elegans*, possibly due to binding dependence with the C-terminal of Bcl-2, which varies in the homologue CED-9. Further experimentation is warranted in a *C. elegans* system where Bcl-2 replaces CED-9 to determine BetA effects concerning Bcl-2.

Introduction

Betulinic acid (3b-Hydroxy-lup-20(29)-en-28-oic acid) (BetA) is a naturally occurring pentacyclic lupane-type triterpenoid originally derived from white birch (*Betula pubescens*) as betulin (lup-20(29)-ene-3β,28-diol), which composes up to 30% of the dry weight of the extract (Pisha *et al.* 1995, Green *et al.* 2007). Betulin and BetA is widely distributed through the plant kingdom and may be isolated from *Tryphyllum peltatum, Ancistrocladus heyneaus, Diospyros* spp, *Ziziphus* spp, *Syzygium* spp, *Paeonia*, and *Tetracera boliviana* (Kim *et al.* 2001, Mukherjee *et al.* 2006, Zuco *et al.* 2002, Cichewicz and Kouzi 2004, Yogeeswari and Sriram 2005). BetA is derived from betulin in a simple two-step process (Cichewicz and Kouzi 2004) involving the substitution of a carboxyl for a hydroxyl group on C-28 (Fig. 1). Betulin has no reported medical properties, unlike BetA, indicating that BetA's effects rely on the carboxyl group on C-28 (Mukherjee *et al.* 2006). BetA is reported to have anti-inflammatory properties countering skin inflammation and ear edema in mice via the inhibition of phospholipase A_2, and antiviral, antiseptic, antimalarial, weak antimicrobial, antileshmanial, antihelmentic, and antifeedant activities in other model systems (Sami *et al.* 2006, Yogeeswari and Sriram 2005, Cichewicz and Kouzi 2004). The two properties of BetA most studied are its anti-HIV and anticancer activity. BetA inhibits HIV-1 replication by preventing syncytium formation between HIV-1-infected cells and uninfected CD4 cells, disrupting the cellular entry of HIV-1 (Cichewicz and Kouzi 2004, Mayaux *et al.* 1994). BetA exhibits cytotoxicity towards human melanoma (Pisha *et al.* 1995), neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor, leukemia, and several carcinomas of various tissues including head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian, and cervix. Moreover, BetA limits the growth of drug resistant cell

lines (Fulda 2008). BetA is not cytotoxic or cytostatic towards normal cell lines and has been found to be highly safe even at the dose of 500mg/kg body weight (Zuco *et al.* 2002, Cichewicz and Kouzi 2004). BetA's cytotoxicity involves the activation of cellular apoptotic (programmed cell death) pathways. It has been found to activate two human apoptotic pathways: the mitochondrial apoptotic pathway and NF-κB pathway (Fulda 2008). It induces apoptosis independently of CD95 (AP0-1/FAS) and p53 (Walczak and Krammer 2000, Mukherjee *et al.* 2006).

Apoptosis is activated through the extrinsic and intrinsic pathways. Extrinsic apoptosis is mediated by death receptors, which are part of the tumor necrosis factor receptor gene superfamily, and which include TNFR-1, Fas/CD95, and Trail receptors DR-4 and DR-5 (Ashkenazi 2002). Signals sent from the death receptors are amplified via the mitochondrial apoptotic pathway (Fig. 2) by tBid, which changes the permeability of the outer mitochondrial membrane. Intrinsic apoptosis is induced by internal factors including DNA damage, oxidative stress, starvation, and chemotherapeutic drugs. Intrinsic apoptosis is induced by a disruption of the mitochondrial outer transmembrane potential, through the opening of permeability transition pores, changing the permeability of the outer membrane, and releasing cytochrome c, AIF (apoptosis inducing factor), the endonuclease endoG, Smac/Diablo, and Htr/Omi into the cytoplasm (Adams and Cory 2007). The changes in the outer mitochondrial membrane leads to reduced ATP synthesis, a cytoplasmic increase in the redox molecules NADH and NADPH, oxidation of glutathione (which normally protects the cell from free radicals), and synthesis of reactive oxygen species (ROS). ROS oxidate lipids, proteins, and nucleic acids in the mitochondrial membrane creating a positive feedback loop. Mitochondrial membrane potential is regulated by the Bcl-2 family proteins, which are defined by Bcl-2 homology domains (BH1, BH2, BH3, BH4) and are composed of prosurvival proteins and proapoptotic proteins. Prosurvival proteins include Bcl-2, Bcl-X_l, Bcl-w, A1, and Mcl-1 and consist of all four homology domains. Proapoptotic proteins are divided into the Bax subfamily, which are found on the mitochondria, and BH3 only proteins. The Bax subfamily (BH1, BH2, and BH3) are normally inhibited by the prosurvival proteins and include Bax, Bak, and Bok. BH3 only proteins, when activated by transcriptional upregulation, subcellular relocalization, dephosporylation, or proteolysis, inhibit the prosurvival proteins and allow the Bax subfamily to form pores and change the membrane permeability of the mitochondrial membrane. BH3 only proteins include Bid, Bim, Bik, Bad, Bmf, Hrk, Nova, Puma, Blk, BNIP3, and Spike (Galluzzi *et al.* 2006, Saelens *et al.* 2004).

