Antimicrobial properties of phytohormone (gibberellins) against phytopathogens and clinical pathogens

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Abstract

The *in vitro* antimicrobial potential of physiologically active diterpenoid plant-derived gibberellins (gibberellic acids; GAs) was tested on microbial pathogens of significance to plant and human health. The racemic enantiomer GA3 produced varying inhibitory effects against a wide range of plant host disease causal agents (phytopathogens) comprising fungi, oomycetes and bacteria. The results showed that GA3 effected either strong growth arrest of phytopathogenic fungi or holistic biocidal effects on oomycete and phytopathogenic fungi at higher concentration (>10–50 mM) and increased hyphal extension growth when the concentration of GA3 was lowered (<10–0.1 mM). When human clinical pathogenic bacteria cohorts were challenged with gibberellin enantiomers, viz GA1, GA4, GA5, GA7, GA9 and GA13 (50 mM), employing Kirby–Bauer disc bioassay methods for assessment of their efficacies, no inhibitory effect was seen with gibberellin enantiomers, viz GA1, GA3, GA5 and GA13, while GA4 inhibited all human clinical bacterial organisms examined, with GA_7 and GA_9 showing limited activity. The antibiotic effects of enantiomeric diterpenoid phytohormones evinced in our preliminary study raise prospects for further studies to fully examine their potential therapeutic value for human healthcare and their compliance with cytotoxicity and other ethical considerations in the future.

INTRODUCTION

Gibberellins (gibberellic acids – GAs) are diterpenoid plant-derived hormones that have been studied most commonly in agriculture for nearly 100 years. GAs mediate various plant developmental and growth processes, including seed germination, shoot elongation and flower initiation and development, and ~136 different gibberellin structures have been identified, named numerically in order of discovery [1]. Gibberellins are present in varying quantities and concentrations in diverse plants and fungi. In terms of plant health, there is worldwide interest in phytohormone biostimulant applications. Biostimulants and the recent 'bio-effectors' (BEs) (www.biofector.info) comprise bio-active natural compound extracts from seaweed, plants and composts, as well as micro-organisms (bacteria, fungi), also collectively known as plant-growth-promoting microbes (PGPMs), and have the ability to improve growth, nutrient acquisition and stress tolerance of crops, being utilized for either crop production or protection [2]. Recent reviews [3, 4] have highlighted the increasing trend for utilizing biostimulants for their functional benefits against the backdrop of climate change and the concomitant pressures of biotic and abiotic stress.

Foliar application of GAs has been known for its role in plant stem elongation and mitigation of abiotic stress under the growing demands of global climate change mitigation. Whilst the antimicrobial effects of one of the phytohormones, gibberellic acid GA3, on plant colonizing phytopathogens is highlighted in our study, it would be interesting to examine the effects of phytohormones upon plant-growth-promoting fungi such as *Gliocadium* spp. and other commercial microbial alternative control agents [5], which are poorly understood for plant health improvement.

In recent years, gibberellins have been studied as possible therapeutic options in a wide range of medical fields, particularly with anti-tumour activity for the treatment of lung

cancer [6]. Antibiotic resistance presents a significant challenge to clinical, veterinary and plant health and is now recognized by the World Health Organization (WHO) as a major emerging problem of global significance. Most common fungi themselves are also known to produce large amounts of GA3 (e.g. *Fusarium fujikuroi*) [7]. Our primary aim was restricted to plant-derived diterpenoid phytohormones and we thus focused upon investigations into natural antimicrobials, especially from plants, in our present investigations. Historically, plants have been a rich source of medicines, ranging from chemotherapeutics compounds, anti-inflammatories to antimicrobial agents, where such therapeutic activity has been recognized and exploited by traditional medicine in many countries, particularly in Asia. For example, in an earlier study [8] we demonstrated antimicrobial activity of *Callendula officinalis* petal extracts against fungi, as well as Gram-negative and Gram -positive clinical pathogens.

