Testing a sustainable source of Taxol for the treatment of RUNX cancers using *Caenorhabditis elegans*.

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ABSTRACT
RUNX proteins, master regulators of development, are frequently dysregulated in cancers, particularly leukaemia and breast cancer. A common drug for RUNX cancers, Paclitaxel, is derived from the bark of the Taxus plant. We tested compounds derived from leaves and needles of Taxus as a sustainable source of compounds to treat RUNX cancers. We accessed the effectiveness of Paclitaxel, Cephalomannine and 10-Deacetylbacatin III (10-DAB), in a *Caenorhabditis elegans* RUNX cancer model. Our results show Taxol derivatives have nuanced potency, affect development and fertility as well as reduce hyperproliferation. This research paves the way for further *C. elegans* based research using Taxol compounds against RUNX.

Keywords
C. elegans, RUNX, cancer, Taxol, fertility, Paclitaxel, Cephalomannine, 10-DAB.

Introduction
Cancer-related mortality is considered a leading cause of death, with 8.8 million deaths in 2015[1]. The family of Runt-related transcription factors, RUNX, are master regulators of various developmental pathways[2] and are often described as possessing both an oncogenic and tumour suppressive function[3,4]. Mutations in all mammalian RUNX genes have been shown to result in various cancers including breast, prostate, intestinal, lung and epithelial cancers[3], as well as acute lymphoblastic leukaemia and acute myeloid leukaemia[5]. Therefore, developing drugs that specifically target RUNX may prove effective against a wide variety of cancers[5,6].

A novel family of drugs, the Taxanes, have proven to be effective in the treatment of RUNX cancers[7,8]. One, Paclitaxel, functions by binding and stabilising ß-tubulin, ultimately resulting in cell cycle arrest[9]. Studies with human osteosarcoma cell lines (Saos-2) show the mechanism of action of Paclitaxel is to inhibit microtubule-associated shuttling of RUNX between the nucleus and cytoplasm[7]. Paclitaxel is a natural product found in the bark of the Taxus plant which is commonly grown in the Netherlands. However, extraction of Paclitaxel is detrimental for the plant as well as being economically costly. Consequently, the supply of Paclitaxel is limited and this has prompted efforts to identify renewable sources of Paclitaxel. Cephalomannine and 10-Deacetylbaccatin III (10-DAB) are two derivatives of Paclitaxel which are found in the needles and twigs of the Taxus plant, these could potentially provide a sustainable and economically viable source of a cancer drug. However, Cephalomannine and 10-DAB have not yet been evaluated for their anti-cancer potency. Thus, the aim of this study is to evaluate the effectiveness of Paclitaxel and its derivatives to treat RUNX related cancer using a *Caenorhabditis elegans* model system.

C. elegans is a unique platform to study the effects of Paclitaxel as the nematode has conserved disease pathways and genetic compatibility to mammals[11,12]. Indeed, the 1mm long nematode has a single RUNX homolog, *rnt-1,* that is required for proliferation in the stem cell-like seam cells[6]. Adult *C. elegans* have 16 neuroectodermal seam cells on each side of the animal (Figure 1A)[13,14]. In contrast, a nematode that lacks *rnt-1* shows a reduced number of seam cell nuclei (Figure 1B), whereas overexpression of *rnt-1* and/or *bro-1* causes hyperproliferation of seam cells, which is effectively seen as being tumour-like[6] (Figure 1C). *C. elegans* provides an opportunity to investigate drugs (and other compounds) in a simple whole-organism system that lacks RUNX gene redundancy and is amenable to the 3Rs (the Reduction, Replacement and Refinement of animal testing models).

