Bioactive diterpenoid metabolism and cytotoxic activities of genetically transformed *Euphorbia lathyris* roots

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Abstract:

Plants in the genus *Euphorbia* produce a wide variety of pharmacologically active diterpenoids with anticancer, multidrug resistance reversal, and antiviral properties. Some are the primary industrial source of ingenol mebutate, which is approved for treatment of the precancerous skin condition actinic keratosis. Similar to other high value phytochemicals, *Euphorbia* diterpenoids accumulate at low concentrations *in planta* and chemical synthesis produces similarly low yields. We established genetically transformed root cultures of *Euphorbia lathryis* as a strategy to gain greater access to diterpenoids from this genus. Transformed roots produced via stem explant infection with *Agrobacterium rhizogenes* strain 15834 recapitulated the metabolite profiles of field-grown plant roots and aerial tissues. Several putative diterpenoids were present in transformed roots, including ingenol and closely related structures, indicating that root cultures are a promising approach to *Euphorbia*-specific diterpenoid production. Treatment with methyl jasmonate led to a significant, albeit transient increase in mRNA levels of early diterpenoid biosynthetic enzymes (farnesyl pyrophosphate synthase, geranylgeranyl pyrophosphate synthase, and casbene synthase), suggesting that elicitation could prove useful in future pathway characterization and metabolic engineering efforts. We also show the potential of transformed *E. lathyris* root cultures for natural product drug discovery applications by measuring their cytotoxic activities using a panel of human carcinoma cell lines derived from prostate, cervix, breast, and lung.
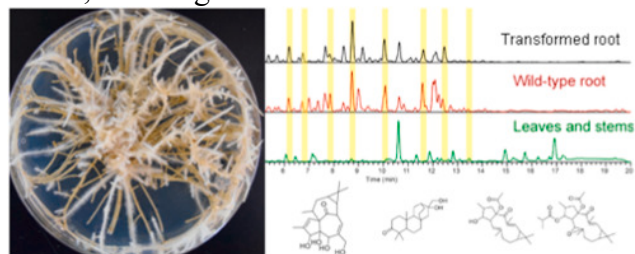
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1. Introduction

Diterpenoids from plants in the genus *Euphorbia* (family: Euphorbiaceae) are the focus of natural product drug discovery due to their broad range of pharmacological activities. Over 650 unique structures have been reported (Vasas and Hohmann, 2014; Ernst et al., 2019), a number of which have shown potential as drug leads (Ernst et al., 2016; Saklani and Kutty, 2008). Multiple plant species within the genus *Euphorbia* contain the diterpenoid ingenol, which is generally present in conjugated forms and substituted by various carboxylic acids (Béres et al., 2018). Some naturally occurring ingenol conjugates possess anticancer and anti-HIV properties (Abreu et al., 2014; Vasas et al., 2012), whereas semi-synthetic derivatives show potential for increased efficacy (Silva et al., 2019). Ingenol mebutate, a naturally occurring ester derivative containing angelic acid, has gained significant attention due to its approval by the Food and Drug Administration in 2012 and the European Medicines Agency in 2013 for treatment of the precancerous skin condition, actinic keratosis (Braun et al., 2018; Martin and Swanson, 2013; Ogbourne and Parsons, 2014; Ortega Del Olmo and Salido-Vallejo, 2018). Originally isolated from *Euphorbia peplus* L., ingenol mebutate has been extensively studied for its potent antitumor and antileukemic activities (Alchin, 2014; Doan et al., 2012). Current supply is limited because of its low-yield extraction from *E. peplus* biomass (Hohmann et al., 2000), but it is also produced semi-synthetically starting from ingenol (Liang et al., 2012). An early total synthesis of ingenol involved 43 steps and yielded 0.007% (Winkler et al., 2002), whereas a recent 14-step semi-synthesis yields around 1% (Jørgensen et al., 2013). As such, the low yields via plant extraction and challenging synthetic routes have prompted investigation of alternative, economically viable sources of ingenol. One strategy for improving access to ingenol and other pharmaceutically relevant *Euphorbia* diterpenoids is to apply biotechnological approaches (Atanasov et al., 2015).

The precursor of all plant diterpenoids is geranylgeranyl pyrophosphate (GGPP), a C_{20} branched hydrocarbon chain bound to an ionizable diphosphate group (Cheng et al., 2007). Casbene synthase catalyzes the first step in taxonomically-restricted Euphorbiaceae diterpenoid biosynthesis via the cyclization of GGPP into casbene (Kirby et al., 2010). From casbene, the downstream biosynthetic steps involved in producing bioactive *Euphorbia* diterpenoids are poorly understood, but apparently proceed through intermediates such as jolkinol via cytochrome P450-catalyzed oxidations and a short-chain alcohol dehydrogenase (King et al., 2014; Luo et al., 2016; Zerbe et al., 2013). Engineering metabolic pathways or reconstructing them in heterologous hosts requires characterization of the biosynthetic steps that underlie metabolite production in the source plant (Gandhi et al., 2015; Kitaoka et al., 2015). Microbial hosts are attractive production platforms, however diterpenoid production in microbes is lagging behind other phytochemicals, with fewer studies and lower yields. Plant tissue cultures express biosynthetic pathways of the intact plant, are amenable to metabolic studies, and can generate uniform plant biomass for screening biological activities (Gandhi et al., 2015; la Parra and

Quave, 2017; Ricigliano et al., 2015; Ricigliano et al., 2016; Yonekura-Sakakibara and Saito, 2009).

Euphorbia lathyris L., a plant native to the Mediterranean region, accumulates cytotoxic and anticancer diterpenoids called *Euphorbia* factors. Some of these compounds inhibit p-glycoprotein transporters involved in multidrug resistant cancers (Jiao et al., 2009; Lee et al., 2018; Lin et al., 2017). Hydrolysis products of ingenol-based *Euphorbia* factors from *E. lathyris* seeds have been used in the semi-synthesis of ingenol mebutate (Liang et al., 2012). Transcriptomic and metabolomic analyses of *E. lathyris* seeds identified biosynthetic machinery involved in the conversion of casbene to jolkinol, but the full pathway leading to ingenol and structurally related diterpenoids remains to be elucidated (Luo et al., 2016). Nevertheless, *E. lathyris* cells and tissues possess the biosynthetic potential for a variety of *Euphorbia* diterpenoids, including ingenol (Adolf et al., 1984; Hou et al., 2011; Bicchi et al. 2001; Jiao et al., 2013).