To date, there have been no reports on the antibacterial properties of gibberellins from plants and therefore it was the aim of this study to examine the potential antibacterial properties of gibberellins against Gram-negative and Gram-positive clinical pathogens. Further, while plant and/or fungal production of gibberellic acids has been described, the antimicrobial effects of gibberellic acids on plant pathogenic fungi, oomycetes and bacterial causal agents of disease on a wide range of host plants have been scarcely reported. Amongst the racemic forms of GAs, the most bioactive is GA3, followed by GA4, and they have been known to play an exponential role in promoting plant growth [9]. In this study, we have focused on GA3's antimicrobial potential, seeking to obtain information and knowledge concerning its antimicrobial effects on phytopathogenic microbes that plant cells would encounter in an infected plant cell environment. Some physiological evidence demonstrates that plants infected with endophytic fungi often have a distinct advantage against biotic and abiotic stress over their endophyte-free counterparts [10]. Gibberellins are defined by their enantiomeric (ent–) structure rather than their biological activity. They are all cyclic diterpenes with an ent–gibberellane ring structure. Two main types of GAs are recognized, those with the full complement of 20 carbon atoms, the C20 GAs, and the C19 GAs, which have lost one C atom and possess a lactone. The biologically active GAs in higher plants are C19 compounds. The gibberellins are tetracyclic diterpenoid acids, strictly related, representing an important group of plant growth hormones. All known gibberellins are identified by subscribed numbers, GAn, where n corresponds, approximately, to the order of discovery. Gibberellic acid, which was the first gibberellin to be structurally characterized, is GA$_3$ [11]. Many structural modifications can be either made or may be encountered in cells as the ent–gibberellane ring system diversity accounts for the large number of known GAs [12]. This study therefore aims to understand some of the known GAn (n=1, 3, 4, 5, 7, 9 and 13) cyclic diterpenes with an ent–gibberellane ring structure, with a view to understanding their antibiotic effects on either clinical or plant-associated pathogens. Bearing in mind the potential cytotoxicity of some or all of these gibberellins, the antimicrobial assays were performed at sublethal concentrations in this study, which is intended to be a first information report to ascertain whether or not the gibberellin structures possess antibiotic-like activity against clinical and plant pathogens per se.
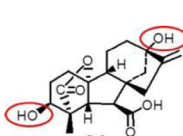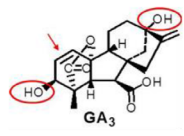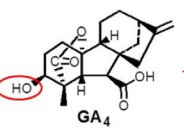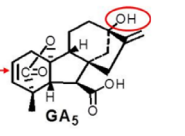
In order to obtain an insight into the antimicrobial inhibitory effects of ent–gibberellic acids, in this study we examined the biocidal/biostatic effects upon a wide range of plant host disease causal agents (phytopathogens) comprising fungi, oomycetes and bacteria, and with a view to finding the extended range of the anticancer chemotherapeutics of terpenoids already known in human healthcare, we concomitantly challenged cohorts of human clinical pathogenic bacteria with gibberellins to evaluate their antibiotic effects.
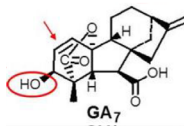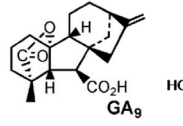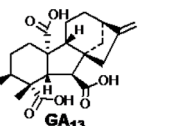
METHODS

Antifungal effects of gibberellic acid GA3 against phytopathogens

In order to obtain an insight into the antibiosis between plant pathogens and gibberellic acids, GA3 was selected as a test case, in that, this bioactive natural racemic form, which is commercially available in abundance, is more amenable than the others to Kirby–Bauer disc assay [13] for assessing its antimicrobial effects on phtyopathogens as follows. Fungal and oomycete cultures comprising ~6 mm diameter plugs were excised and transferred individually to the Cartesian co-ordinate centre of four directional segments marked previously using a fine-tip marker pen on potato dextrose agar (PDA) medium incorporating GA3 at concentrations of 0, 0.1, 1, 5, 10, 20 ad 50 mM on fresh plates of PDA, and incubated for 3 days at ambient temperature to facilitate natural contours of hyphal growth. The culture plates were examined using a binocular microscope and the outline of the perimeter of the hyphae was carefully traced by marker pen. At 3-day intervals, the extent of the hyphal growth (mm) was marked up to 3 weeks. The final area covered by each culture was measured using the bio-imaging technique [14] to assess growth vigour using the Autochemisystem UVP Bioimaging system (UVP Products, Cambridge, UK), supported by the LabWorks software package, which yields the 'area of irregular contours' of fungal growth, providing a more accurate estimate of the *in vitro* growth intensity of the test fungi measured in comparison to their respective controls in which no GA (0 mM) was supplied.

