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Tubulin modulating antifungal and antiproliferative pyrazinone derivatives

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ABSTRACT

A novel class of synthetic tubulin polymerization disruptors, based on a substituted pyrazin-2-one core, has been discovered. These molecules have proven to be potent broad spectrum fungicides, with activity on agriculturally important ascomycete and basidiomycete pathogens. They have also been found to be particularly potent against human rhabdomyosarcoma cells. Using an efficient synthetic route, the agricultural and medicinal activity was explored.

1. Introduction

It is projected that the world will need to increase food production by 60% in order to feed a global population expected to be in excess of 9 billion people by 2050. At the same time, it is estimated that every year global crop yields are reduced by 20–40% by agricultural pests.[1] Because of the ability of plant pathogens to rapidly develop resistance, it is imperative that new classes of broad spectrum disease control agents, with novel modes of action, be discovered in order to prevent catastrophic yield losses, which can approach 80% in some circumstances.[2] In order to discover a fungicide with activity on a broad range of taxonomic classes of fungi, one must target an essential pathway that is fairly well conserved across the pathogens of interest. Historically, some of the most successful fungicides have inhibited critical processes such as mitochondrial electron transport,[3] sterol biosynthesis,[4] and tubulin polymerization.

Tubulin is a member of a superfamily of globular proteins which exist in six subtypes, including the most common α- and β-tubulin. α- and β-tubulin polymerize into heterodimers and then ultimately into larger structures which govern critical cellular processes, such as mitosis. Interference with the dynamic equilibrium between polymerization and depolymerization of tubulin has historically been an effective strategy for treating diseases characterized by rapid cellular proliferation, such as cancer.[5] Conceptually, there is much similarity between the proliferation of cancer cells and the rapid cellular propagation of a fungal infection. It is therefore not surprising that tubulin polymerization disruptors have been successfully used in agricultural settings since the 1960s, starting with methyl benzimidazole carbamates (MBCs)[6], and more recently with zoxamide[7] and the quinolinyloxyacetamides[8] (which have yet to be exploited commercially).

With tubulin polymerization modulators playing such an important role in agriculture and medicine, it is imperative that novel chemotypes of known classes of molecules, as well as molecules that bind to completely different binding sites, be discovered and studied. In this paper, we will discuss the synthesis and biological evaluation of a new class of tubulin modulating molecules, based on the pyrazin-2-one core, for both medicinal and agricultural use.

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0968-0896/

2. Results and discussion

2.1. Chemistry

When we started our work in the area of tubulin polymerization modulators, we were aware of the triazolopyrimidines (**1**, Fig. 1) from American Cyanamid and eventually BASF[9] as well as subsequent research programs at Bayer[10] and Syngenta.[11] In particular, we were drawn to the 2-pyrazolyl-pyrimidines (**2**),[12] as they had opened a new vein of opportunity by moving away from the bicyclic core, into a linear arrangement. Additionally, the internal imine in the pyrido[2,3-*b*]pyrazines (**3**) suggested the possibility of a carbonyl to replace one of the pyrimidine nitrogens. To satisfy the need for another sp^2 center in the core, which would be the attachment point for the alkyl side chain, the pyrimidine nitrogen was conceptually moved over by one position, resulting in the pyrazin-2-one core (**4**).[13]

