

Meeting abstract

Nitric oxide, cell death and increased taxol recovery

Don J Durzan*

Address: Department of Plant Sciences, University of California, One Shields Ave. Davis, CA 95616, USA

Email: Don J Durzan* - djdurzan@ucdavis.edu

* Corresponding author

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Background

With NASA support this research aimed at evaluating early opportunities in Microgravity Sciences to commercialize space and to develop the biotechnology facility for the International Space Station [1]. The main task was to evaluate the production of taxol (generic name: paclitaxel) with cell suspensions in bioreactors designed for the Space Shuttle. Unexpectedly, this work led to the early demonstration of L-arginine-dependent nitric oxide (NO) bursts in mechanically and gravitationally stressed plant cells, to NO-induced programmed cell death (apoptosis) (reviewed in [2]), and to a model describing how these factors contribute to increased taxol and taxane recovery from conifers.

Earlier, when intermediates of the Krebs-Henseleit or urea cycle (See Figure 1) were fed to conifers, several substituted guanidino compounds were derived from uniformly labeled ^{14}C -L-arginine, and less so from ^{14}C -L-citrulline [3,4]. At that time, the substituted guanidines were considered mainly as respiratory inhibitors [5,6]. Today, they are natural inhibitors of plant, animal and human nitric oxide synthases (NOSs). NOS substrates are L-arginine and oxygen. NOS products are L-citrulline and NO.

Since nitrate and nitrite reductases were also known sources of NO, we obtained an *Arabidopsis* nitrate reductase double mutant with the aim of finding out if cells could produce NO in the absence of nitrate, nitrite, and their reductases [7]. With this mutant we reaffirmed that the source of NO was putative NOS activity. The production of NO from L-arginine was blocked by D-arginine, and by the NOS inhibitor, N^G -monomethyl-L-arginine (L-NMMA) ensuring that NO was produced in the absence of any residual nitrate reductase activity. NOS-dependent

NO production in cells was inhibited by other guanidino compounds but not by D-arginine. Subsequently, the discovery by others of two plant NOS genes provided evidence that plant genomes code for NOS. In our work with *Taxus* cell suspensions, the substituted guanidines offered protection against mechanically induced stress and cell-death or apoptosis.

Taxol is an effective anti-cancer agent that was first isolated from the bark of *Taxus brevifolia* [8]. Taxol binds to microtubules thereby offering a novel mechanism of blocking cell proliferation. It became the best-selling anti-cancer drug in history. By 2000, commercial sales of taxol were well over \$1.5 billion. New models for taxol and taxane biosynthesis emerged [9] so that taxol biosynthesis could be followed at the subcellular level by immunocytochemistry, and by laser confocal and scanning electron microscopy [10-13,20]. The use of NO donors, NOS substrates, products, inhibitors (substituted guanidines), and NO traps provided new opportunities to control the citrulline-NO cycle (See Figure 1), apoptosis, and taxol production in unit gravity, simulated microgravity, and in hypergravity.

Haploid egg cells from female trees of *T. brevifolia* were selected as the experimental material [12,19]. These cells are easily screened without the effects of dominance, recessive, and epistatic interactions characteristic of diploid cells. Lethal genes are directly expressed, and removed by apoptosis making cell populations genetically more uniform. Diploid cell suspensions were also established from *T. cuspidata* [13] needles on 3-year-old stock obtained in 1995 from Zelenka Nursery (Grand Haven MI), and from seeds of *T. chinensis*. This provided better comparisons with all other published work with using diploid cells.