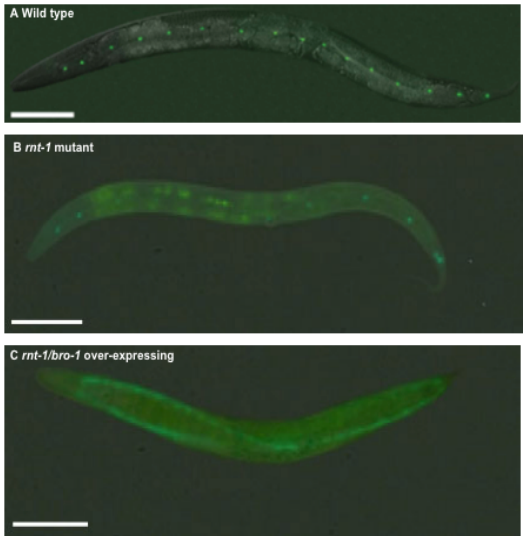

Figure 1: Representative image of worms that carry the seam cell marker. (A) Wild type L4 stage *C. elegans* showing the nuclei of 16 seam cells. The strain is *JR667* and carries an integrated GFP reporter in every seam cell. (B) *rnt-1* mutant, strain *AW187*, which has 10 seam cells per side. (C) *rnt-1 bro-1* over-expressing strain, *AW257*, that carries an integrated *rnt-1::gfp* and *bro-1::gfp* reporter. This strain has more seam cells per side, a tumour-like phenotype. All scale bars are 100µm.

The aim of this study is to evaluate the effectiveness of Paclitaxel and its derivatives to treat RUNX related cancer, using a *C. elegans* RUNX model system. Firstly, the toxicity of the Taxol derivatives are explored using the standard outputs of development and brood size (fertility). Then, we evaluate the effects of Taxol derivatives on the number of stem cell-like seam cells and its effects in our *C. elegans rnt-1* cancer model.

Materials and methods

Worm maintenance: C. elegans strains were maintained on Nematode Growth Media (NGM) agar prepared according to standard protocols and plates seeded with *OP50 E. coli* as a bacterial food source[13] and maintained at 20°C. The following strains were used: Wild-type Bristol *N2* strain; *JR667* (wIs51 [SCMp::GFP+*unc-119*$^+$]) the wild type animal carrying an integrated seam cell GFP reporter[15]; the *rnt-1* mutant *AW187 (rnt-1*(*tm388*)I;*him-5*(*e1489*)*wIs51[*SCMp::GFP+ *unc119*$^+$]V); *AW257*(*msIs114[rnt-1*::GFP+*rol-6];msIs344 [bro-1*::GFP]*)* the over-expressing strain with a tumour-like phenotype.

Taxol and its derivatives: Taxol derivatives were acquired from the Avans Hogeschool and stored at either -80°C (solid) or -20°C (in solution). Taxol derivatives were added to the molten NGM media to the desired concentrations.

Reproduction assays: To determine brood size, L4 larvae (*n*=10) were maintained individually under standard conditions[16]. At the onset of egg-laying, animals were transferred to a fresh plate daily until egg laying ceased (3-4 days at 22ºC). Progeny from each plate were counted and added together.

Developmental assays: Developmental fitness was assed using the method described by Xiong *et al*[17]. L1 animals (*n*=24) were maintained individually under standard conditions and the number of animals in each developmental stage was noted every 12 hours for 3 days, until egg laying began. The concentrations used were 100µg/ml Paclitaxel, 50µg/ml Cephalomannine and 250µg/ml of 10-DAB.

Seam cell assays: Strains carrying the integrated seam cell specific GFP marker were used to observe seam cell number at the L4 stage[15]. To take images, worms were mounted onto 2% agarose pads in 0.1% sodium azide. Fluorescent imaging was carried out using a Zeiss Imager. M2 microscope and photomicrographs were taken using a x40 objective (Zeiss) and Zeiss Zen 2012 Blue Software. Figures were compiled using R software package and the statistical analysis applied was a 2-tailed unpaired t-test.

Results

Developmental and reproductive toxicity (DART) assays were performed to evaluate a dose response to all 3 compounds (Paclitaxel, Cephalomannine and 10-DAB). Control animals laid 276 viable offspring, a similar number to animals exposed to 0.2% DMSO (Figure 2). Exposure to the Taxol derivatives did not affect brood size at low concentrations (data not shown). When *C. elegans* were exposed to the Taxol derivatives at concentrations of 100µg/ml for Paclitaxel, 50µg/ml Cephalomannine and 250µg/ml 10-DAB, the result was a significant reduction in the number of viable offspring compared to the control (respective cumulative brood size is 79, 68 and 78, $p<0.001$).