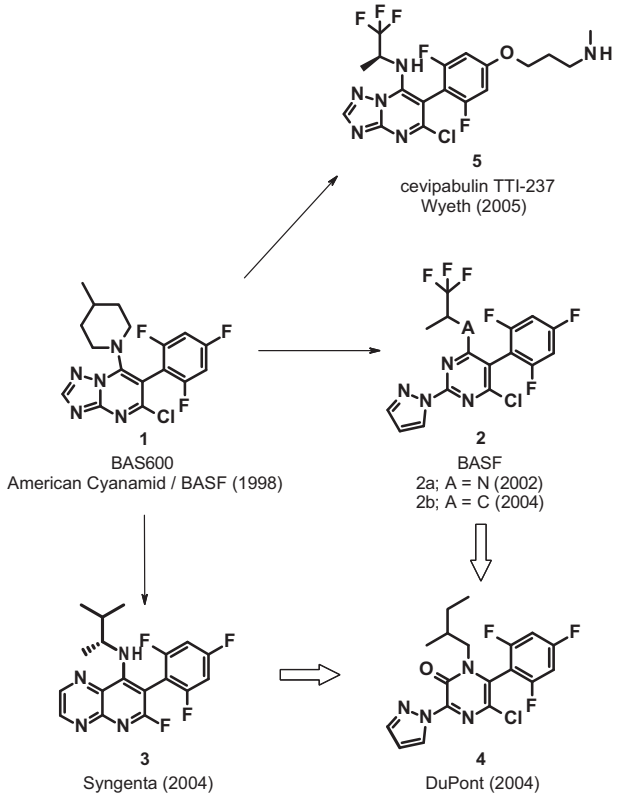
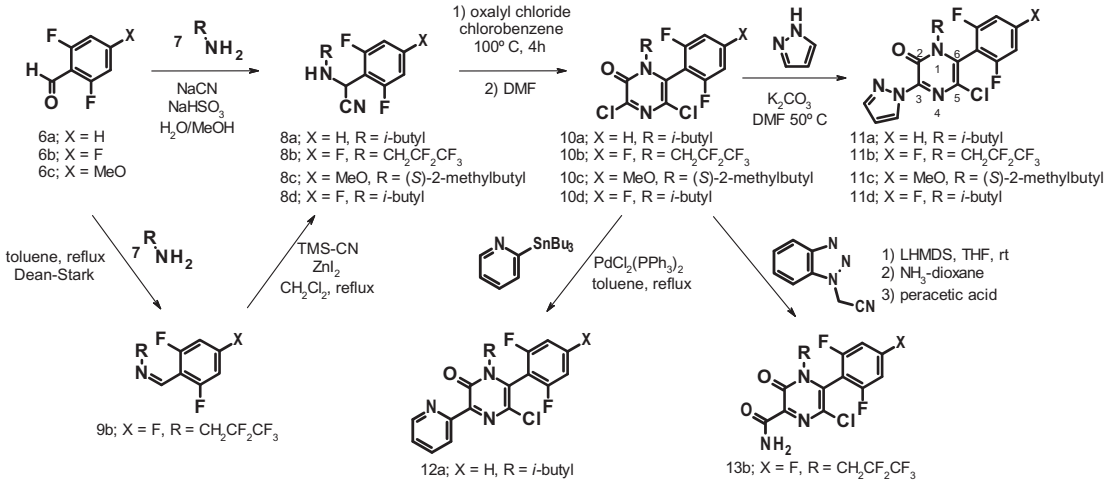


Figure 1. Different mono- and bicyclic tubulin modulating fungicides.

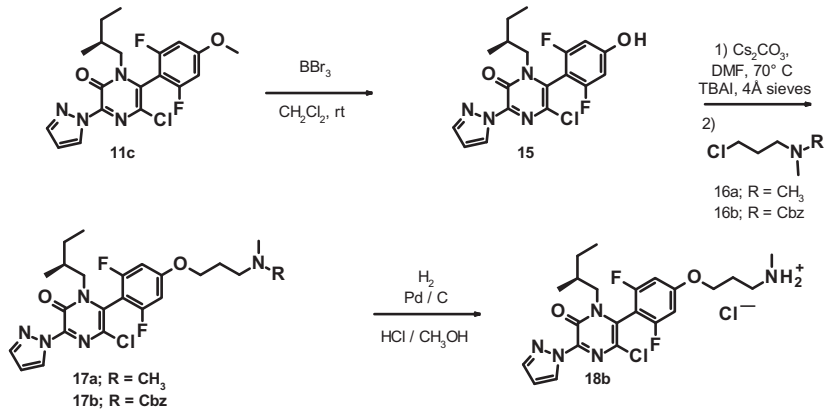
The optimization and evaluation of the pyrazin-2-ones was greatly simplified by the early adoption of a modular synthesis, which allowed us to specifically modify the four pendant functionalities in order to explore their biological relevance (Scheme 1). The synthesis usually began with a Strecker reaction between an aldehyde, an amine, and cyanide to establish the functionalities that would ultimately be in the 1 and 6 positions of the pyrazinone core. If necessary, the Strecker reactions could be catalyzed with sodium bisulfite or a Lewis acid such as indium(III) chloride.[14] In cases where the amine had poor nucleophilicity, such as anilines or fluorinated alkyl groups, a two-step procedure was employed where the imine was prepared under dehydrating conditions, and then subsequently allowed to react with a cyanide source to generate the α-aminonitrile.