We established genetically transformed root cultures of *E. lathyris* to assess their diterpenoid biosynthetic capabilities and suitability for use in natural product drug screens. Transformed, or "hairy" root cultures have been established in plants that are susceptible to infection by *Agrobacterium rhizogenes*, a phytopathogen capable of horizontal genetic transfer of bacterial root-inducing DNA into the host plant genome (Nilsson and Olsson, 1997). *Agrobacterium*-derived roots exhibit many advantages compared to studying whole plants. Genetically transformed roots express the metabolite profiles of wild type plants, maintain high growth rates, and are amenable to functional genomic studies (Georgiev et al., 2007). Herein, transformed *E. lathryis* roots and field-grown plant tissues were analyzed by UPLC-PDA-HRMS (Ultra-Performance Liquid Chromatography-Photodiode Array-High Resolution Mass Spectrometry) to compare their metabolic equivalence. Culture treatment with the classical elicitor methyl jasmonate upregulated mRNA transcript levels of early diterpenoid biosynthetic enzymes, suggesting the utility of chemical elicitation to identify diterpenoid biosynthetic genes under similar regulation. We also tested the cytotoxic activities of transformed root extracts against a panel of human carcinoma cell lines derived from prostate, cervix, breast, and lung.

2. Results

2.1. Genetic transformation and establishment of *E. lathyris* root cultures

Transformed roots of *E. lathyris* were established via *A. rhizogenes*-mediated infection of greenhouse grown plants. Stem explants of *E. lathyris* were used as the target tissue for infection and root induction (Fig. 1a). Root primordia were observed at the infection sites, emerging from callus as early as 15 days after inoculation (Fig. 1b). Fully developed adventitious roots showed an abundance of root hairs along the axis that are characteristic of the "hairy root" phenotype (Fig. 1c). An isogenic root line displaying vigorous growth was propagated on hormone-free solid media (Fig. 1d), and then grown in quadruplicate liquid culture flasks on an orbital shaker to generate the experimental biomass used in subsequent experiments (Fig. 1e).
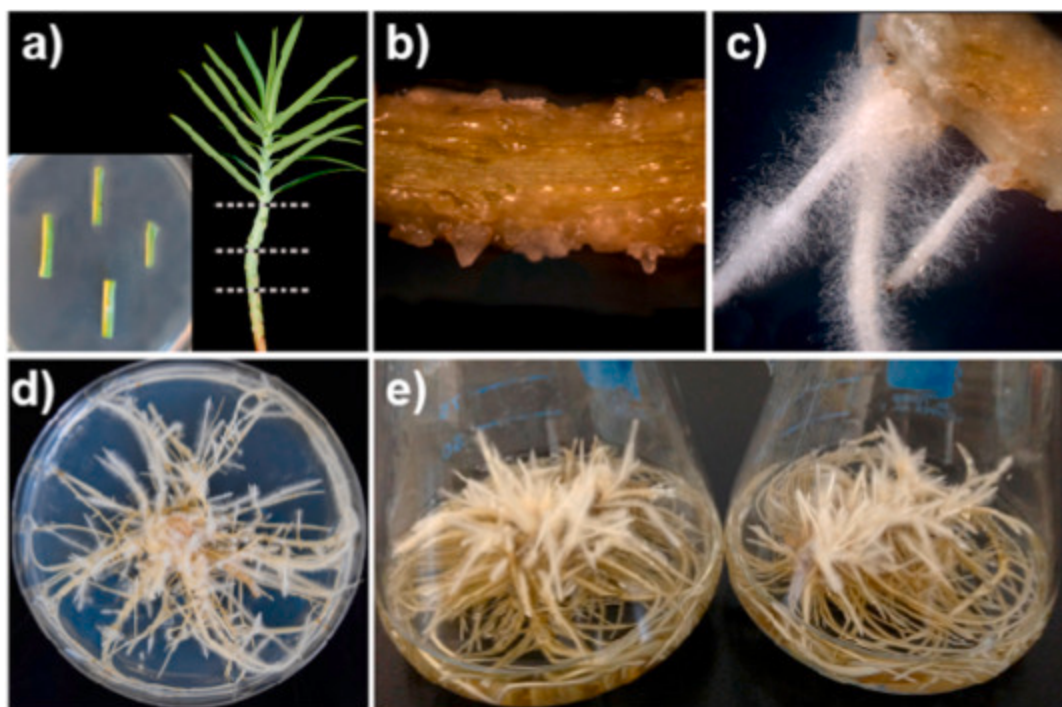
Fig. 1. Establishment and culture maintenance of transformed *E. lathyris* roots. (**a**) Stem explants of 3-week-old greenhouse grown plants were used for co-culture with *A. rhizogenes.* (**b**) Roots emerged from callus at the site of infection after 2–3 weeks.(**c**) Adventitious roots displaying the characteristic of the "hairy root" phenotype. (**d**) Growth characteristics of the isogenic root line used in this study on agar media and in (**e**) liquid media.

2.2. Metabolite profiling

As a metabolic qualification of the transformed root cultures, chemical profiles of roots stably transformed with *A. rhizogenes* harboring Ri plasmid pRi15834 were compared to wild-type plant roots and aerial tissues (leaves and stems) from soil-grown *E. lathyris* plants. Samples were extracted and analyzed via a UPLC-PDA-HRMS system, including biological triplicates (Fig. S1); representative chromatograms are shown in Fig. 2. Chemical profiles of transformed roots closely resembled those of wild-type plant roots and shared some overlap with compounds detected in aerial tissues (Fig. 2).