A developmental fitness assay was performed to investigate the effects of Paclitaxel and its derivatives on the rate of larval development. The fitness assay showed the percentage of worms within a specific larval stage of development, every 12 hours (Figure 3, hours 12 and 60 not shown).

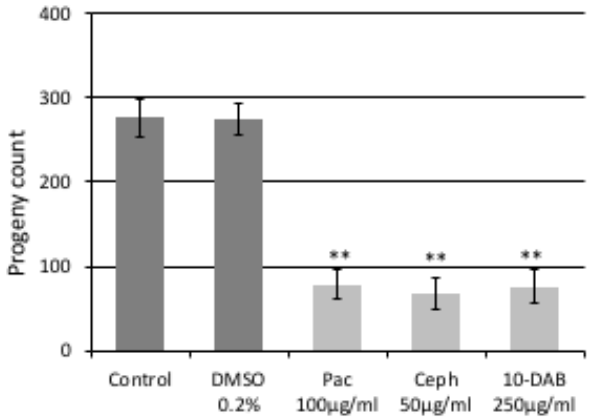

Figure 2: DART assay results from exposed Taxol derivatives. Graph indicating cumulative number of offspring (*n*=10). Wild type animals lay 276 and 275 viable offspring under control and DMSO vehicle conditions respectively (dark grey bars). Exposure to Paclitaxel (Pac), Cephalomannine (Ceph) and 10-DAB significantly reduced cumulative brood size (light grey bars). Error bars represent S.E.M. and ** represents $p<0.001$, a 2-tailed unpaired t-test where all compounds were compared to the wild type control.

After 24 hours, there was a distinct change in the rate of development with Cephalomannine and 10-DAB exposed worms developing a lot slower compared to those exposed to Paclitaxel and the control animals (Figure 3A). This pattern continued after 36 hours (Figure 3B) and after 48 hours, over 80% of control animals were laying eggs, compared to 30% of Cephalomannine tested animals (Figure 3C). Around 60% of animals exposed to Paclitaxel and 10-DAB were laying eggs at 48 hours (Figure 3C). Moreover, 10-DAB potency showed some loss of potency between 24 and 48 hours, as over 90% of 10-DAB exposed worms were in L1-L3 larval stage after 24 hours, opposed to less than 30% of control animals.

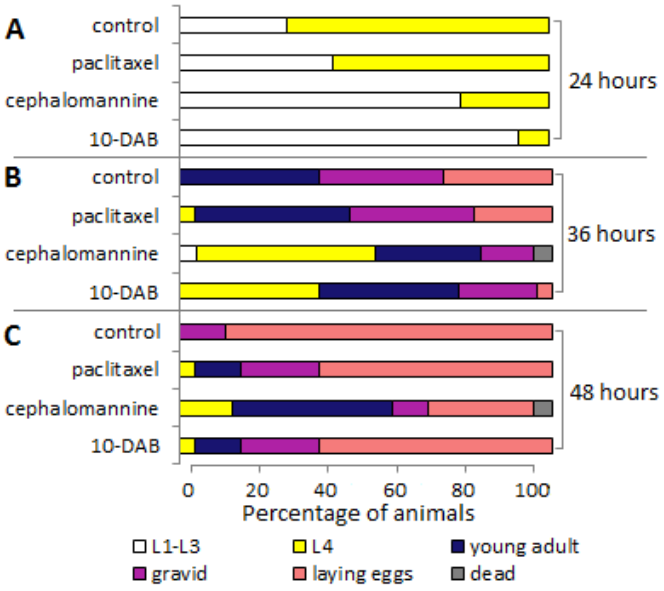

Figure 3: Developmental fitness of *N2* worms on Paclitaxel. Graph indicating the percentage of worms in each developmental stage at (A) 24 hours, (B) 36 hours and (C) 48 hours at 22ºC (hours 12 and 60 not shown). Developmental speed of animals exposed to paclitaxel is similar to control animals. While 10-DAB exposed worms are initially slow in development, they catch up to control animals after 48 hours. At all-time points, animals exposed to Cephalomannine have significant developmental delay. *n*=24.