The pyrazinone core was then formed by treating the α-aminonitrile (**8**) with oxalyl chloride at elevated temperatures. This reaction often suffered from yields in the range of 40–60%, with the remaining mass balance being composed of mono-chlorinated pyrazinone. It was subsequently found that a more efficient conversion to the dichloro pyrazinone could be achieved by the addition of a small amount of DMF to the reaction mixture *after* the α-aminonitrile had reacted with the oxalyl chloride. This procedure resulted in increased product yields, approaching 90% in some cases. Under the same conditions, use of oxalyl bromide resulted in the 3,5-dibromo-pyrazin-2-one, which could be used to explore alternate substitutions at the 5 position. Finally, the 3-chloro group could be displaced with a variety of nucleophiles, including NH containing heterocycles, such as pyrazole to form the final targets. The same chlorine could also be subjected to transition metal catalyzed cross-coupling reactions to yield targets with C-linked heterocycles. Amides could also be prepared from the same synthon by reaction with *N*-cyanomethyl-benzotriazoles and base followed by oxidation with peracetic acid.[15]

While in related art, the alkylaminoalkoxy side chain of cevipabulin (**5**) could be added through the nucleophilic displacement of the 4-fluoro on a 2,4,6-trifluorophenyl,[16] this was not possible in the pyrazinone system due to the concomitant displacement of the N-linked pyrazole. To overcome this difficulty,



Scheme 1. The general synthesis of pyrazin-2-one fungicides.



Scheme 2. Further elaboration of the substitution on phenyl.

4-methoxy substituted analogs were prepared. The methyl group was then cleaved using boron tribromide and the phenolic oxygen was alkylated with the appropriate side chain. It was important that the alkylation be conducted under strictly anhydrous conditions as it was found that any hydroxide present in the reaction would displace the N-linked pyrazole (Scheme 2).

2.2. Mode of action

The effect of analogs **11a** and **11d** on the dynamics of tubulin polymerization was investigated relative to paclitaxel, a known tubulin polymerization enhancer, along with a DMSO control. Figure 2 shows the effect on polymerization of bovine tubulin as measured by the change in absorbance at 340 nm in the presence and absence of **11a** and **11d** at the indicated concentrations. The progress curve indicates an action similar to paclitaxel by dramatically enhancing and stabilizing the polymerized state over the course of the assay relative to the solvent control. The superior potency of **11d** over **11a** in the bovine tubulin assay was consistent with its superior antifungal activity on plants (Table 4, entries 1 and 2), supporting the idea that the antifungal activity is derived from disrupting the dynamic equilibrium between polymerized and depolymerized tubulin. Ultimately, using mutation studies,[17] it was determined that while the compounds caused tubulin to behave in a similar manner to paclitaxel, the pyrazinones actually bind at a location similar to the vinca alkaloids,[18] which is consistent with the related cevipabulin (**5**).[16]

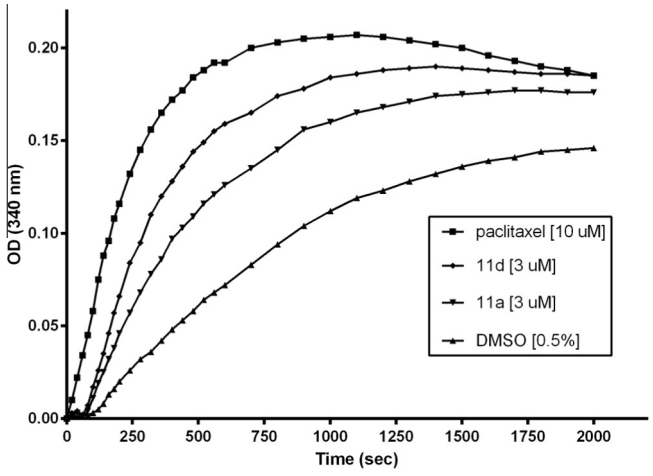


Figure 2. The enhanced rate of polymerization of bovine tubulin by **11a** (3 μM) and **11d** (3 μM), relative to solvent (DMSO) control. The progress of tubulin polymerization in the presence of paclitaxel (10 μM) is shown for comparison.

