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Short communication

Target-directed discovery and production of pharmaceuticals in transgenic mutant plant cells

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1-methy-4-phenylpyridinium (MPP+: Pubmed CID: 39484)
[^{3}H]GBR12935 (Pubmed CID: 3455)
Lobinaline (1-Methyl-5,7-diphenyl-6-(3,4,5,6-tetrahydro-2-pyridinyl)decahydroquinoline (Pubmed CID: 419029)

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ABSTRACT

Plants are a source of complex bioactive compounds, with value as pharmaceuticals, or leads for synthetic modification. Many of these secondary metabolites have evolved as defenses against competing organisms and their pharmaceutical value is "accidental", resulting from homology between target proteins in these competitors, and human molecular therapeutic targets. Here we show that it is possible to use mutation and selection of plant cells to re-direct their "evolution" toward metabolites that interact with the therapeutic target proteins themselves. This is achieved by expressing the human target protein in plant cells, and selecting mutants for survival based on the interaction of their metabolome with this target. This report describes the successful evolution of hairy root cultures of a *Lobelia* species toward increased biosynthesis of metabolites that inhibit the human dopamine transporter protein. Many of the resulting selected mutants are overproducing the active metabolite found in the wild-type plant, but others overproduce active metabolites that are not readily detectable in non-mutants. This technology can access the whole genomic capability of a plant species to biosynthesize metabolites with a specific target. It has potential value as a novel platform for plant drug discovery and production, or as a means of optimizing the therapeutic value of medicinal plant extracts.

1. Introduction

The pharmaceutical industry is increasingly abandoning plants as a source of drugs (see Littleton, 2007). One reason is that many bioactive plant metabolites are too complex for convenient chemical synthesis, making them difficult to optimize for interaction with the molecular target. Another, is that production of such compounds requires purification from the variable and miniscule amounts in intact plants. A solution to the latter (pioneered by Phyton for taxanes) is to use cultured plant cells as production systems (Howat et al., 2014). Here we show that mutant plant cells in culture can be used both as optimized production systems, and as sources of metabolites with optimized activity at the target. The concept is simple. First a plant species is identified that contains complex metabolites with activity at a molecular target protein. Then transgenic plant cells are created that stably express this target protein. Mutants of these transgenic cells are then selected under conditions that favor survival of individuals containing metabolites with the desired interaction with the target. The surviving sub-population is enriched in mutants containing metabolites with the appropriate pharmacological activity. These may be metabolites that are present in the wild-type plant or, if a mutation alters a relevant biosynthetic pathway, then "novel" active metabolites with enhanced activity at the target may be generated (Rogers et al., 2003). Target proteins have previously been expressed in plant cells as screens (Littleton, 2007; Doukhanina et al., 2007; Zhao et al., 2012; Gunjan et al., 2015), but this is the first report in which survival of mutant plant cells expressing a foreign target protein has been used to direct secondary metabolism toward a specific pharmacological phenotype. Proof of concept uses

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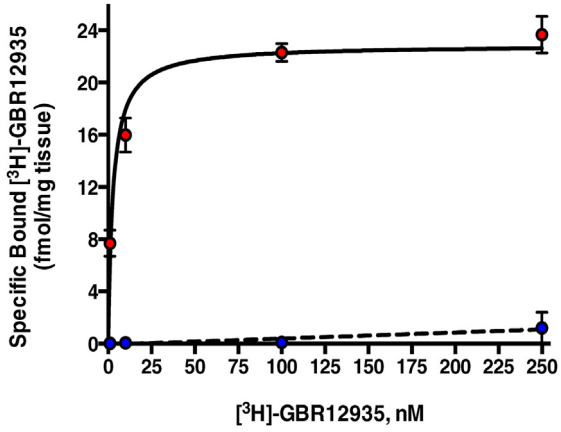

Fig. 1. Binding of [^{3}H]GBR12935 to membranes derived from hairy root cultures of *Lobelia cardinalis*.
The upper curve shows saturable specific, one-site binding of [^{3}H]GBR12935, a selective radioligand for the DAT, to membranes from transgenic *hDAT* hairy roots. Binding parameters were Kd 7.33 nM and Bmax = 1.04 + 0.9 pmol/ mg protein (n = 4)]. Non transgenic hairy roots (lower curve) showed no specific binding. Each data point represents mean ± standard error of the mean (SEM) of 4 replicates and the curve represents best fit to a one-site binding model using Graphpad nonlinear regression software.