The other apoptosis pathway affected by BetA is the NF-κB pathway. DNA damage activates NF-κB (Dobrovolskaia and Kozlov 2005). NF-κB leads to inflammation, the synthesis of ROS, cytokines and chemokines including TNF, lymphotoxins, IL-6, and IL-8, and growth and angiogenic factors (Dobrovolskaia and Kozlov 2005). NF-κB can lead to malignant proliferation, the prevention of apoptosis, and an increase in metastasis and angiogenesis (Karin 2006, Naugler and Karin 2008). NF-κB consists of 5 homologous subunits (RelA/p65, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2); these normally dimerize and are held in the cytoplasm by IκBs (inhibitors of NF-κB). IκB kinase (IKK), which is composed of 2 catalytic units (IKKα and IKKβ)

and one regulatory unit (IKKγ/NEMO), phosphorylizes IκB, allowing NF-κB to enter the nucleus and act as a transcription factor (Naugler and Karin 2008). Two activation pathways of NF-κB related to cancer have been described (Keats *et al*. 2007, Annunziata *et al*. 2007, Dobrovolskaia and Kozlov 2005). The first, canonical, is activated in response to microbial, viral infections, and pro-inflammatory mediators. These activate NEMO and IKKβ, leading to inflammation. The noncanonical pathway is hypothesized to be stimulated by kinase NIK, which activates IKKα, leading to lymphoid organogenesis (Naugler and Karin 2008). NF-κB is thought to inhibit apoptosis via the activation of members of the Bcl-2 family (Bcl-X_L and Bcl-2), MnSOD, c-IAP, and cFLIP. It neutralizes ROS, decreasing the efficiency of mitochondrial apoptosis. NF-κB's inhibition of apoptosis leads to cancer cell growth, but can limit cancer growth in epidermal neoplasia and squamous cell carcinoma by decreasing proliferation in response to cell death (Karin 2006, Dobrovolskaia and Kozlov 2005). Therefore, NF-κB can act both as a pro-apoptotic and an anti-apoptotic protein.

Andre *et al.* 2002 found that mitochondria treated with BetA released cytochrome *c* independently of downstream caspases and dependent on the permeability of the transition pore. Yogeeswari and Sriram 2005 confirmed these results. BetA releases cytochrome *c* and AIF (apoptosis inducing factor), leading to the cleavage of caspase-8 and caspase-3, and producing ROS, possibly affecting the permeability of the outer mitochondrial membrane (Fulda 2008, Yogeeswari and Sriram 2005, Mullauer *et al.* 2009, Wick *et al.* 1999). BetA's activation of the mitochondrial apoptotic pathway is reportedly based on its modulation of expression levels of different Bcl-2 family proteins, although Mullauer *et al.* 2009 reports one case of independent effects. Members of the Bcl-2 family affected by BetA include Bax, Bcl-X_S, Bcl-2, Mcl-1, and BetA's effect on each member differs based on the type of carcinoma being treated (Fulda 2008). The other apoptotic pathway BetA has been reported to effect is the NF-κB pathway (Fulda 2008). According to Kasperczyk *et al.* 2005, BetA activates NF-κB via increasing IKK activity in cancer cell lines. However, BetA also interferes with NF-κB activation in response to carcinogens and inflammatory stimuli (Fulda 2008, Takada and Aggarwal 2003, Rabi *et al.* 2008). In this experiment, I will be using a *Caenorhabditis elegans* model to determine how BetA demonstrates context-dependent activities within different types of carcinomas and why BetA induces apoptosis in carcinomas, not normal cells.

Even though all known experiments concerning betulinic acid's cytotoxic effects have used human cell lines and mice carcinomas *in vivo,* in this experiment, I will use *C. elegans* as a model organism. Its mapped genome and available mutations allows for the manipulation of genes and proteins. *C. elegans* also provides a simpler system than a human cell. Worms have a short lifecycle and a large number of progeny, and may replicate either sexually or asexually, preventing genetic recombination. *C. elegans* also allows for easy observation of apoptosis, which may be directly observed in the germ or somatic cell line (Saito and Heuvel 2002). *C. elegans* also allowed for a forward genetics approach (introducing chemicals and observing random mutations) rather than reverse genetics (removing gene and investigating the mutant) for the identification of proteins integral to the apoptosis pathway. Therefore, promoters, inhibitors, and proteins have been investigated in terms of cancer in *C. elegans*. Apoptosis in *C. elegans* follows three

stages: specification, killing, and execution (Fig. 4). Both germline and embryonic apoptosis require EGL-1, CED-13, CED-9, CED-4, CED-3, and CED-1 (proteins necessary for the killing and execution stages) (Fig. 3). These proteins and their corresponding genes have human homologous: EGL-1 and CED-13 (BH3), CED-9 (Bcl-2), CED-4 (Apaf-1), and CED-3 (caspases). Proteins involved in specification, including CEP-1 and CES-1 vary between embryonic and germline apoptosis (Conradt and Xue 2005, Gartner *et al*. 2008). Betulinic acid has been hypothesized to target one of the Bcl-2 family receptors causing apoptosis in cancer cell lines (Fulda 2008). If this hypothesis describes betulinic acid's actions accurately and CED-9 mimics Bcl-2 in *C. elegans* cells, I treat *ced-1(e1735)* and *ced-1(e1735); ced-9(n1653ts)* worms with BetA and a DMSO control, and I compare the numbers of apoptotic cells in the germline and the embryonic cell line, then the numbers of apoptotic cells will be significantly higher in *ced-1* treated with BetA than *ced-1* treated with the DMSO control, while all treatments in *ced-1; ced-9(ts)* worms will have statistically indistinguishable numbers of apoptotic cells.