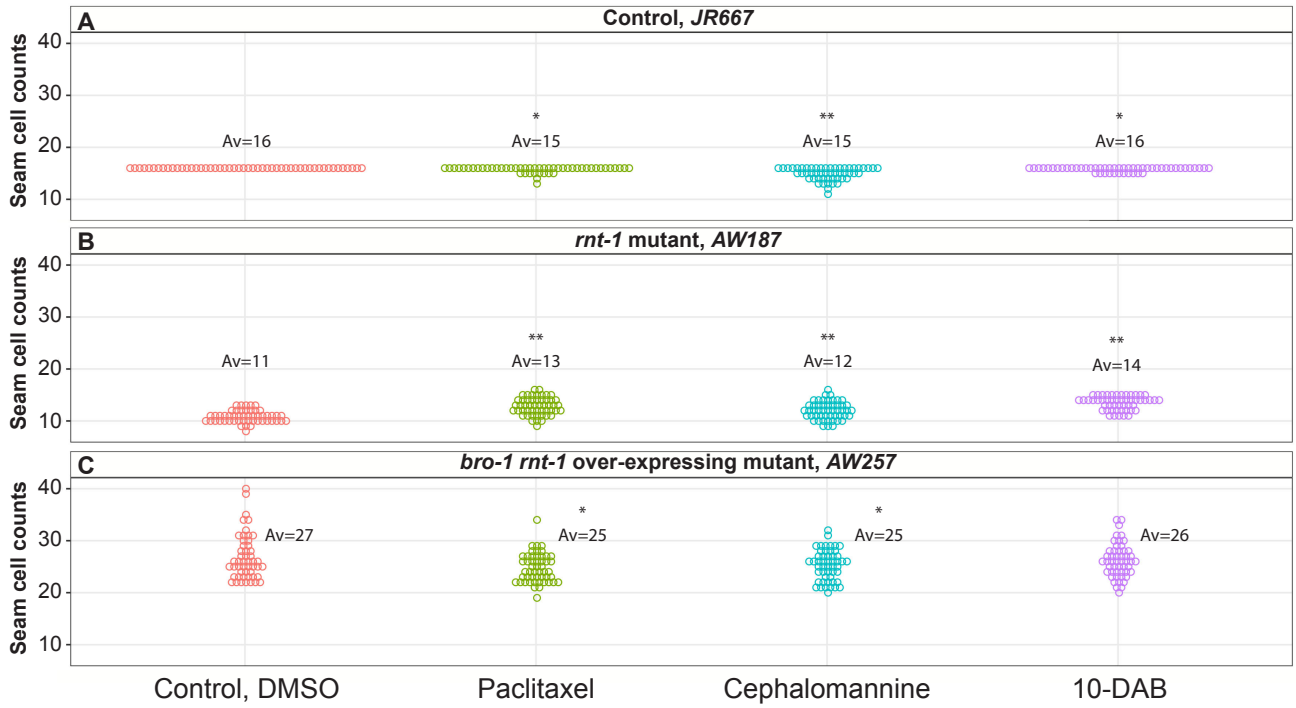

Figure 4: Seam cell count results from strains exposed to Taxol derivatives. (A) Wild type L4 larvae (strain *JR667*), with a control showing an average of 16 seam cells per side. There is a significant reduction in seam cell counts in the Cephalomannine exposed animals. (B) *rnt-1* mutant L4 larvae (strain, *AW187*), with a control showing 11 seam cells per side. There is a significant increase in seam cell number following exposure to all paclitaxel derivatives. (C) *rnt-1/bro-1* overexpressing L4 larvae (strain *AW257*) with a control showing 27 seam cells per side. Animals exposed to the paclitaxel and Cephalomannine derivatives show significantly reduced seam cell number. Each circle represents one worm and for each condition 50 worms were counted. Red circles are control DMSO vehicle exposed animals, green circles are 100µg/ml Paclitaxel, blue circles represent 50µg/ml Cephalomannine and purple circles are 250µg/ml 10-DAB. The 2-tailed unpaired t-test was used, where * $p<0.05$ and ** $p<0.01$.