Several putatively assigned diterpenoid compounds were present in transformed roots that are structurally related to ingenol **(1)**. The structures were identified by comparing their accurate mass to metabolites that were isolated from *Euphorbia* spp. then analyzing the fragmentation patterns (MS/MS). This was carried out by searching the molecular formula identified from the mass spectra, identifying metabolites from *Euphorbia* spp. that have been reported in the literature, and then using the MS/MS data to assign the structures. Based on the UPLC-PDA-HRMS data, several compounds were putatively assigned. Several metabolites correlated to an identified accurate mass, and these metabolites were further narrowed down and putatively assigned based on the MS/MS fragmentation pattern as shown in Fig. 3. Based on these fragmentation patterns, nine compounds were putatively assigned to be structurally related to ingenol. These compounds are ent-16α,17-dihydroxyatisan-3-one **(2)**, jolkinol A **(3)**, 3,15-diacetoxy-5,6-epoxylathyr-12-en-14-one **(4)**, jolkinol D **(5)**, 3-*O*-benzoylingenol **(6)**, jolkinol B **(7)**, 15-*O*-acetyl-3-*O*-*iso*-butyryljolkinol-5*β*,6*β*-oxide **(8)**, jolkinoate M **(9)**, and euphoractin

J **(10)** (Fig. 2). The peak areas of compounds 1–10 revealed higher abundance in wild-type roots relative to aerial tissues. When peak areas from transformed roots were compared to wild type root and aerial tissues, relative diterpenoid abundances were between 1 and 4 times higher in transformed roots (Table 1).
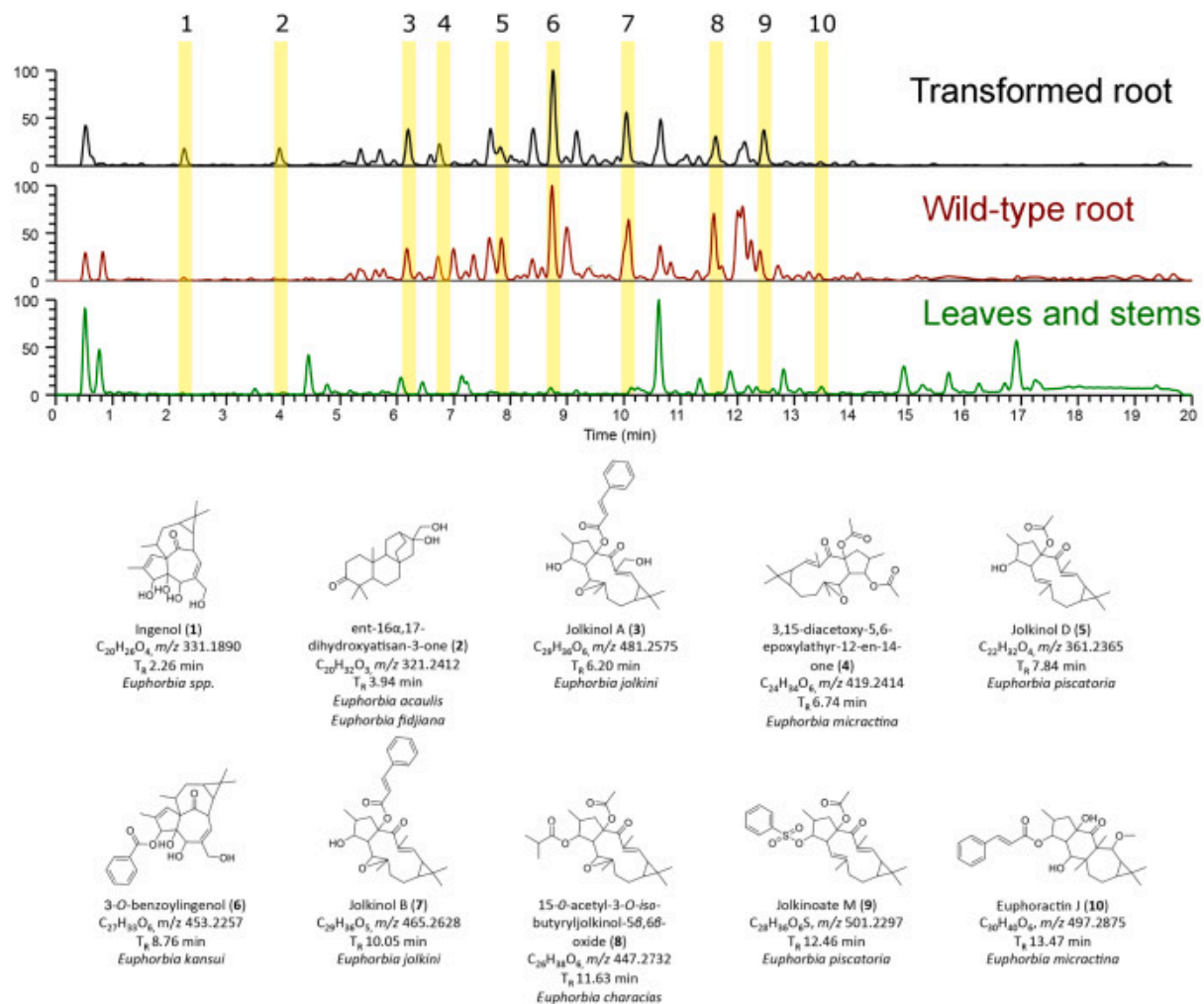