Materials and Methods

Chemical treatment of Caenorhabditis elegans

BetA was dissolved in DMSO, then diluted in oil to 20μg/mL and DMSO was dissolved in oil as a control (20μg/mL). 2mL of oil was placed on each Petri dish. This method of treatment was nontoxic and ensured a stronger treatment than dissolving BetA in DMSO then in the agar. BetA exhibits limited solubility in organic alcohols such as MeOH, EtOH, $CHCl_3$, and ether, a low solubility in polar solvents including H_2O, petroleum ether, DMF and benzene, and high solubility in pyridine and acetic acid (Cichewicz and Kouzi, 2004). However, experiments examining the cytotoxicity of BetA for human cancer cells use DMSO as a solvent almost exclusively (Zuco *et. al*., 2002; Liby *et. al*., 2007; Melzig and Bormann, 1998; Pisha *et. al*., 1995). DMSO may disrupt solidification of the agar and is reported to have negative consequences on *C. elegans* (Goldstein and Magnano 1988, Anderson *et al.* 2003), so the smallest concentration of DMSO is preferred. Research investigating BetA's cytotoxic and cytostatic properties in human and vertebrate cell lines use a concentration from 0.03 to 20μg/mL BetA, stabilized in DMSO, and dissolved in the matrix used for culturing cells (Fulda and Debatin 2005; Fulda 2008; Zuco *et al.* 2002; Pisha *et al*. 1995; and Mukherjee *et al.* 2006). Therefore, I used 20μg/mL BetA, which treated the worms, yet did not kill them.

C. elegans *procedures*

C. elegans were cultured on a Petri dish containing E. coli OP50, a uracil auxotroph with limited growth on NGM plates, providing nutrients for the nematodes and providing better observation and mating of the worms. Worms were maintained by standard procedures (Brenner 1974, Schertel and Conradt 2007). The wild-type strain was N2. *C. elegans* is classified as BSL-1 and will be obtained from Caenorhabditis Genetic Center. E. coli OP50 will be obtained from the Department of Molecular, Cellular, and Developmental Biology at University of Colorado at Boulder. E. coli OP50 is classified as BSL-1 and will be handled as such. Neither organisms may be considered harmful, and both will be handled and disposed of as described in Wormbook.

Determining effect of betulinic acid on apoptosis

I examined apoptosis in the germline and somatic cells in embryos, and I compared apoptosis in *C. elegans* treated and untreated with BetA. The development of *C. elegans* in optimum conditions from a zygote to a reproducing adult requires an average of 55 hours. The embryo develops in 9 hours from a gastrula, to a comma, to 2-fold, 2.5-fold, 3 fold, 4 fold, then hatches to become an L_1 adult. An L_1 adult then develops into a L_2, L_3, L_4, young adult, then a reproducing adult in the remaining 46 hours (WormBook). Apoptosis occurs naturally in embryonic cell development and may be observed with using DIC microscopy using a light microscope by collecting slides of embryos (Fig. 9, obtained from Conradt and Xue 2005). Slides were prepared on an agar buffer with 8μL of 25mM sodium azide, which euthanizes *C. elegans*. I scored the number of apoptotic cells in treated and untreated embryos across all six stages of development. To score apoptosis in the germline, I counted the number of apoptotic cells in the gonad arm (Gumienny *et al.* 1999). Germ cells may naturally acquire genetic mutations through non-disjunction; therefore they may be more representative of a human cancer cell and I was more likely to observe an effect of BetA on apoptosis in the germline in *C. elegans*. To score germline apoptosis, two generations of worms were cultured and treated, then L_4 worms were picked and apoptotic cells were scored 24 and 48 hours post the L_4 stage. The strain of *C. elegans* which I used in this part of the experiment were *ced-1(e1735)* and *ced-1(e1735); ced-9(n1653ts),* which are engulfment mutants. Engulfment mutants increase the number of observable apoptotic cells, increasing the sensitivity of the system. *ced-1;ced-9(ts)* provides a highly sensitized system without an active CED-9 (Bcl-2 homologue) at temperatures higher than 24°C (Wormbase).

Statistics

I used a Student's t-test with an $\alpha=0.05$ comparing the number of cells which underwent apoptosis in each treatment to determine whether BetA has an effect on apoptosis in the germline. I performed a Mann-Whitney U test ($\alpha=0.05$) for experiments examining the effect of BetA on apoptosis in the somatic cell line in embryos because the sample size was smaller and was not indicated to come from a Gaussian distribution.