2.3. Structure–activity relationships

Our chemical optimization program allowed us to explore the structure-activity relationships for substitution in all four quadrants of the pyrazinone core. In Tables 1–5, we will discuss these relationships in terms of the control of wheat glume blotch (WGB, *Stagonospora nodorum*), wheat brown rust (WLR, *Puccinia recondita f.* sp. *Tritici*), tomato gray mold (TBT, *Botryotinia fuckeliana*) and ultimately a human rhabdomyosarcoma cell line (RMS; ATCC CCL-136).

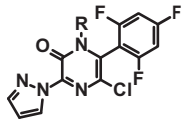
2.3.1. Influence of the nitrogen substituent on fungicidal activity

Substitution at N1 tolerated the greatest structural diversity including alkyl, cycloalkyl and aryl groups. While straight chain alkyl and alkenyl groups (Table 1, entries 1 and 5) were potent in our whole plant assays, we quickly found that branching, and in particular branching on the beta carbon (entries 2–4), enhanced potency and spectrum, as seen with *iso*-butyl, *sec*-butyl and cyclopropylmethyl. Ultimately, it was found that all of the activity resided with the (*S*)-enantiomer of the *sec*-butyl side chain. Branching could also be achieved using halogenated alkyl groups (entries 6–8), which exhibited some improvement in activity over the *n*-butyl analog (entry 1). Branching alpha to the pyrazinone ring nitrogen was also tolerated, which allowed for the examination of cycloalkyl and aryl groups (entries 9–13). A variety of substituents in the *ortho*, *meta* and *para* positions of the aryl ring were also acceptable.

2.3.2. Influence of substitution at the 3-positon of the pyrazinone on fungicidal activity

Having established both *iso*-butyl and *sec*-butyl as excellent nitrogen substituents, we chose to examine a broad range of substitution at the 3-position. Our initial hit in this area of chemistry (**11a**) had established that an N-linked pyrazole (Table 2, entry 1) resulted in excellent activity. We subsequently found that substitution in the 3-position of the pyrazole was tolerated, though not necessarily beneficial (entry 2). The addition of a methyl group in the 5-position (entry 3) eliminated nearly all spectrum and potency. Examination of additional N-linked azoles found that 1,2,4 and 1,2,3-triazoles were effective, although it appeared preferable that there not be two nitrogens flanking the point of connection as in the 1,2,3-triazol-2-yl (entries 4–6). An N-linked imidazole was found to be completely inactive, suggesting that

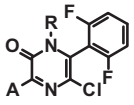
Table 1
Influence of the nitrogen substituent on fungicidal activity[a]



Entry	R	WGB				WLR				TBT			
		200	40	10	2	200	40	10	2	200	40	10	2
1	*n*-butyl	-	100	98	0	-	100	88	26	-	100	0	0
2 (**11d**)	*i*-butyl	100	100	90	0	100	100	100	92	100	100	100	100
3 (**4**)	*s*-butyl	100	100	98	0	100	100	100	89	100	100	100	100
4	*c*-propyl-CH_2-	-	100	100	0	-	100	100	80	-	99	98	98
5	allyl	-	100	97	0	-	99	89	0	-	99	99	70
6	$CF_3CF_2CF_2CH_2$-	-	99	78	0	-	99	96	68	-	100	99	38
7 (**11b**)	$CF_3CF_2CH_2$-	-	100	89	0	-	100	99	91	-	100	100	99
8	2-CF_3-n-propyl	-	100	0	0	-	100	100	92	-	99	99	99
9	*c*-hexyl	100	99	73	-	99	97	89	-	99	99	99	-
10	Ph	-	99	98	60	-	99	87	45	-	100	100	100
11	4-Cl-Ph	-	100	100	78	-	100	100	96	-	99	99	92
12	3-Cl-Ph	-	99	99	73	-	100	99	86	-	100	99	97
13	2-Cl-Ph	-	100	94	0	-	100	100	97	-	100	100	99

[a] WGB, WLR and TBT results are given as percent control relative to the untreated control plants. Rates are given in ppm. Gray shading represents control in excess of 70%.