In the case of bacterial phytopathogens, the Kirby–Bauer Petri plate assay, 200 µl of an overnight bacterial broth culture was spread on each PDA plate with a sterile glass hockey stick and allowed to dry to form a 'lawn' of the test bacterial growth on the agar surface. Sterile and void micro-antibiotic discs were saturated using a maximum 20 µl GA3 at various concentrations. Using sterilized tweezers, the discs were transferred to the Petri dishes containing the microbial lawns. The cultures were incubated overnight at 30 °C to facilitate bacterial growth. Next day, the cultures were examined for sensitivity as shown by clear areas visible on the lawns surrounding the discs where microbial growth had been prevented, i.e. the

Bacteria	Gibberellin			

Gram-negative	GA₁	GA₃	GA₄	GA₅
Escherichia coli NCTC 9001	Growth*	Growth	No Growth**	Growth
Raoultella planticola NCTC 9528	Growth	Growth	No Growth	Growth
Pseudomonas aeruginosa NCTC 10662	Growth	Growth	No Growth	Growth
Salmonella Nottingham NCTC 7832	Growth	Growth	No Growth	Growth
Gram-positive				
Bacillus cereus NCTC 7464	Growth	Growth	No Growth	Growth
Listeria monocytogenes NCTC 11994	Growth	Growth	No Growth	Growth
Staphylococcus aureus NCTC 6571	Growth	Growth	No Growth	Growth

Bacteria	Gibberellin		

Gram-negative	GA₇	GA₉	GA₁₃
Escherichia coli NCTC 9001	Growth	Growth	Growth
Raoultella planticola NCTC 9528	Growth	Growth	Growth
Pseudomonas aeruginosa NCTC 10662	No Growth	No Growth	Growth
Salmonella Nottingham NCTC 7832	No Growth	Growth	Growth
Gram-positive			
Bacillus cereus NCTC 7464	No Growth	No Growth	Growth
Listeria monocytogenes NCTC 11994	No Growth	No Growth	Growth
Staphylococcus aureus NCTC 6571	No Growth	Growth	Growth

Fig. 1. Activity of seven gibberellins against Gram-negative and Gram-positive clinical pathogens. *growth, detectable and active proliferation of bacteria on agar surface. **no growth, complete absence of detectable and active proliferation of bacteria on agar surface.

zone of inhibition and the area of microbial antibiosis were measured against a control that did not receive GA3 in the media.

Antibiotic effects of racemic forms of gibberellins (GAs) on human pathogenic bacteria

Seven species of clinical pathogens were included in this study, as detailed in Fig. 1. These included four Gram-negative and three Gram-positive organisms. All isolates were obtained from the Northern Ireland Health and Social Care (HSC) Trust Microbiology Culture Respository, MicroARK (identifier for microbiological archives), housed at the Northern Ireland Public Health Laboratory, Belfast City Hospital. All isolates were recovered and passaged twice on Columbia agar base (Oxoid CM0331, Oxoid Ltd, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood, which was incubated at 37 °C for 48 h, prior to employment in the current study. The antimicrobial properties of the gibberellins on human clinical pathogens was determined as previously described [15]. A fresh culture of each isolate was prepared as described above and harvested into 0.1% (w/v) peptone saline (CM0733) diluent to yield a 0.5 McFarland inoculation

standard. Each inoculum was streaked onto fresh agar base (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood and allowed to dry. Gibberellins (GA1, GA3, GA4, GA5, GA7, GA9 and GA13) as detailed in Fig. 1 were reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK) to a concentration of 50 mM. GA1, GA4, GA5, GA7, GA9 and GA13 were a gift from Peter Hedden, Rothamsted Research Station, and GA3 was purchased commercially (Sigma, UK). Gibberellins (10 µl) were added separately onto the surface of the preinoculated bacterial plates and allowed to dry. Plates were subsequently incubated at 37 °C for 48 h prior to reading. As a control, DMSO was also examined for inhibitory properties. Inhibition was defined as a zone of no growth, surrounded by confluent bacterial growth.