Results

BetA had no statistically significant effect on germline apoptosis in *ced-1(e1735)* worms at 24 and 48 hours-post the L_4 stage. Worms were cultured at 25°C for 24 hours, then counted at 24hr post- L_4, then cultured at 20°C for 24 hours, then counted at 48hr post- L_4. These data were obtained in a 6-blind test, where 6 slides were obtained, containing at least one control and at least one treatment. At 24-hr post- L_4, the mean number of germline apoptotic cells in worms treated with BetA dissolved in DMSO in oil (20μg/mL) was 2.654 (SD=2.208) and was 3.481 (SD=2.082) for a DMSO control in oil. A t-test indicated that the means were statistically indistinguishable, and that BetA had no effect on germline apoptosis ($t_{obs}=1.403$, $p=0.167$, $n_1=27$, $n_2=26$). At 48-hr post- L_4, the mean number of germline apoptotic cells in worms treated with BetA dissolved in DMSO in oil (20μg/mL) was 40.296 (SD=8.085) and was 36.063 (SD=9.922) for a DMSO control in oil. A t-test again indicated that the means were statistically indistinguishable,

and that BetA had no effect on germline apoptosis (t_{obs}=1.806, p=0.0763, n_1=32, n_2=27) (Fig. 5).

BetA was also found to have no statistically significant effect on germline apoptosis in *ced-1(e1735); ced-9(n1653ts)* worms at 24 and 48 hours-post the L_4 stage. Worms were exposed for at least 96 hours. These data were obtained in a 4-blind test, where 4 slides containing at least one treatment and one control were counted. At 24-hr post-L4, the mean number of germline apoptotic cells in worms treated with BetA dissolved in DMSO in oil (20µg/mL) was 12.905 (SD=4.182) and was 13.591 (SD=3.432) for a DMSO control in oil. A t-test indicated that the means were statistically indistinguishable, and that BetA had no effect on germline apoptosis in *ced-1; ced-9 (ts)* (t_{obs}=0.587, p=0.561, n_1=22, n_2=21). At 48-hr post- L_4, the mean number of germline apoptotic cells in worms treated with BetA dissolved in DMSO in oil (20µg/mL) was 41.571 (SD=7.193) and was 44.536 (SD=9.070) for a DMSO control in oil. A t-test again indicated that the means were statistically indistinguishable, and that BetA had no effect on germline apoptosis (t_{obs}=1.355, p=0.181, n_1=28, n_2=28) (Fig. 6).

BetA had no statistically significant effect on somatic cell apoptosis, observed in embryonic cell death, in *ced-1(e1735)* worms. Apoptosis was documented in at least six embryonic stages: comma/bean, 1-fold, 2-fold, 2.5-fold, 3-fold, and 4-fold. Worms were exposed to BetA dissolved in DMSO in oil (20µg/mL) or DMSO in oil for 36 hours. These data were obtained in a 4-blind test, where 4 slides containing at least one treatment and one control were counted. The mean number of apoptotic cells for BetA was 26.09 (SD=4.011), 34.8 (SD=3.084), 28.6 (SD=3.169), 24.6 (SD=2.797), 23.27 (SD=3.552), and 17.71 (SD=2.289), for each stage, respectively. The mean number of apoptotic cells for DMSO was 28 (SD=3.621), 34.6 (SD=3.836), 25.6 (SD=3.239), 25.4 (SD=1.838), 22 (SD=3.127), 18 (SD=1.449). A Mann-Whitney U test indicated that BetA had no effect on apoptosis in any of the stages: U=73.0, p=0.223; U=52.5, p=0.853; U=75.0, p=0.0630; U=63.0, p=0.353; U=64.0, p=0.556; U=18.0, p=0.999 (Fig. 7).

BetA had no statistically significant effect on somatic cell apoptosis, observed in embryonic cell death, in *ced-1(e1735); ced-9(n1653ts)* worms. Apoptosis was documented at comma/bean, 1-fold, 2-fold, 2.5-fold, and 3-fold. Worms were exposed to BetA dissolved in DMSO in oil (20µg/mL) or DMSO in oil for 48 hours and were incubated at 25°C for 24 hours. These data were obtained in a 4-blind test, where 4 slides containing at least one treatment and one control were counted. The mean number of apoptotic cells for BetA was 27.5 (SD=2.68), 29.8 (SD=3.65), 24.1 (SD=3.93), 22.2 (SD=4.37), and 21.8 (SD=3.96) for each stage, respectively. The mean number of apoptotic cells for DMSO was 27.4 (SD=2.37), 30.4 (SD=1.72), 26.8 (SD=4.74), 21.6 (SD=3.74), and 18.7 (SD=2.69). A Mann-Whitney U test indicated that BetA had no effect on apoptosis in any of the stages: U=51.0, p=0.971; U=36.5, p=0.887; U=59.0, p=0.278; U=44.5, p=0.696; U=26.0, p=0.202 (Fig. 8).