The variability in the stem cell-like seam cell number in an isogenic *C. elegans* individual within a population was assessed to investigate the effects of the Taxol derivatives on stem cells. A wild type, (strain *JR667*), a *rnt-1* mutant (strain *AW187*) and a *bro-1 rnt-1* over-expressing (*AW257,* the cancer model) strain were used. In all cases, the parents were exposed to either a negative control (DMSO) or Taxol derivatives, and the seam cells in the offspring (L4-stage larvae) counted. Animals tested under control conditions showed an average of 16 seam cells per side, as expected (Figure 4A). In contrast, worms exposed to Taxol derivatives show a small but significant decrease in seam cell number ($p<0.01$) averaging 15 seam cells per side in Paclitaxel and Cephalomannine exposed worms.

rnt-1 mutant animals (Figure 4B) showed an average of 11 seam cells per side, as expected. Exposure to the Taxol derivatives resulted in a significant increase in seam cell number ($p<0.001$), with an average of 13 and 12 in Paclitaxel and Cephalomannine respectively, and an average of 14 seam cells in 10-DAB exposed worms. The *C. elegans* RUNX cancer model where *rnt-1* and *bro-1* are overexpressed, resulted in animals having an average of 27 seam cells per side (ranging between 21-40) and a tumorous appearance (Figure 4C). Exposure to 10-DAB did not affect the seam cell number. In contrast, Paclitaxel and Cephalomannine exposed *bro-1 rnt-1* overexpressing worms displayed an overall reduction in seam cell number ($p<0.05$) with an average of 25 seam cells, ranging between 20-34 and 20-32 respectively.

Discussion

Extraction of Paclitaxel is costly, inefficient and limited as it is derived from the bark of the Taxus plant, a non-sustainable source. Recently, there has been investigation towards synthetic synthesis of Paclitaxel and to identify renewable sources of (the metabolites of) Paclitaxel. The metabolites Cephalomannine and 10-DAB are found in the needles and twigs of the Taxus plant[10], and are a likely sustainable source, but their potency and effectiveness as a cancer treatment has yet to be fully elucidated. Here we demonstrate the use of *C. elegans* as a platform for the evaluation of Taxol derivatives, specifically showing a nuanced potency between Paclitaxel and its derivatives. The use of *C. elegans* in such tests highlights how an invertebrate can be used in place of vertebrates in cancer studies as well as other testing studies[18].

In all our assays, we establish Cephalomannine as a potent derivative of Paclitaxel since it exerts similar effects but at much lower concentrations. 10-DAB however, is less potent compared to Paclitaxel. The differences in potency can be explained by their chemical structure, as Cephalomannine has a conformational change of the phenyl ring-bearing side chain as well as a structural compartmentalization of the bottom side chain (Figure 5). Moreover, 10-DAB shows an exclusion of two of the phenyl ring-bearing side chains and acetoxy moieties, effectively forming only half the structure of Paclitaxel (Figure 5).

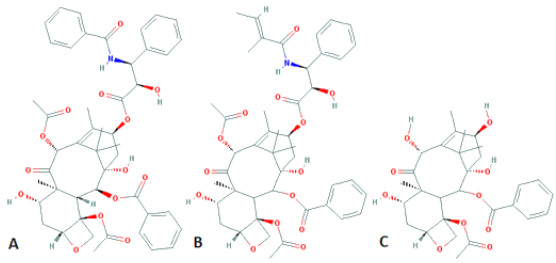

Figure 5. Chemical structure of (A) Paclitaxel, (B) Cephalomannine and (C) 10-DAB. (A) Paclitaxel structure includes a dipertene core, 3 phenyl bearing side chains and 2 acetoxy moieties. (B) Cephalomannine structure shows a conformation change of one phenyl ring-bearing side chain and a compartmentalisation of the bottom side chain, when compared to Paclitaxel. (C) 10-DAB structure shows the exclusion of the 2 top phenyl ring-bearing side chains, when compared to Paclitaxel. Figures were all derived from Pubchem sources.