Fig. 2. Base peak chromatograms of transformed *E. lathyris* roots compared to wild-type plant roots and aerial parts with putatively assigned metabolites that are structurally related to ingenol.
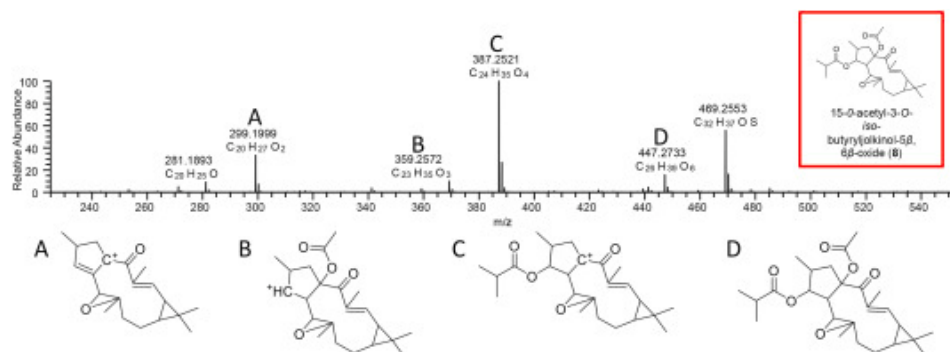

Fig. 3. The MS/MS (fragmentation) data of *m/z* 477.2732 aided in the putative assignment of compound **8** as 15-O-acetyl-3-O-iso-butyryljolkinol-5β,6β-oxide. The fragmentation structures and *m/z* values correspond to each other (i.e. structures and peaks A-D).

Table 1. Peak areas of the putatively assigned diterpenes (**1**–**10**) in transformed roots, wild type, and leaves/stem. Peak area data were generated via an extracted ion chromatogram (i.e. XIC) followed by summing the area under the curves (i.e. AUC).

m/z	Putative Molecular Formula	T_R (min)	Putative ID	Peak Area (Transform-ed Root)	Peak Area (Wild Type)	Peak Area (Leaves and Stem)
331.1890	$C_{20}H_{26}O_4$	2.3	ingenol (**1**)	1.27E+08	2.68E+07	4.76E+06
321.2412	$C_{20}H_{32}O_3$	3.9	ent-16α,17-dihydroxyatisan-3-one (**2**)	4.62E+07	1.71E+06	ND*
481.2575	$C_{29}H_{36}O_6$	6.2	jolkinol A (**3**)	1.72E+08	1.49E+08	3.41E+05
419.2414	$C_{24}H_{34}O_6$	6.7	3,15-diacetoxy-5,6-epoxylathyr-12-en-14-one (**4**)	1.29E+08	1.46E+08	ND*
361.2365	$C_{22}H_{32}O_4$	7.8	jolkinol D (**5**)	6.16E+08	1.14E+08	ND*
453.2257	$C_{27}H_{33}O_6$	8.8	3-O-benzoylingenol (**6**)	4.87E+08	4.66E+08	9.36E+06
465.2628	$C_{29}H_{36}O_5$	10.1	jolkinol B (**7**)	1.41E+09	6.56E+08	7.18E+05
447.2732	$C_{26}H_{38}O_6$	11.6	15-O-acetyl-3-O-iso-butyryljolkinol-5β,6β-oxide (**8**)	1.52E+08	3.29E+08	ND*
501.2297	$C_{28}H_{36}O_6S$	12.5	jolkinoate M (**9**)	9.11E+08	ND*	3.39E+04
497.2875	$C_{30}H_{40}O_6$	13.5	euphoractin J (**10**)	1.14E+08	1.23E+08	5.12E+05

ND* Not detected.