Discussion and Conclusions

These data indicate that BetA does not induce apoptosis in *C. elegans*, indicating that BetA targets a protein which has no homologue in *C. elegans* or that BetA targets a specific domain which differs between the *C. elegans* and human homologues. Because BetA has been hypothesized to target Bcl-2 family proteins in human cancer cells (Fulda 2008) and because CED-9 and Bcl-2 differ strongly on the C-terminal (α3, α6, and α7), I hypothesize that BetA's effect on human cancer tissue relies on the C-terminal of Bcl-2. Further study using a mutant of *C. elegans* where CED-9 is replaced by Bcl-2 is warranted to determine BetA's reliance on the C-terminal of Bcl-2. Though this explanation is likely, BetA might target a protein without a *C. elegans* homologue. Possible proteins include a majority of the BH3 family (Puma, Noxa, Mcl-1, and Bcl-X_L), which have few homologues in the *C. elegans* apoptotic pathway, and NF-κB, which does not have a *C. elegans* homologue and is often dysregulated in cancers and inflammation. BetA might also act as a transcription factor: if BetA influences expression of Bcl-2 family proteins as a promoter or inhibitor in DNA, rather than directly binding to Bcl-2 family proteins, its effects will be negated in an organism with strong differences in DNA transcriptional organization. BetA could affect ROS, which has been reported in Wick, *et al*. 1999, either by synthesizing them or by amplifying their effects. Finally BetA could induce non-classical apoptosis, in which it would enter the outer mitochondrial membrane, opening the permeability transition pores, inducing apoptosis (Mullauer *et al*. 2009). Unfortunately, little is known about the direct function of the mitochondria in apoptosis in *C. elegans*, though CED-9 is known to co-localize on the surface of the mitochondria with CED-4 and mediators of DNA fragmentation, CPS-6 and WAH-1, are known to be released from the mitochondria (Conradt and Xue 2005). However, the apoptotic function of mitochondrial membrane permeability may vary between *C. elegans* and humans, resulting in a loss of BetA's effect in *C. elegans* if BetA relies on ROS or non-classical apoptosis.

Possible sources of error in this experiment include the use of oil as method of worm exposure and the lack of certainty regarding treatment effectiveness. The worms are treated with BetA in DMSO or DMSO dissolved in oil, which then covers the agar. This method of treatment increases the chance that the worms maintain exposure to the treatment, yet it also introduces an apoxic environment. Apoxia slows the aging process, changing the time points used to count germline apoptosis. Gumienny *et al*. 1999 counted germline apoptosis at 24hr and 48hr post- L_4, yet 24hr and 48hr post- L_4 in worms under normal conditions are different from those in apoxic worms. These data, as a result, are difficult to compare to reported data, removing a control. I am unable to ensure that other confounding variables, normally determined through controls which are compared to reported data, are not affecting apoptosis. Furthermore, the worms ingest oil particles, changing the appearance of the worm, introducing error into scoring of apoptotic cells. The oil reduces the resolution power on microscope when DIC microscopy is used, so these data are less precise and therefore the experiment is less sensitive to slight effects of BetA. Even with the use of oil, I cannot ensure that the worms and the embryos are treated. BetA might slowly precipitate out of oil, reducing the treatment concentration and reducing the effects of BetA on apoptosis. Because only mammals have been treated

with BetA, the concentration I used might not be strong enough to document an effect, possibly explaining the slight, though statistically indistinguishable, effects seen in Fig. 5 and Fig. 7. The embryos are protected by a chitinous shell; therefore, even if BetA is in high enough concentrations to effect the worms, it might not effect the embryo. Even if BetA induced apoptosis in somatic cells, I would not observe an effect using this procedure.

Further experiments are suggested to test the hypothesis that BetA's effect relies on terminal-C of Bcl-2 and to determine the mechanism of BetA's selective cytotoxicity. An experiment as follows is suggested: *C. elegans* mutants, where Bcl-2 replaces CED-9, should be treated with BetA and a DMSO as described above and both germline and embryonic apoptosis should be scored. If BetA targets the C-terminal of Bcl-2, an increase in apoptosis will be observed in worms treated with BetA dissolved in DMSO. If BetA continues to have no effect on apoptosis, either germline or embryonic in both *ced-1* and *ced-1; ced-9(ts),* then experiments reported and suggested here are suggested to be repeated using pyridine as the vehicle of exposure as opposed to DMSO. Pyridine has a weaker effect on *C. elegans* and might not reduce BetA's effect on apoptosis.

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Appendix

Figure 1. Betulin and BetA.

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Figure 2. Apoptosis Pathway: A General Overview. Obtained from Calbiochem.