Statistical scrutiny of data emerging from disparate cultural methods for estimating clinical bacterial, plant pathogens and fungal isolates

The standard error calculations emerging from each data set comprising clinical bacterial cohorts tested using different methodological or media regimes, fungi (cultural and using plugs on potato dextrose media for fungal hyphal inhibition

assays) and microbiological (plate culture) analyses would have variant measurement protocols and also unequal replicates. To statistically validate the assumptions made for standard errors and the linear correlation (magnitude of variance) between the above disparate methods, an Altman–Bland estimation [16] was used for drawing comparative relationships of agreement between variant measurements and unequal replicates whilst achieving the end point titre of growth observations, as previously described and adapted by Carmichael and Rao [17].

RESULTS AND DISCUSSION

The phytopathogenic fungi or oomycetes (e.g. *Phytophthora*) and bacterial disease causal agents of a wide range of plant hosts were grown on PDA incorporating GA3 at low (0.1, 1, 5 mM), medium (10, 20 mM) and high (50 mM) concentrations (Table 1). All taxa of phytopathogenic fungi or oomycetes showed increased hyphal extension growth as the concentration of GA3 decreased. To this end, it is observable that low concentrations appear to invigorate fungal hyphal growth. It is noteworthy that, interestingly, it is a very common feature amongst phytohormones such as gibberellic acid GA3 to elicit host cellular growth and/or cell elongation at physiologically lower concentrations. Only two of the 10 taxa were able to make any outgrowth into PDA incorporating 50 mM GA3 (*Cryptocline taxicola* and *Dreschlera graminea*). By contrast, *Microdochium nivale majus*, *Phytophthora kernoviae* and *Phytophthora ramorum* only began to show extension growth at 5 mM GA3. Ultimately, 0.1 mM GA3 acted as a growth promoter *for P. ramorum, Pseudonectria buxicola* and *Sclerotinia homeocarpa*. The concentration-dependent antifungal effects of gibberellins tested in our laboratory against a number of *Fusarium* wilt phytopathogens (unpublished data) indicated that fungistasis activity of the phytohormones against phytopathogens may still be more widespread than those shown in Table 1. In the case of bacterial phytopathogens, none could grow regardless of the concentration. A low level of bacterial cells appear to have survived at very low micromolar (0.1 mM) concentration, but the phytopathgenic bacteria tested succumbed to all other higher concentrations of GA3, indicating that plant pathogenic bacterial cells may be hypersensitive to phytohormones compared to fungal pathogens. In the light of the above observations, it will be interesting to see the response of plant-beneficial bacteria (e.g. *Rhizobium*, *Azotobacter,* phosphorus-solubilizing *Rahnella* spp. and fungi (*Trichoderma* spp.)).

Amongst the racemic forms of GAs, the most bioactive is GA3, followed by GA_4, and they have have been known to play an exponential role in promoting plant growth [9]. In this study we have focused on GA3's antimicrobial potential, seeking to obtain information and knowledge regarding its antimicrobial effects on phytopathogenic microbes that plant cells encounter in an infected plant cell environment. Some physiological evidence indicates that plants infected with endophytic fungi often have a distinct advantage against biotic and abiotic stress over their endophyte-free

counterparts [10]. GA3 is normally used in European Union territories at concentrations of 10–200 p.p.m. in horticulture, for example to increase flower bud size (10–50 p.p.m.) and to break the dormancy of seeds (200–500 p.p.m.). GA3 can be bioactive at levels as low as 3–5 p.p.m. (www.Triplantanol.com). Foliar application of GAs has been known for its role in plant stem elongation and mitigation of abiotic stress under the growing demands of global climate change mitigation. Whilst the antimicrobial effects of one of the phytohormones gibberellic acid GA3 on plant-colonizing phytopathogens is highlighted in our study, it would be interesting to examine the effects of phytohormones upon plant-growth-promoting fungi such as *Gliocadium* spp. and other commercial microbial control agents [5], which are poorly understood for plant health improvement.