The developmental rate of animals exposed to Paclitaxel and 10-DAB was not significantly affected, while in contrast, Cephalomannine exposure resulted in striking developmental delay. Interestingly, while 10-DAB showed an initial delay in development during the first 24 hours, this was not the case after 48 hours, suggesting 10-DAB is more susceptible to degradation or metabolic conversion. Studies show Paclitaxel is predominantly metabolized by cytochrome P450s, and it is possible that 10-DAB is metabolized to a greater extent than Paclitaxel, possibly due to its chemical structure[19].

We used *C. elegans* to observe the effects of Paclitaxel, as the solo homolog *rnt-1* is the target of Paclitaxel in the same way as in mammalian RUNX cancers. In a wild type background, all three compounds significantly reduced seam cell number, suggesting that the Taxol derivatives are affecting the post-embryonic seam cell divisions. Strikingly, as *C. elegans* is eutelic (the developmental patterning is highly stereotypical) and as the complete lineage of all cells is known, a slight change in seam cell number from 16 can be of significant interest. In a wild type animal, "extreme" seam cell numbers (a 10% deviation from 16 i.e. less than 15 or greater than 17 seam cells) are very rare[20]. Indeed, as all strains show more than 20% variation in seam cell number, confirming that the effects seen were indeed due to Taxol derivative exposure.

While the specific changes to the seam cell division were not investigated here, it is likely that the reduction in seam cell number is a result of failure to maintain the seam cell (proliferative) fate or a failure in execution of the seam cell divisions[21,22]. It is known that *rnt-1* is crucial in licensing the seam cells to divide[6,23], and as Paclitaxel is known to inhibit RUNX shuttling[7], it is likely that these compounds are inhibiting RNT-1 localisation to the nucleus of the proliferative daughter in the nematode seam cells. The nematode cancer model, with over-expression of *rnt-1* and *bro-1* displays seam cell hyperproliferation, and this is reduced following the exposure to Paclitaxel and Cephalomannine. However, it is not yet clear how this reduction is achieved, and exploring the reasons behind this is extremely challenging and beyond the scope of this project.

Conclusion

The object of this study was to evaluate Paclitaxel and its derivatives as an effective treatment for RUNX cancer using a *C. elegans* platform. We have shown that the fertility and development of *C. elegans* is affected by Taxol derivatives, but we also observed a significant reduction in the number of stem cell-like seam cells. In addition, we have shown differences in the potency of the 3 compounds extracted from the Taxus plants. Together, we demonstrate a basis for testing anti-cancer drugs using *C. elegans*. The advantage of *C. elegans* over a cancer stem cell line, is that it is possible to link perturbations of molecular and cellular events to a whole-body adverse outcome, such as development or fertility. Finally, we suggest that Cephalomannine, extracted from the twigs and needles of the Taxus plant, is a potential drug for the treatment of RUNX cancers that can be obtained in a sustainable and cost-effective manner. *C. elegans* is an effective and simple model system for DART testing[17], thus screening the potency and toxicity of Cephalomannine and 10-DAB from sustainable sources as a potential RUNX cancer treatment will contribute to the reduction in the use of vertebrates in cancer studies. Indeed, by using this *C. elegans* cancer model it will be possible to explore the molecular interactions of Paclitaxel and its derivative's in a system that is not hampered by genetic redundancy[13] and form the basis of more informed, targeted (cancer) cell culture assays.

Role of the student

David van de Klashorst was a final year undergraduate student working in the laboratory of Dr. Samantha Hughes at the HAN BioCentre. David was responsible for the design and the execution of the experiments, subsequent analysis of the data and interpretation of the results, including writing of this paper. This was done under the supervision of Dr. Hughes.

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