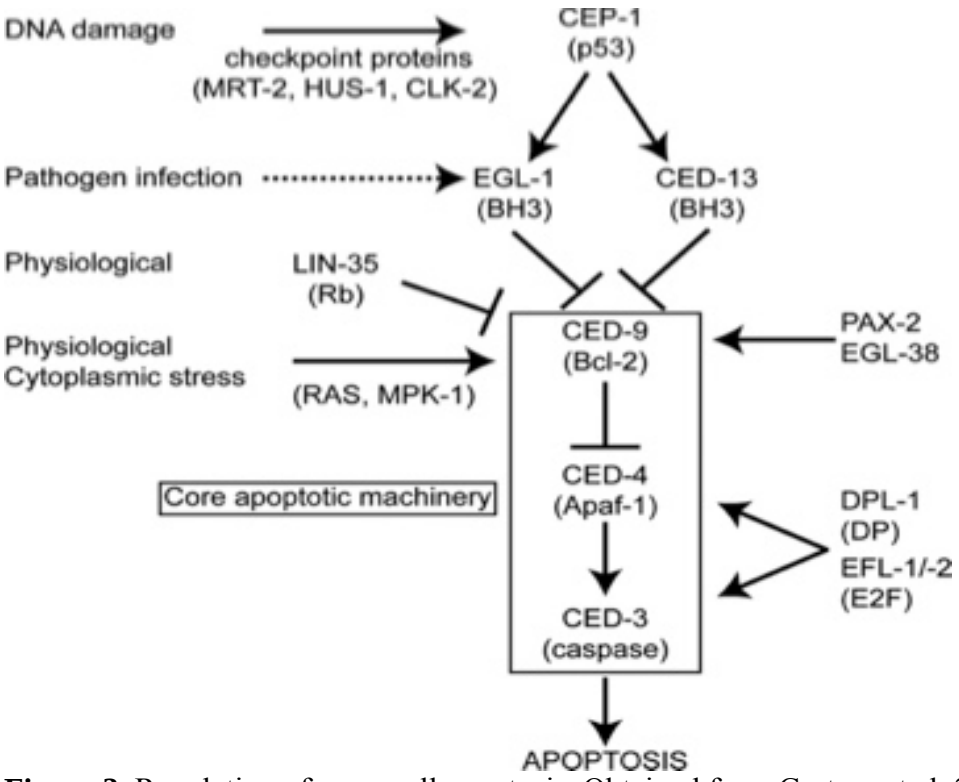

Figure 3. Regulation of germ cell apoptosis. Obtained from Gartner et al. 2008. Human homologues are shown in parentheses.

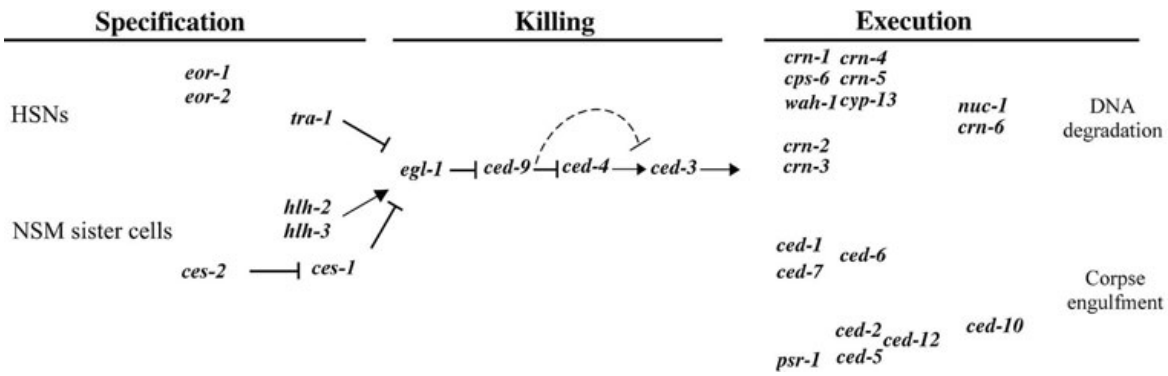

Figure 4. Genes regulating embryonic apoptosis, obtained from Conradt and Xue 2005.

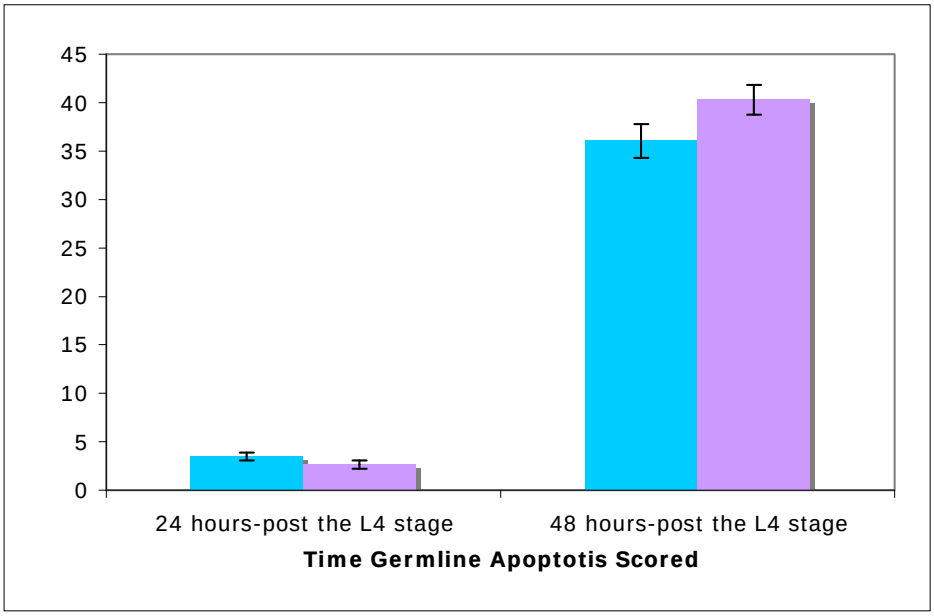

Figure 5. The effect of betulinic acid on germline apoptosis in *ced-1* worms at 24 and 48 hours-post the L_4 stage. Germline apoptosis score for worms treated with either BetA in DMSO in oil (20µg/mL) or DMSO in oil. Error bars indicate the standard error of the mean. The blue bars indicate DMSO control; the purple bars indicate BetA and DMSO treatment. P-values from t-tests indicate BetA had no significant effect on germline apoptosis (t-test for 24hrs post L4 p=0.167, t-test for 48hrs post L4p=0.0763). Bars have N=27, 26, 32, 27 from left to right.