2.3. Bioinformatic analysis and elicitation of early diterpenoid biosynthetic genes in transformed roots

GGPP is derived from the activities of isoprenyl diphosphate synthases. These enzymes are classified according to the chain lengths of their end products, catalyzing the alkylation of one, two, or three units of isopentyl diphosphate (IPP; C5), with an allylic diphosphate such as dimethylallyl pyrophosphate (DMAPP; C5) to produce geranyl diphosphate (GPP; C10), farnesyl diphosphate (FPP; C15), and geranylgeranyl diphosphate (GGPP; C20). GGPP synthase (GGPS) alone or in regulated coordination with FPP synthase (FPS) may use DMAPP, GPP, or FPP as its allylic substrate to produce GGPP. In view of the key role of these enzymes in early diterpenoid biosynthesis, FPS and GGPS transcripts were amplified from *E. lathyris* transformed root mRNA for bioinformatic and gene expression analyses. The predicted amino acid sequences showed high similarity to other members of the Euphorbiaceae family and *Arabidopsis*. The deduced *ElFPS* sequence exhibited 98.9%, 93.1, 93.0%, 92.2%, and 83.4% identity to *Euphorbia pekinensis* (ACN63187), *Hevea brasiliensis* (ABR09548), *Jatropha curcas* (KDP35531), *Ricinus communis* (XP_002534338), and *A. thaliana* (U80605), respectively. The deduced *ElGGPS* sequence exhibited 89.7%, 88.5%, 84.2%, 83.4%, and 70.1% identity to *Euphorbia esula* (PRJNA411945), *Hevea brasiliensis* (BAF98302), *Jatropha curcas* (KDP31808), *Ricinus communis* (XP_002525096), *A. thaliana* (AAB67730), respectively. Two conserved aspartate-rich domains [DDxx(xx)D], FARM (First Aspartate Rich Motif) and SARM (Second Aspartate Rich Motif) are characteristic of prenyl diphosphate synthases (Gupta et al., 2011; Ohnuma et al., 1996). The FARM and SARM regions act as binding sites for allylic substrates and IPP, and are crucial to catalytic activity. Amino acid alignment of *ElFPS* with known FPP synthase accessions revealed the conserved FARM and SARM motifs at positions 93–97 aa and 232–235 aa, respectively (Fig. 4a). Sequence alignment of *ElGPS* with known GGPP synthase accessions indicated the FARM and SARM motifs at positions 162–168 aa and 303–308 aa, respectively (Fig. 4b).
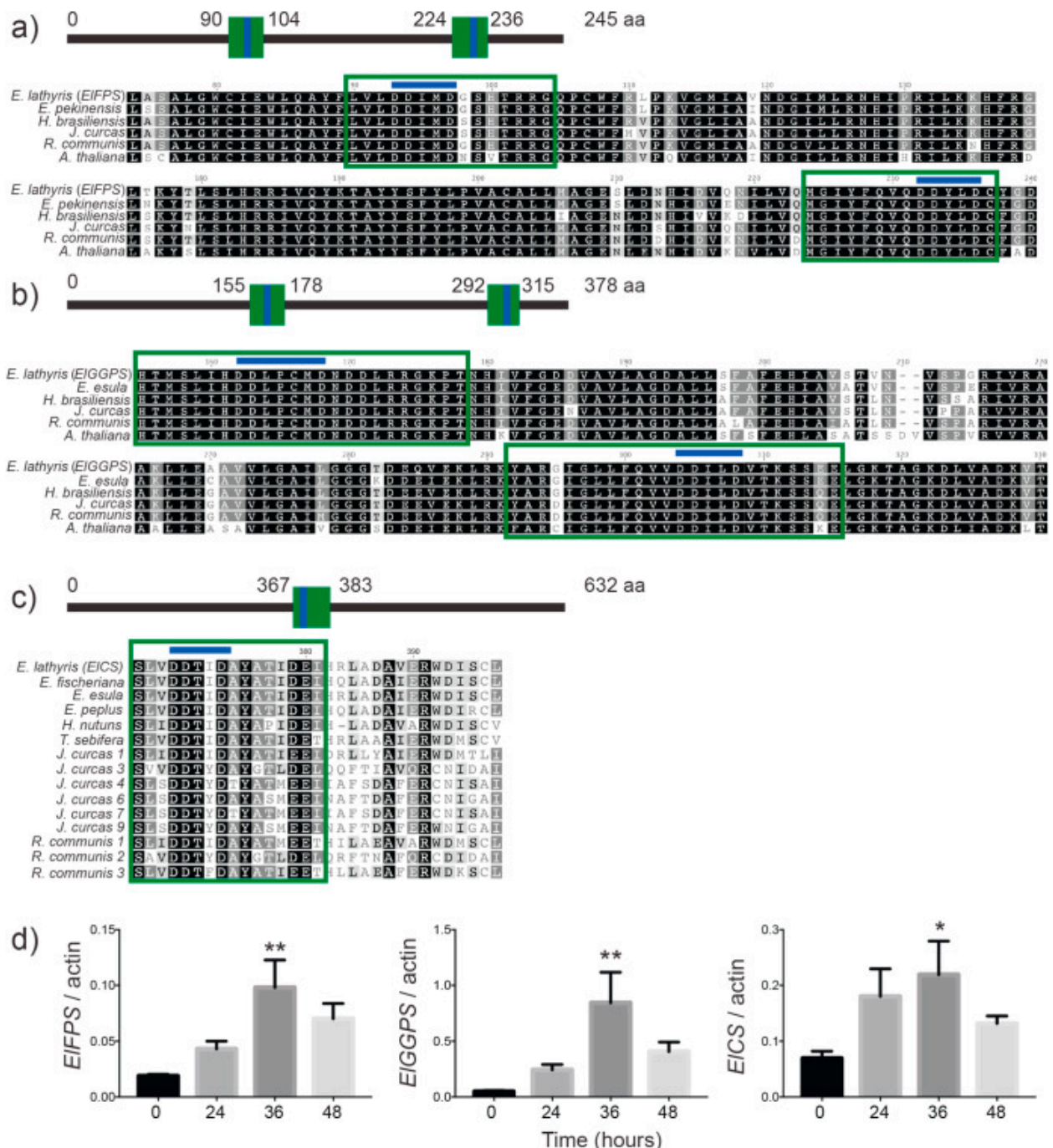
Fig. 4. Bioinformatic analysis and chemical elicitation of early diterpenoid biosynthetic genes expressed in transformed *E. lathyris* roots. Comparison of the deduced amino acid sequences of (**a**) *ElFPS* and (**b**) *ElGGP* highlighting two conserved aspartate-rich domains [DDxx(xx)D]. (**c**) Comparison of the deduced amino acid sequence of *ElCS* highlighting a conserved [DDxxD] motif that is essential to the cyclization functionalities of terpene synthases. (**d**) Time course of *E. lathyris* diterpenoid biosynthetic gene transcript levels in transformed root cultures treated with 100 μM methyl jasmonate. Asterisks indicate statistical significance in comparison to 0 h control assessed by one-way ANOVA (**, $P < 0.01$; *, $P < 0.05$).

Casbene synthase catalyzes the first committed step in the formation of diterpenoid backbones taxonomically restricted to the Euphorbiaceae family via the cyclization of GGPP into casbene. A casbene synthase transcript (*ElCS*) was amplified from transformed root mRNA. The deduced

amino acid sequence of *ElCS* exhibited 90.8%, 89.7%, and 86.4% identity to *Euphorbia fischeriana* (JN862821), *Euphorbia esula* (GU332591), and *Euphorbia peplus* (AGN70884), respectively. Within the Euphorbiaceae family, *ElCS* shared the following pairwise amino acid identities: 76.4% with *Triadica sebifera* (GU332590), 67.9% with *Homalanthus nutans* (GU332592), 62.8–46.2% with *J. curcas* (BAJ53213, BAJ53216, BAJ53218, BAJ53219, BAJ53220, BAJ53221), and 73.9–47.5% with R. communis (L32134, XM_002513323, XM_002513288). Protein alignment revealed a conserved [DDxxD] motif at positions 370–374 aa that is essential to the cyclization functionalities of terpene synthases, likely through association with Mg^{2+} (Aubourg et al., 2002). Taken together, these results suggest that *ElFPS*, *ElGPS, and ElCS* are catalytically active, possessing functional attributes that are comparable to homologous genes.

The transcript expression patterns of *ElFPS*, *ElGPS*, and *ElCS* were profiled in transformed roots treated with methyl jasmonate (MeJA), a classical elicitor of plant secondary metabolism. Gene expression was monitored over a 48-h period after addition of 100 μM MeJA. At 36 h, the *ElFPS*, *ElGGPS*, and *ElCS* mRNAs were transiently induced, 5.4-, 17.1-, and 3.2-fold, respectively (Fig. 4c). These results indicate that *ElFPS*, *ElGGPS*, and *ElCS* are coordinately regulated at the transcriptional level in response to chemical elicitation.