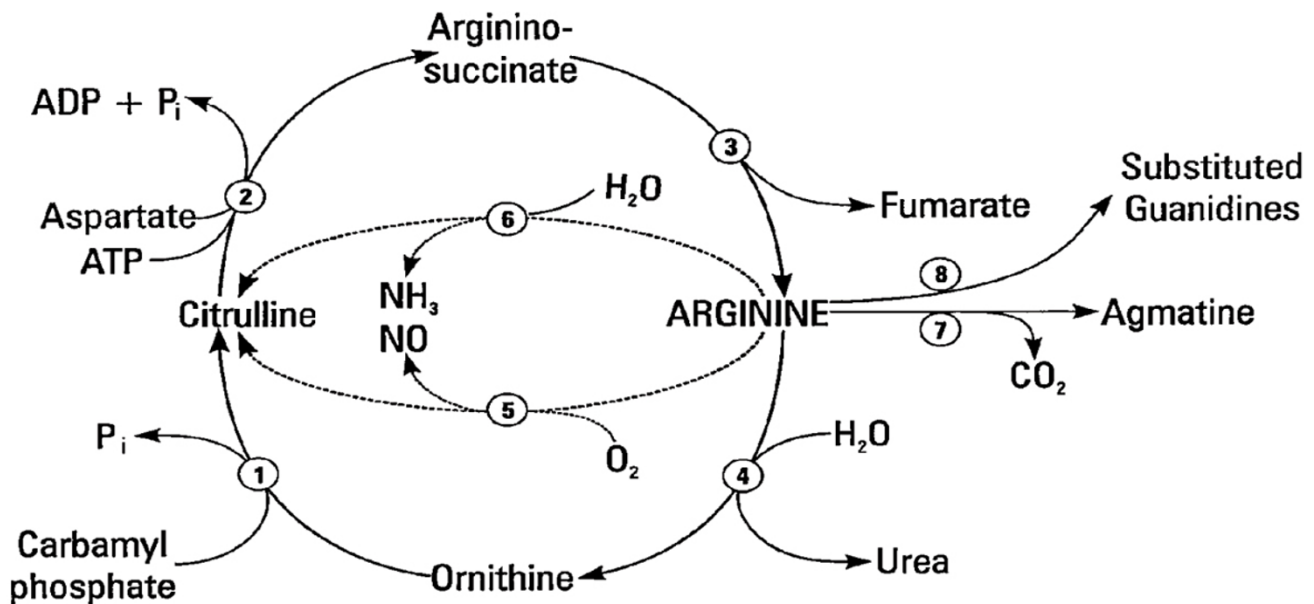


Figure 1

The reactions of the Krebs-Henseleit (urea) cycle, and their relations to NOS activity. Enzymes: **1.** ornithine carbamoyl transferase, **2.** argininosuccinate synthetase, **3.** argininosuccinate lyase, **4.** arginase, **5.** nitric oxide synthase, **6.** arginine deiminase, **7.** arginine decarboxylase, **8.** numerous enzymes contributing to the formation of substituted guanidino compounds. Reactions 1 to 4 comprise the urea cycle. Reactions 2, 3, 5, may account for NOS activity in plants (citrulline-NO cycle). Arginine deiminase was reported in chloroplasts but is mostly found in microorganisms. Reactions 7 and 8 comprise decarboxylation, oxidation, methylation, transamidation, phosphorylation, keeping the guanidino group intact or modifying it by methylation, phosphorylation, etc. They remove L-arginine as a substrate from the urea cycle, and from reactions 5 and 6. L-arginine represents an important branch point that links nitrate and ammonium nutrition to protein synthesis and turnover (not shown), to the urea cycle, to a postulated citrulline-NO cycle, and to the formation of guanidino compounds (substituted and non substituted). Through oxygen requirements, the stress-induced NOS activity links respiration to NO, ROS and RNS production, their signaling pathways and damaging reactions, e.g., the nitration of phenols and tyrosines residues in cell regulatory proteins, and to apoptosis.

Cell suspensions were maintained in darkness at $25 \pm 2^\circ\text{C}$ on semisolid media, in 125 ml Erlenmeyer flasks (60 rpm), 1 L nipped flasks on a clinostat (1 rpm simulating $2 \times 10^{-4} \times g$, but with significant convective mixing of the gaseous and liquid environment), 100 ml high-aspect rotating vessels (HARV 12.5 cm dia.), and rotating cylindrical culture vessels (RCCV 7.5 cm dia.) both at ca. $10^{-2} \times g$ (Synthecon, Houston TX). The HARV and RCCV were used by NASA in early space shuttle experiments, and in a mini-payload integration center, designed as an in-flight laboratory.

In all cell assays, apoptotic cells were distinguished morphologically and by the TUNEL reaction [14,15]. Free taxol, taxanes, and baccatin III in cells and the culture medium, or bound taxol and taxanes, released after xylanase activity, were determined with competitive inhibition enzyme-linked immunoassays kits from Hawaii Biotechnology. Cells were examined by laser confocal

microscopy (Zeiss LSM 410 Invert Scan Microscope) using single or double-labeling immunocytochemical fluorescence (FITC, Cy3) and colloidal gold to reaffirm the sub-cellular locations of taxol, the taxane ring (baccatin III), the C-13 side chain of taxol, and taxanes in general. For samples larger than 10 g fresh biomass, these compounds were determined by HPLC using authentic standards and taxil columns (MetaChem Technologies Inc.) [12].

Results

With *T. cuspidata* suspension in RCCV bioreactors ($10^{-2} \times g$), and in 1 L nipped flasks (3 wks in darkness at 24°C), free taxol comprised 42 and 21 percent of the total taxanes (1.1 and 2.4 mg taxol/kg air-dry biomass weight), respectively [13]. Centrifugation of cells at 3 and $24 \times g$ greatly increased taxane content but reduced taxol recovery. *T. cuspidata* and *T. brevifolia* responded similarly to mechanical stresses, simulated microgravity, and hypergravity by

producing NO bursts, apoptosis, and by the overproduction of taxol and taxanes.

T. brevifolia heat-killed cells did not produce NO. Generally 19% of the live cells (unit gravity in shaker flasks for 3 h) were always positive for NO as assayed with DAF-2DA. With the addition of 10^{-4} mM SNP, a NO donor, or after centrifugation at $150 \times g$ for 3 h, nearly all cells produced NO. However, if 0.5 mM L-NMMA (NOS inhibitor) was added, only 26% of the cells produced NO. When 0.5 mM D-NMMA, a non inhibitor of NOS, was added, 92% of the cells produced NO [14].