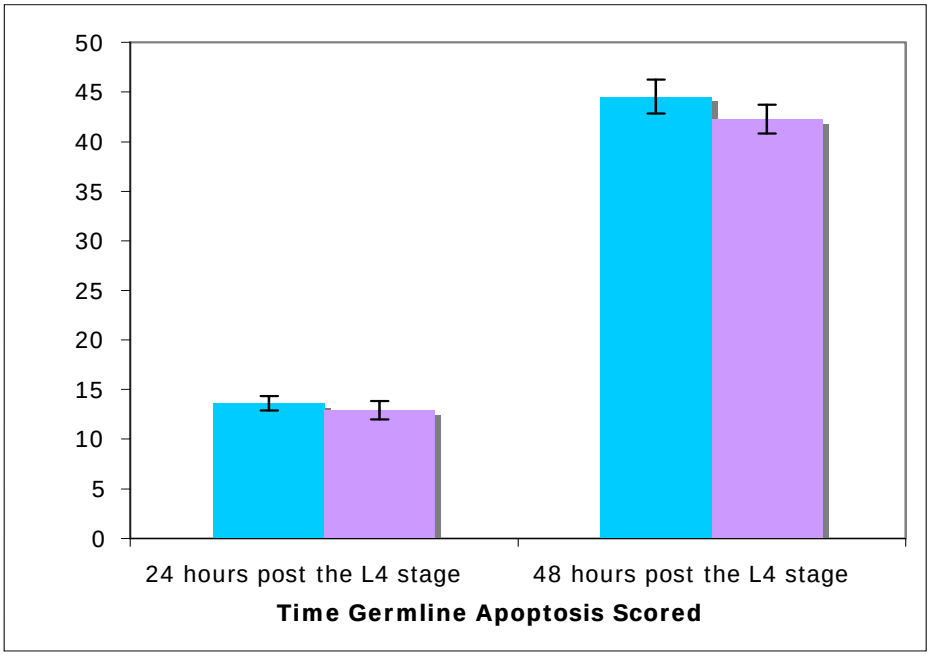

Figure 6. The effect of betulinic acid on germline apoptosis in *ced-1; ced-9(ts)* worms at 24 and 48 hours-post the L_4 stage. Germline apoptosis score for worms treated with either BetA in DMSO in oil (20µg/mL) or DMSO in oil. Error bars indicate the standard error of the mean. The blue bars indicate DMSO control; the purple bars indicate BetA treatment. P-values from t-test indicate BetA had no significant effect on germline apoptosis (t-test for 24hrs post L4 p=0.561, t-test for 48hrs post L4 p=0.181). Bars have N=21, 22, 28, 28 from left to right.

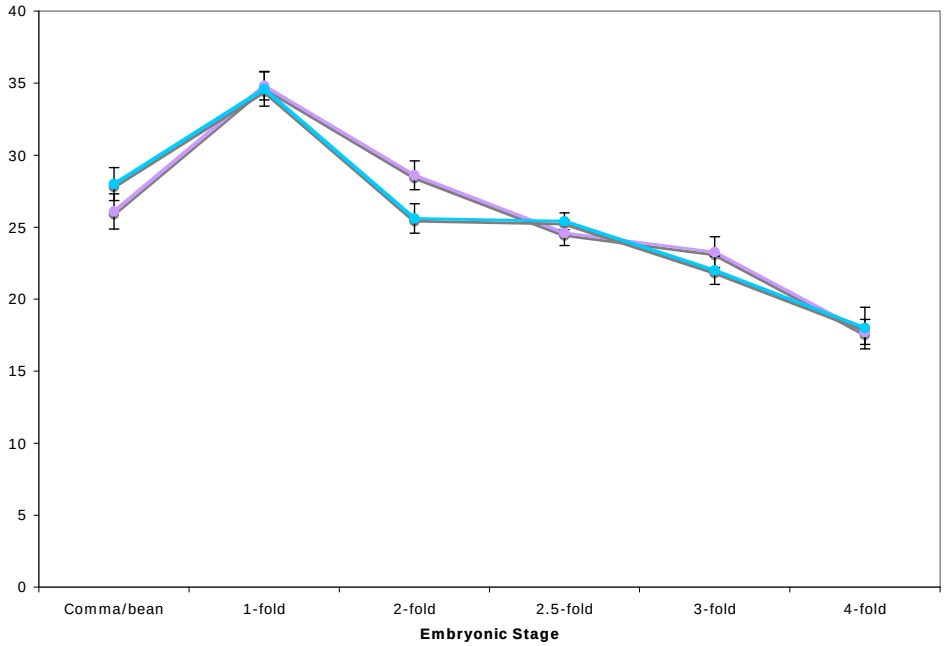

Figure 7. Effect of BetA on somatic cell apoptosis in embryonic stages in *ced-1*. Embryonic apoptosis score for worms treated with BetA in DMSO in oil (20µg/mL) or DMSO in oil. The purple line indicates BetA treatment; the blue line indicates DMSO

control. Error bars indicate the standard error of the mean. P-values from the Mann-Whitney U test indicate that no significant effect of BA is observed (comma 0.223, 1-fold 0.853, 2-fold 0.0630, 2.5-fold 0.353, 3-fold 0.557, 4-fold 0.999). 4-fold points have a sample size of at least five. All other points have a sample size of ten or larger.