No inhibitory effect was seen with the gibberellins, GA1, GA3, GA5 and GA13. GA4 inhibited all bacterial organisms examined, with GA_7 and GA_9 showing limited activity (Fig. 1). DMSO did not show any antibacterial activity. Structurally, plant-derived diterpenoid gibberellins comprise four linked isoprenyl units (19, 20 carbons) and are enantiomers [12] of the same molecule, i.e. with + or – spatial orientations associated with the variable bending and stretching nature of the complex inter-atomic bonding between their carbon–carbon, carbon–hydrogen and carboxylic carbon–hydroxyl groups. Fourier transform infra-red spectroscopy (FT-IR) of plant phytohormone biostimulants [18, 19] have indicated that inter- and intra-specific IR absorptions vary for phytohormones. Amongst the GAs tested in this study, it is likely that on account of the innate chemical structural flexibility, GA_4 may have differed from others in their bacterial cell surface 'molecular docking' vis-à-vis membrane permeation/protein ligand modification capabilities, transcriptional pathways, culminating in contrasting host cell cycles.

In this study, gibberellins were employed in antimicrobial susceptibility tests at a concentration of 50 mM for human bacterial pathogens and 0.1–50 mM for plant phytopathogens. With these results, the next stage would be to include a preliminary cytotoxic assay, for example, a haemolytic assay, to determine how cytotoxic these molecules would be, which could help deduce their potential for inclusion as a human medicine, as well as considering what systemic concentration would be required theoretically in the human body to achieve a microbiological effect. It was not the experimental design of the current investigation to compare the antimicrobial activity of the gibberellins with that of conventional antibiotics, such as the beta-lactam, amoxicillin. However, for comparative purposes, the pharmacodynamics/pharmacokinetics (PK/PD) of amoxicillin when used in human medicine shows that a concentration of $3.3 \pm 1.12 \,\text{mg} \, l^{-1}$ is achieved [20]. As gibberellins are not traditionally regarded as antibiotics, the PK/PD values required to achieve a therapeutic effect in humans may be too large to facilitate such molecules as potential therapeutic molecules in human medicine.

Although conventional antibiotics are among the most successful drugs used for human therapy and may have

Table 1. Antimicrobial effects of phytohormone gibberellic acid (GA3) challenged with phytopathogens

Phytopathogen		GA$_3$ (mM) in substrate (p.p.m.) Figures represent % microbial growth of control (no GA3)					
Organism tested	Host	50 mM (17320)	20 mM (6928)	10 mM (3464)	5 mM (1732)	1 mM (346)	0.1 mM (35)
Fungi/oomycete[1]							
Cryptocline taxicola CBS255.77	Yew	27.8	50	58.3	70.8	75.6	75.6
Dreschlera graminea CBS100.866	Barley	7.1	8.9	20.2	28.5	63.9	81
Hymenoschyphus fraxinea (local isolate)	Ash	0	0	11.2	12.8	54.7	90.2
Microdochium nivale majus CBS11.78	Cereals	0	0	0	8.4	67.8	85.5
[1]Phytophthora kernoviae ATCC2444	Beech	0	0	0	11.9	62.9	77.2
[1]Phytophthora ramorum ATCC3678	Oak	0	0	0	33	108.2	145.1
[1]Postia placenta (local isolate)	Brown rot (Timber)	0	4.4	17.6	31.3	86.9	88.6
Pythium irregulare ATCC96196	Root rot of multiple hosts	0	10.4	17.6	25	62.5	75.5
Pseudonectria buxicola CBS252.69	Box trees	0	0	13.2	15.1	64.8	154.4
Sclerotinia homeocarpa CBS510.89	Grasses	0	7	14.6	28	63.1	102.9
Bacteria							
Erwinia amylovora ATCC100	Apple, pear	0	0	0	0	0	>35 mm
Erwinia amylovora (local isolate)	Apple, pear	0	0	0	0	0	>35 mm
Pseudomonas syringae aesculi ATCC4436	Horse chestnut	0	0	0	0	0	>35 mm
Xanthomonas arboricola pruni ATCC924	Cherry, hazelnut, walnut	0	0	0	0	0	>35 mm