The greater the NO produced and released by cells, the more cells became apoptotic. Additions of SNP (10^{-6} to 10^{-2} mM) increased apoptosis from 18 to 75%. Centrifugation at $150 g$ yielded 35% apoptosis. This was reduced to less than 10% by the addition of the NOS inhibitor, L-NMMA. Substituting D-NMMA for L-NMMA resulted in apoptosis (32% cells), but this was not significantly different from the cells exposed to $150 g$ [14]. The NOS requirement for oxygen connected NO production to the stress-induced and damaging increases in free radicals, *viz.*, reactive oxygen species and reactive nitrogen species [2]. The lesson learned offered guanidino compounds as countermeasures to NO-mediated stress and apoptosis.

At unit gravity and after 10 d, egg cells exposed to SNP (10^{-4} mM) produced 18 mg taxol/Kg fresh weight. This taxol yield was now 64% higher than the controls without SNP. The number of cells producing NO with SNP was 86.7% higher than the controls without SNP. By contrast, the addition of L-NMMA (0.5 mM) decreased NO production by 87.3%. Taxol yield was reduced by 75% (4.1 mg/Kg fresh weight) (unpublished results).

In a separate study at unit gravity with cells supported on filter paper, the effects of SNP (10^{-4} mM), sodium tungstate, (a nitrate reductase inhibitor at 1 mM), and L-NMMA (0.5 mM) were used to evaluate taxol yields after 5 d. SNP added alone, or tungstate with SNP together, yielded taxol at 8.0 and 8.1 mg/Kg fresh weight, respectively, *i.e.*, the differences were not significantly different. This reaffirmed that nitrate reductase activity was not a contributor to NO-dependent taxol production. L-NMMA alone, or together with added tungstate, reduced taxol recovery to 2.1 and 2.0 mg/kg fresh weight, respectively. Here again, NOS was responsible for the NO that increased taxol production without a significant contribution from nitrate reductase. Removing and starving the control egg cells only slightly increased taxol recovery.

The tetracyclic diterpenoid ring of taxol is synthesized by cyclization of geranylgeranyl diphosphate to give taxadiene, 4(5),11(12(-diene) in plastids and in light and darkness

[9,11]. This basic ring structure gives rise to over 300 different taxanes. One of these, baccatin III, can provide the ring structure for the biosynthesis of taxol. Although the mechanisms are not yet clear, taxol formation also requires the addition of a C-13 side-chain originating from phenylalanine. Additional and finishing structural changes for taxol involve reaction in the endoplasmic reticulum and Golgi stacks.

Using specific antibodies, the biosynthesis of the taxol ring in plastids, and the cytoplasmic assembly of bound taxol and related taxanes were visualized by laser confocal and scanning electron microscopy. Gold labeling was especially useful to identify taxol and taxanes in transport vesicles, the plasmalemma, cell walls, and in the culture medium. Colloidal 40 nm-gold-immunolabeled antibodies to taxanes, taxol, and baccatin III were visualized with 7 to 21 nM sensitivity, with and without gold backscatter (laser confocal and scanning electron microscopy).

In egg cells, baccatin III (taxane ring) was detected in gravisensing amyloplasts and vesicles associated with plastid membranes. Baccatin III, taxol, and other taxanes were transported through the cytoplasm by these vesicles, which then released their contents to the plasma membrane. Plastids, that moved and docked at the plasma membrane, also released bound taxol and taxanes into the plasma membrane. The bound taxol and taxanes at the cell-surface were deposited in newly formed cell walls or released into the culture medium. The drug-productive cells and materials in the culture medium containing bound taxol, baccatin III, and taxanes were collected with antibody-labeled paramagnetic beads [16]. Immunogold-labeled taxol, taxanes and baccatin III were detected in transport vesicles, plasma membrane fragments, and on materials recovered from the culture medium.

Bound taxol and taxanes were recovered from xylanase (commercial and purified) hydrolysates of cell biomass, debris in the culture medium, and from the wood of trees. Taxol and other taxanes were characterized and identified by HPLC using authentic standards. Results indicated that recovered taxol was bound to xyloglucan oligosaccharides. Probes of cells with an antibody to xyloglucan endotransglycosylases implied that 'touch' genes normally expressed under mechanical forces [18], could have provided additional sites for the attachment of taxol and other taxanes.

In hypergravity (3 to $150 \times g$), the taxol and taxanes released from cells by syneresis, were recovered on hydrophobic PVDF (polyvinylidene fluoride) filters. Cyclodextrins added to the culture medium enhanced biomass yield and altered the solubility of taxanes to improve the recovery of taxol and taxanes. The addition of

cyclodextrins to the culture medium improved cell biomass and taxol recovery [17]. Further details are available in US Patents [16-18,20].

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