2.4. Cytotoxicity bioassay

The cytotoxic properties of wild-type *Euphorbia* plant extracts and isolated diterpenoids are a topic of significant research interest, but studies are limited by low-level accumulation of medicinal compounds *in planta*. Tissue culture technologies enable the production of uniform plant biomass, thus improving access to these compounds for use in chemical and pharmacological studies. To test the antiproliferative effects of *E. lathyris* transformed roots in human cells, DU-145 (prostate), HeLa (cervix), MCF-7 (breast), MDA-MB-231 (breast), and H2347 (lung) carcinoma lines were treated with increasing concentrations of *E. lathyris* transformed root extract. After 48 h post-treatment, MCF-7 and MDA-MB-231 cells exhibited a significant reduction in viability in response to a concentration of 31.3 μg/mL extract (Fig. 5c and d). DU-145 cells exhibited a significant reduction in viability at a concentration of 62.5 μg/mL (Fig. 5a). HeLa cells showed a significant reduction in viability at a concentration of 125 μg/mL (Fig. 5b). In H2347 cells, a significant reduction in viability was observed at a concentration of 250 μg/mL (Fig. 5e). Relative to the carrier control, the 250 μg/mL concentration of root extract inhibited the different cell lines as follows: DU-145 (24.4%), HeLa (57.0%), MCF-7 (66.9%), MDA-MB-231 (38.1%), H2347 (70.6%). These results highlight the potential of *E. lathyris* transformed roots for natural product drug discovery applications.
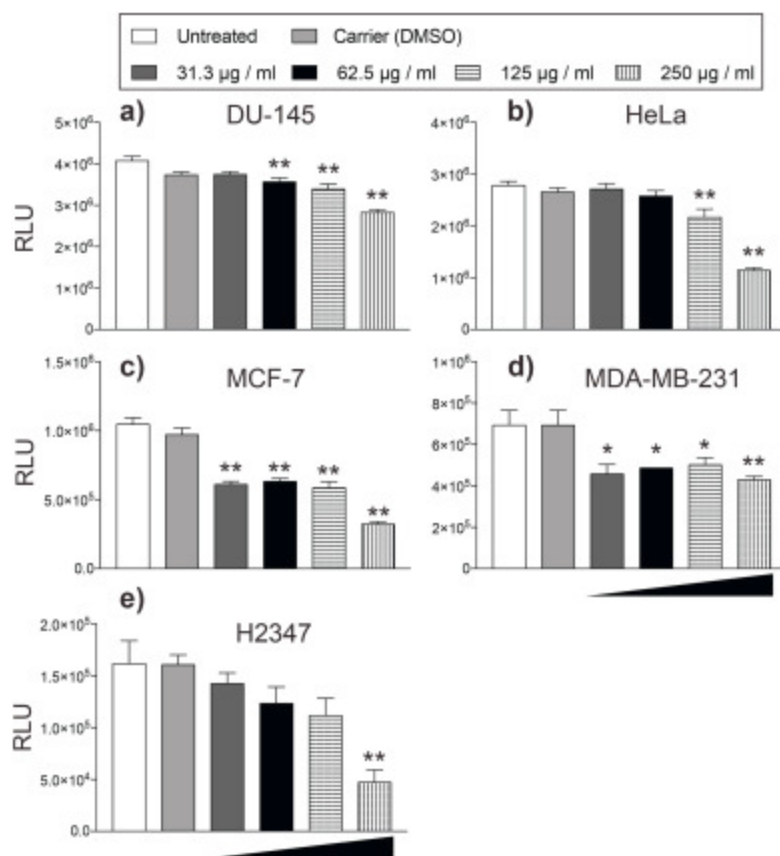
Fig. 5. Cytotoxic activities of transformed *E. lathyris* root extract in human carcinoma and embryonic cell lines. **a)** DU-145 (prostate) **b)** HeLa (cervix) **c)** MCF-7 (breast) **d)** MDA-MB-231 (breast) **e)** and H2347 (lung) were treated with DMSO (carrier), or increasing concentrations of transformed root MeOH extracts (31.3 μg/ml, 52.5 μg/ml, 125 μg/ml, 250 μg/ml). Titer-Glo® (Promega) was used to count cell lines 48 h post-treatment. Each dose and timepoint was performed in triplicate. Asterisks indicate statistical significance in comparison to carrier (DMSO) control assessed by one-way ANOVA (**,$P < 0.01$; *,$P < 0.05$).

3. Discussion

Currently, most *Euphorbia* and Euphorbiaceae family diterpenoids can only be obtained from the source plants. However, many species are slow-growing and accumulate small quantities of medicinal compounds. Our results indicate that *E. lathyris* transformed roots reproduce the metabolism of field-grown plants and are a promising source of *Euphorbia* diterpenoid structures. Additionally, transformed roots are a suitable platform to study *Euphorbia* diterpenoid biosynthesis and may prove useful in functional genomic screens of candidate metabolic genes. Since pharmacological studies are limited by low yields from plants, transformed root cultures could be a source of uniform *Euphorbia* biomass for natural product drug discovery applications.

Alternative sources of ingenol have been a topic of significant research focus due to the interesting pharmacological properties exhibited by naturally occurring conjugates and semi-synthetic derivatives (Abreu et al., 2014; Silva et al., 2019; Vasas et al., 2012). Biotechnological production in microbes is a promising approach to increase the accessibility of high value diterpenoids (Zerbe et al., 2013). Some studies have used heterologous yeast expression systems

to test the contribution of candidate Euphorbiaceae biosynthetic genes to diterpenoid production. This has proved to be useful in identifying casbene synthases responsible for the cyclization of GGPP into casbene (Kirby et al., 2010). From casbene, the routes to highly oxidized casbene derivatives, including ingenol, are less understood but likely proceed through intermediates such as jolkinol via cytochrome P450-catalyzed oxidations and a short-chain alcohol dehydrogenase (King et al., 2014; Luo et al., 2016; Zerbe et al., 2013). Recently, high-titer production of jolkinol by yeast was reported (Wong et al., 2018). Similar to other studies aimed at reconstituting complex oxidized terpenoid biosynthetic pathways in yeast, optimization of heterologous cytochrome P450 expression and precursor flux was a prerequisite (Paddon et al., 2013). Still, the primary bottleneck in heterologous ingenol production is complete pathway elucidation, which has proven difficult due to its structural complexity and the number of casbene modifiers encoded by *Euphorbia* genomes (Zerbe et al., 2013).