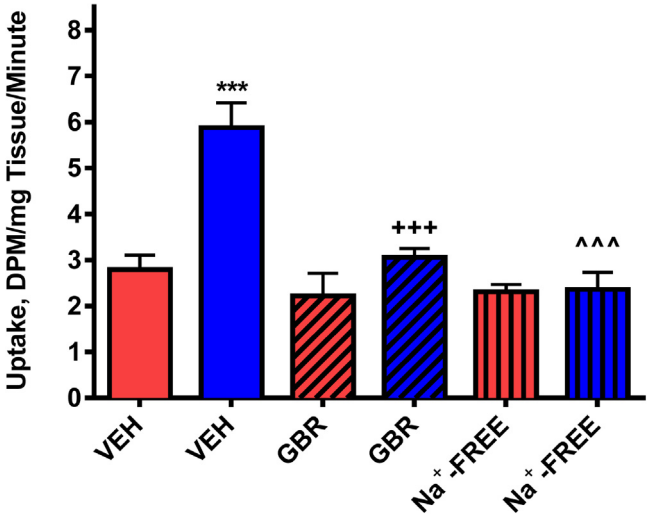

Fig. 2. Uptake of [^{3}H]Dopamine into hairy root cultures of *L. cardinalis*. The first two bars compare [^{3}H]Dopamine uptake into wildtype hairy roots (blue bars) with that into transgenic hairy roots (red bars). Cultures were incubated with [^{3}H]Dopamine for 30 min at 37° C, non-specific uptake was determined at 0 °C. For each replicate, the radioactivity taken up by 10 roots was pooled and specific uptake calculated by subtracting non-specific uptake from total uptake values. Each bar represents four averaged replicates and data is expressed as mean ± SEM DPM/mg tissue. The transgenic *hDAT* hairy roots showed significantly greater uptake of [^{3}H]Dopamine (5.88 ± 0.54^^^, One-way ANOVA, Bonferroni post hoc, $P < 0.001$) than wildtype (2.79 ± 0.31). Pretreatment with the selective inhibitor of the DAT, GBR12909 (100 μM,+++, One-way ANOVA, Bonferroni post hoc, $P < 0.001$), prevented the increased uptake of [^{3}H]Dopamine into transgenic *hDAT* hairy roots, as did substitution of Na^+-containing buffer with Na^+-free buffer (***, One-way ANOVA, Bonferroni post hoc, $P < 0.001$) (the function of the hDAT requires the presence of Na^+), relative to vehicle-treated controls expressing hDAT(blue bar, VEH). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lobelia cardinalis, which contains lobinaline, a novel inhibitor of the human dopamine transporter (DAT) (Littleton et al., 2005; Brown et al., 2016). Metabolites with this activity have evolved in plants to inhibit similar transporter proteins in the brains of herbivorous insects to deter feeding (Chen et al., 2006). This homology makes them valuable as lead compounds in degeneration of dopaminergic

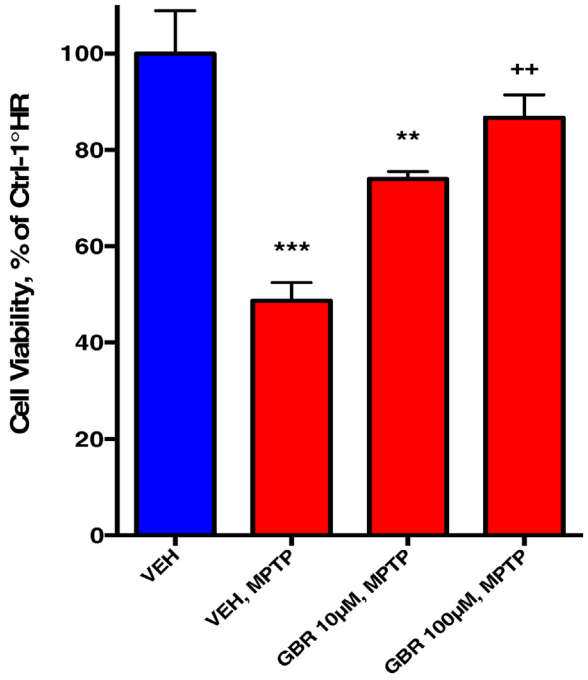

Fig. 3. Increased toxicity induced by dopaminergic neurotoxins in transgenic (*hDAT)* hairy roots of *L. cardinalis* Toxicity in hairy roots was induced by addition of MPTP, MPP+ or 6-hydroxydopamine to the culture medium for 24 h at concentrations that just failed to induce toxicity in wild-type hairy roots. Trypan blue staining was used to assess toxicity. Similar data were obtained for all these neurotoxins, in that toxicity was significantly increased in the transgenic hairy roots, relative to wild-type control. The data shown are means ± SEM of cell viability of hDAT transgenic hairy roots expressed as a percentage of wild type control, where n = 6. 24 h MPTP exposure caused toxicity in ~50% of cells in transgenic *hDAT* hairy roots (One way ANOVA followed by Bonferroni post hoc, $p < 0.001^{***}$). This toxicity was reduced by the selective DAT inhibitor GBR12909 at 10 μM concentrations ($p < 0.01$,** relative to vehicle treated groups) and MPTP toxicity was reversed by 100 μM GBR12909 ($p < 0.01^{++}$, relative to MPTP treatment without GBR12909 pretreatment). These data indicate that cytotoxicity is increased by activity of the DAT, and that overproduction of DAT inhibitors should "rescue" transgenic (*hDAT*) hairy roots. However, MPTP requires intracellular metabolism to the active moiety, MPP+, so the cytotoxicity of MPP+ is more directly related to transport by the DAT. MPP+ (100 μM) was therefore chosen for selection of mutant transgenic (*hDAT*) hairy roots.

neurons in the human brain (Rudnick et al., 2014; Segura-Aguilar and Kostrzewa, 2015). However, lobinaline, is a complex binitrogenous decahydroquinoline alkaloid (Manske 1938; Robison et al., 1964) with 5 chiral centers. Chemical synthesis is difficult, and lobinaline is present in low yields in the wild type plant. Lobinaline is therefore a poor candidate as a conventional lead for DAT inhibitors, but an excellent example for target-directed evolution of biosynthesis (see Fig. 4).

2. Materials and methods

2.1. Expression of the functional target protein

First, full-length cDNA of the human dopamine transporter (*hDAT*) was introduced into hairy root cultures of *L. cardinalis* by *Agrobacterium rhizogenes*-mediated transformation (Tsugawa et al., 2004). The *hDAT* gene was present by PCR and its expression was demonstrated by gel-based electrophoresis. However for this selection procedure it is essential that the expressed protein should be functional, and retain the pharmacology of the native protein in mammalian systems. Therefore, the hDAT protein was shown to be expressed by specific binding of the DAT radioligand [^{3}H]GBR12935 as for mammalian membranes (Reith and Selmeci, 1992) (Fig. 1). The transgenic *hDAT* plant cells showed significantly