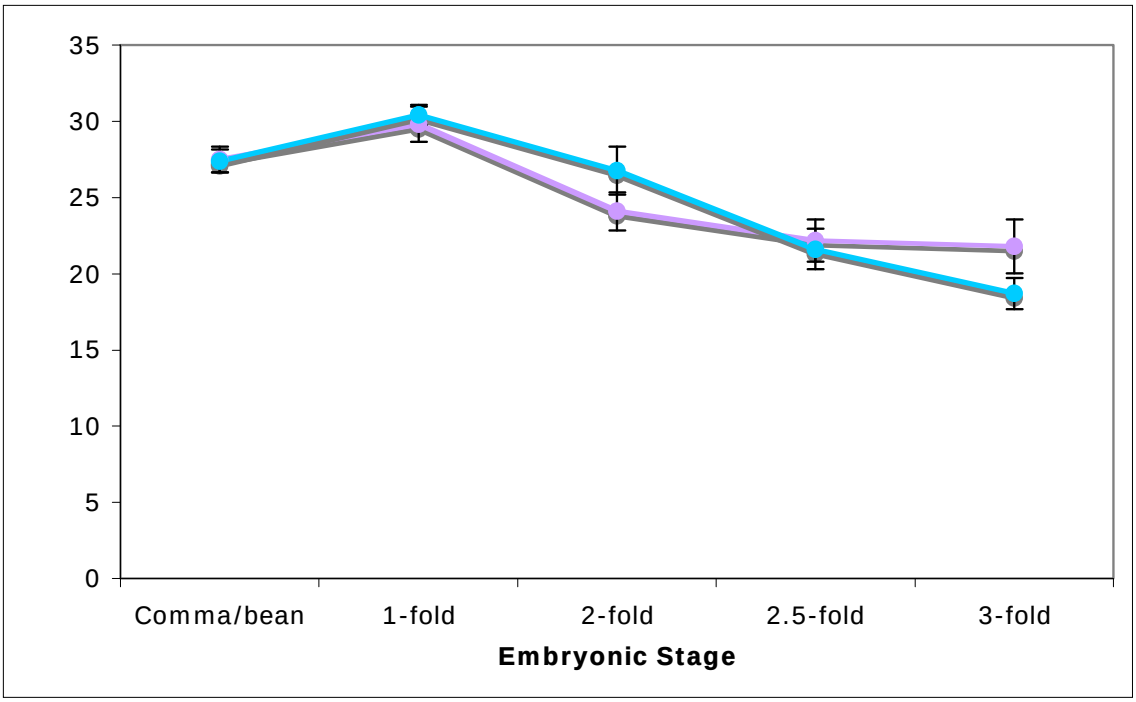

Figure 8. Effect of BetA on somatic cell apoptosis in embryonic stages in *ced-1; ced-9(ts)*. Embryonic apoptosis score for worms treated with BetA in DMSO in oil (20µg/mL) or DMSO in oil. The purple line indicates BetA treatment; the blue line indicates DMSO control. Error bars indicate the standard error of the mean. P-values from Mann-Whitney U test indicate that no significant effect of BA is observed (comma 0.971, 1-fold 0.887, 2-fold 0.278, 2.5-fold 0.696, 3-fold 0.202). All points have a sample size of five or larger.

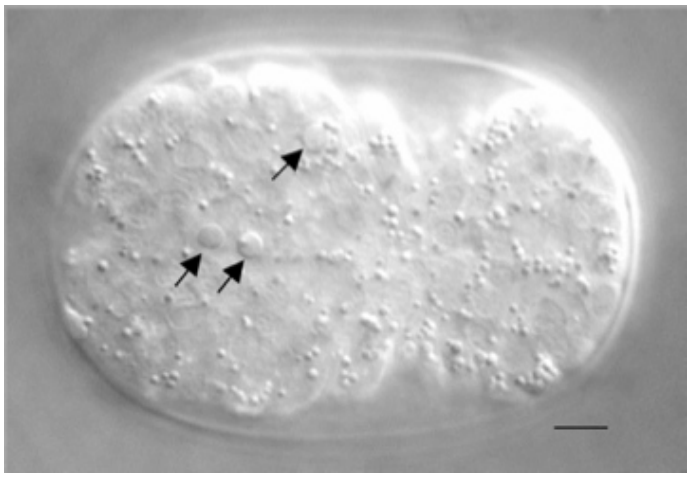

Figure 9. "Nomarski image of an embryo with apoptotic cells. Three cells indicated by arrows underwent programmed cell death in a bean/comma stage embryo and exhibit a refractile, raised-button-like appearance. The bar represents 5µm." Obtained from Conradt and Xue 2005.