4. Concluding remarks

Here, we found that transformed roots derived from *A. rhizogenes*-mediated transformation of *E. lathryis* possess the biosynthetic capacity for ingenol and related diterpenoid structures. A broad diversity of diterpenoids have been identified in aerial tissues and roots of *E. lathryis*, but most studies have focused on seed accumulation of ingenol-based *Euphorbia* factors. Indeed, *E. lathyris* seeds have been used as starting material to obtain ingenol hydrolysis products for the semi-synthesis of ingenol mebutate (Liang et al., 2012). *Euphorbia spp.* roots have been the focus of numerous studies in which diterpenoids were identified and assayed for biological activities (Lee et al., 2016; Liu et al., 2014; Shi et al., 2005; Ul-Haq et al., 2012). These findings are in agreement with our results that transformed roots are a viable approach to *Euphorbia* diterpenoid production *in vitro*. Induction of transformed root cultures in a variety of Euphorbiaceae species has potential to increase the current supply of diterpenoids. Other *Euphorbiacae* species contain diterpenoids of pharmacological significance that could potentially be produced via transformed roots. For example, prostratin from *Homalanthus nutans* (Kulkosky et al., 2001) and resiniferatoxin from *E. resinifera* (Kissin and Szallasi, 2011). Transformed roots are a genetically tractable platform that could be used to test the contributions of candidate biosynthetic genes to metabolite accumulation and pharmacological activities. Combinatorial biosynthesis employing non-native biosynthetic enzymes within the metabolic scaffold of select species could enable the future identification of novel metabolites with augmented pharmacological activities.

5. Experimental

5.1. Establishment and maintenance of *E. lathyris* transformed root cultures

Seeds of *Euphorbia lathyris* were obtained from Plant World Seeds (Newton Abbot, UK) and used to produce the plant materials in this study. Relying on standard procedures described in the literature (Georgiev et al., 2007), stem segments from 6-week-old greenhouse grown *E. lathyris* plants were used for co-cultivation and root induction. *A. rhizogenes* strain 15834 harboring root-inducing plasmid pRi15834 was used to carry out the transformation. Explants were submerged in 1% sodium hypochlorite for 10 min, then rinsed three times with sterile H_2O and 1 cm removed from each end with a sterile scalpel. The explants were submerged in *Agrobacterium*

suspension, blotted dry, and incubated on hormone-free ½ MS medium (Murashige and Skoog, 1962) containing 3% sucrose and solidified with 1% agar for 25 ± 2 °C in the dark. Following 3 days of co-cultivation, the explants were subcultured to MS medium containing 150 mg/L timentin to kill the excess *Agrobacterium*. In approximately 2.5 weeks, hairy roots developed from the cut surfaces and along the axis of the explants. Single root segments were removed from different explants to avoid the unintended propagation of the same clone. For liquid cultures, approximately 200 mg of transformed root biomass was transferred to 30 mL of liquid MS medium and grown in darkness at 25 ± 2 °C on an orbital shaker at 100 rpm with subcultures performed every 3 weeks.

5.2. Metabolite extractions

Separately, biomass from transformed roots, wild-type roots, and wild-type leave/stems were ground using a mortar and pestle. MeOH was added to each sample of ground plant material to a concentration of 0.1 g/mL. Then, the samples were shaken overnight (~16 h) at 100 rpm at room temperature. The samples were filtered using vacuum filtration, and the remaining residues were washed with MeOH. MeOH-H_2O-hexanes (9:1:10) was added to the filtrate. The mixtures were stirred for 0.5 h, and then transferred into separatory funnels. The bottom layers were drawn off into round-bottom flasks, which were evaporated to dryness. The dried MeOH extracts were reconstituted in H_2O-MeOH-chloroform (9:1:10). The biphasic solutions were shaken vigorously. The layers were separated and evaporated to dryness under vacuum.

5.3. Metabolite profiling via UPLC-PDA-HRMS

The QExactive Plus mass spectrometer operated over a range from *m/z* 130 to 1900 at a resolution of 70,000. The voltage for both positive and negative ionization modes were set to 3.7 kV, with a nitrogen sheath gas set to 25 arb and an auxiliary gas set to 5 arb. The S–Lens RF level was set to 50.0 with a capillary temperature at 350 °C. The flow rate of the UPLC was set to 0.3 mL/min using an Acquity UPLC HSS T3 column (2.1 × 50 mm × 1.7 μm) equilibrated at 40 °C. The mobile phase consisted of Fisher Optima LC–MS (Liquid Chromatography-Mass Spectrometry) grade acetonitrile–H_2O (acidified with 0.1% formic acid), starting at 15% acetonitrile and increasing linearly to 100% acetonitrile over 17 min. It was held at 100% acetonitrile for 1.5 min before returning to starting conditions for re-equilibration. The PDA was set to acquire from 190 to 500 nm with 4 nm resolution.

5.4. Metabolite analyses

SciFinder (https://scifinder.cas.org/) was used to putatively assign the diterpenoid compounds structurally related to ingenol **1–10** (Fig. 2) based on mass spectral comparisons to previously reported structures isolated from *Euphorbia* spp. This was carried out by searching the molecular formula obtained from the mass spectra through the database, then identifying compounds that were previously reported from *Euphorbia* spp. Then by using the MS/MS data, the compounds were putatively identified. The peak areas for the compounds were determined from the area under the curve (AUC) of an Extracted-Ion Chromatogram (XIC) with a window of ±5.0 ppm.