Bacteria were also tested at 100 mM of GA3 and with the exception of the *Erwinia amylovora* strain, all others showed no growth on the culture plates.
p.p.m., parts per million.

benefits in many different human interventions, such as agriculture, aquaculture, bee keeping and livestock as growth promoters, the antibiotic compounds are usually detected in µg l⁻¹ and quantitatively expressed in a parts per million (p.p.m.) range in our environments. To this end, for a better grasp of quantitative comparisons between the conventional antibiotics and the concentrations of the phytohormone gibberellins we used in this study for their *in vitro* antibiotic efficacy tests, they are shown (Fig. 1) in both millimolar and the equivalent in p.p.m. The *in vitro* antibiotic effects of the diterpinoid phytohormone gibberellins at 50 mM on the cohorts of clinical pathogens in our preliminary study are quite remarkable in terms of comparable MIC scales for a standard antibiotic such as amoxicillin, referred to elsewhere, and should attract further pharmacological investigations concerning their toxicity and reduced half-lives in human body fluids, in particular given the serious global concerns about the rise of antibiotic-resistant 'superbugs' that normally include the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.), some of which we tested (Fig. 1), and which are now resistant to many conventional antibiotics. A recent study demonstrated a set of novel modified antimicrobial aminopeptide template approaches for addressing antimicrobial resistance challenges [21]. Future work should also include testing the susceptibility of other organisms, including non-pathogenic bacteria, as well as pathogenic and non-pathogenic fungi, with these molecules.

With regard to plants, it is also interesting that investigations (e.g. [22] on plant-growth-promoting endophyte fungi (PGPF) from the roots of crown daisy (*Chrysanthemum coronarium*) have discovered that a new species of *Penicillium*, that produces amoxicillin like antimicrobial secondary metabolites, namely *Penicillium* sp. MH7, used for improving plant health, has been described to produce nine different gibberellins.

In this study, we employed methods to estimate clinical and plant pathogenic bacteria or fungi comprising *in vitro* microbiological, methods. Using a universal Student's *t*-test on each of the methods, the data indicated that they were statistically significant (*P*<0.05) by themselves. However, our laboratory methods for estimating clinical bacteria in this study were quite different from those used on the plant pathogenic bacterial or fungal cohorts tested for inhibitory efficacies. The data sets emerging from individual testing measurements can be therefore be expected to vary not only in terms of unequal replicate numbers, but also in terms of the quantitative underlying values in one type of method or the other. To this end, the measurement gauge and emergent quantitative (replicate) and qualitative (observatory method) differences evinced in different laboratory methods, viz. microbiological tools for comparison studies applied for the targets (i.e. clinical bacteria, plant-associated bacteria or fungi in our present study), are inevitable. To contend with this innate anomaly in our multi-variate

analyses, we used the Altman–Bland test and statistically set a 95% limit on standard error (*P*=<0.05) confidence intervals, and our data provided an agreement of relationship for comparisons of the variant cultural methods for the distinction of inhibitory consistency achieved across the microbial variants. This statistical tool was successfully demonstrated in a previous study in our laboratory [17].

Our study also brings to the fore the practical potential utility of phytohormone gibberellins in addition to their foliar spray formulations for improving plant growth as a control agent on a wide range of tree and arable crop and horticulture pathogens. Interestingly, this study has raised the pharmacognosy and phytotherapeutic possibility of developing plant- and fungus-derived diterpenoid gibberellins as oil emollients, which may offer a new plausible complementary phytoremedial approach, facilitated by their unique, penetrative, highly flexible natural bending and stretching structures that interact effectively with intracellular microbial targets for the desired antibiotic effect on clinical cohorts of human-risk bacterial pathogens, making a helpful contribution in addressing antimicrobial resistance to conventional antibiotics, in an ever-increasing global crisis for healthcare, agriculture and hygiene.

Author contributions
Authors P.T. and D.N. contributed equally to this manuscript and should therefore be considered as joint and equal first authors.

Conflicts of interest
All authors of this article have no conflict of interest to declare.

Ethical statement
This article does not contain any studies with human participants or animals performed by any of the authors.

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