5.5. Sequence analyses of diterpenoid biosynthetic genes

The nucleotide and predicted amino acid sequences of early diterpenoid biosynthetic genes were analyzed using Geneious version 7.0 (Biomatters). Transcripts corresponding to farnesyl diphosphate synthase (FPS), geranylgeranyl diphosphate synthase (GGPS), and casbene synthase (CS) were amplified from a transformed root cDNA library using primers based on the consensus sequence of previously identified *Euphorbiaceae* orthologs. The nucleotide sequences of the *E. lathryis* biosynthetic genes amplified from transformed roots are deposited in EMBL/DDBJ/GenBank under the following accessions: *ElFPS* (MK512674), *ElGGPS* (MK512675), and *ElCS* (MK559432). GenBank accessions used in the *ElFPS* amino acid sequence comparison were as follows: *Euphorbia pekinensis* (ACN63187), *Hevea brasiliensis* (ABR09548), *Jatropha curcas* (KDP35531), *Ricinus communis* (XP_002534338), and *A. thaliana* (U80605). GenBank accessions used in the *ElGGPS* amino acid sequence comparison were as follows: *Euphorbia esula* (PRJNA411945), *Hevea brasiliensis* (BAF98302), *Jatropha curcas* (KDP31808), *Ricinus communis* (XP_002525096), *A. thaliana* (AAB67730). GenBank accessions used in the *ElCS* amino acid sequence comparison were as follows: *Euphorbia fischeriana* (JN862821), *Euphorbia esula* (GU332591), *Euphorbia peplus* (AGN70884), *Triadica sebifera* (GU332590), *Homalanthus nutans* (GU332592), *J. curcas* (BAJ53213, BAJ53216, BAJ53218, BAJ53219, BAJ53220, BAJ53221), and Ricinus communis (L32134, XM_002513323, XM_002513288).

5.6. Gene expression analyses

To test the effects of elicitation on biosynthetic gene expression, transformed roots were maintained in liquid ½ MS medium containing 3% sucrose in darkness on an orbital shaker at 25 ± 2 °C and 100 rpm. A 10 mM solution of MeJA was prepared in EtOH:H_2O (1:1 v/v) and was added to 30 mL of liquid ½ MS to a final concentration of 100 μM. A solution of 1:1 v/v EtOH:H_2O was added to the cultures as a control. Three-week-old vigorous cultures were sampled at 0, 24, 36, and 48 h time points following MeJA treatment. Total RNA was isolated using an RNeasy plant mini-kit (Qiagen) according to the manufacturer's instructions.

Primers with annealing temperatures between 60 and 64 °C and amplicons of 185–292 bp were designed using Geneious version 7.0 (Biomatters) based on the sequence of biosynthetic gene transcripts amplified from *E. lathyris* transformed root cDNA. Primer specificity was confirmed by cloning and sequencing of the PCR products. Quantitative PCR was performed on an iCycler iQ™ Real-Time PCR system (Bio-Rad), using iScript™ One-Step RT-PCR Kit with SYBR® Green as per manufacturer instructions (Bio-Rad) with gene-specific primers. *E. lathyris* actin (GenBank: KC182753.1) was used as an internal reference for normalization. Three biological and three technical replicates were measured to confirm the accuracy of the results, and to determine standard error. Relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Statistical analyses was performed using one-way ANOVA. Primers used in the qPCR expression analysis are as follows: *actin* (5′-AGCCGTTCTGTCATTGTATGCC-3′ and 5′-AAGTTTCTCCTTCATGTCACGG-3′), *ElFPS* (5′-AGACGTGGTCAGCCTTGTTGG-3′ and 5′-ATCATCTGTCCTGAAGCTGTTTGG-3′), *ElGGPS* (5′-TGCACATGGATGAACTCAAGCG-3′ and 5′-ACGATGACCTCCCGTGTATGG-3′), *ElCS* (5′-TCAAACAACACATAACGAACGCC-3′ and 5′-TTCGGCCATTCTGTCTCTTGCG-3′).

5.7. Human carcinoma cell culture and cytotoxicity bioassay

DU-145 (prostate), HeLa (cervical), MCF-7 (breast), MDA-MB-231 (breast), H2347 (lung) carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with FBS (5% v/v), nonessential amino acids (100 mM), L-glutamine (5 mM), streptomycin (100 μg/mL), and penicillin (100 units/mL); all from BioWhittaker, Walkersville, MD. Cells were grown at 37 °C, in a 5% CO_2, 95% air humidified tissue culture incubator.

For the transformed root extract used in the cytotoxicity bioassays, 3 g of air-dried transformed root biomass was ground using a mortar and pestle. MeOH was added to the ground plant material to a concentration of 0.1 g/mL. The sample was shaken overnight at 100 rpm at room temperature. The sample were filtered using vacuum filtration and the MeOH was removed under vacuum to yield a yellow semi-solid that was dissolved in DMSO. To elucidate the antiproliferative activities of the root extract, carcinoma cell lines were treated with either DMSO (carrier), or increasing concentrations of *E. lathyris* root MeOH extracts (31.3 μg/mL, 52.5 μg/mL, 125 μg/mL, 250 μg/mL). Dilutions were prepared with cell culture medium in 1.5 mL tubes, then vortexed thoroughly each time immediately prior to adding them to cell culture plates. After 48 h post-treatment, cells were trypsinized, harvested and vortexed to produce a single-cell suspension. Cells from each treatment condition were counted using CellTiter-Glo® Luminescent Cell Viability Assay (Promega). For each sample, a volume of CellTiter-Glo® Reagent equal to a volume of cell culture medium containing cells was placed into a standard 12 mm luminescence tube and vortexed. Each tube was incubated for 10 min at room temperature to stabilize luminescent signal. Luminescence was determined using a Sirius single tube luminometer (Berthold). All CellTiter-Glo® luciferase results were reported as relative light units (RLU). Each dose and timepoint was performed in triplicate, and statistical analyses was performed using one-way ANOVA.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at
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