Table 2
Influence of substitution at the 3-positon of the pyrazinone on fungicidal activity[a]

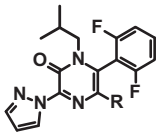


Entry	A	R	WGB				WLR				TBT			
			200	40	10	2	200	40	10	2	200	40	10	2
1 (**11a**)	pyrazol-1-yl	*i*-butyl	-	99	92	0	-	100	100	28	-	99	97	75
2	3-Me-pyrazol-1-yl	*i*-butyl	-	95	0	0	-	100	94	25	-	100	100	95
3	3,5-di-Me-pyrazol-1-yl	*s*-butyl	0	0	0	-	73	22	0	-	0	0	0	-
4	1,2,4-triazol-1-yl	*s*-butyl	100	94	-	-	98	98	-	-	100	100	-	-
5	1,2,3-triazol-1-yl	*s*-butyl	73	0	0	-	73	67	67	-	98	81	33	-
6	1,2,3-triazol-2-yl	*s*-butyl	0	0	-	-	86	19	-	-	97	0	-	-
7	imidazol-1-yl	*s*-butyl	0	0	0	-	0	0	0	-	0	0	0	-
8	1-Me-imidazole-4-yl	*i*-butyl	97	67	0	0	99	97	0	0	100	99	0	0
9	1-Me-imidazole-2-yl	*i*-butyl	0	0	0	-	0	0	0	-	0	0	0	-
10 (**12a**)	pyrid-2-yl	*i*-butyl	99	0	-	-	99	85	-	-	100	99	-	-
11	pyrid-3-yl	*i*-butyl	-	0	0	0	-	0	0	0	-	0	0	0
12	pyrid-4-yl	*i*-butyl	-	0	0	0	-	0	0	0	-	0	0	0
13	pyrazin-2-yl	*i*-butyl	98	0	0	-	99	89	0	-	98	98	83	-
14	thiazol-2-yl	*i*-butyl	-	0	0	0	-	0	0	0	-	0	0	0
15	CN	*s*-butyl	0	0	-	-	96	74	-	-	99	99	-	-
16	$H_2NC(=O)$-	*s*-butyl	-	95	0	0	-	100	99	80	-	99	97	92
17	MeHNC(=O)-	*s*-butyl	-	51	0	0	-	99	74	0	-	99	90	57
18	MeC(=O)NH-	*i*-butyl	0	0	0	-	100	90	0	-	99	92	15	-

[a] WGB, WLR and TBT results are given as percent control relative to the untreated control plants. Rates are given in ppm. Gray shading represents control in excess of 70%.

having one nitrogen adjacent to the point of connection was possibly required (entry 7). To further explore this, we turned our attention to C-linked azoles like 1-methyl-imidazole-4-yl (entry 8), which returned a nitrogen to a flanking position, and restored activity in the whole plant assays. While the alternate isomer 1-methyl-imidazole-2-yl has the required flanking nitrogen, it has a methyl in the same location that was previously seen to eliminate nearly all activity (entries 3 and 9). The requirement that a flanking nitrogen be present carried through to six membered rings as evidenced by the activity of 2-pyridyl and 2-pyrazinyl and the inactivity of 3- and 4-pyridyl (entries 10–13). It was surprising, however, that the 'pseudo' six-membered 2-thiazole did not show activity at the rates tested (entry 14). Perhaps a foreshadowing of future discoveries,[19] it was found that the presence of a pendant heterocycle was not a requirement, and it could be replaced with simple groups such as cyano, and N- and C-linked

Table 3
Influence of substitution at the 5-positon of the pyrazinone on fungicidal activity[a]



		WGB				WLR				TBT			
Entry	R	200	40	10	2	200	40	10	2	200	40	10	2
1 (**11a**)	Cl	-	99	92	0	-	100	100	28	-	99	97	75
2	Br	100	100	86	-	100	99	88	-	100	100	99	-
3	Me	-	98	0	0	-	100	92	0	-	100	99	0
4	H	0	0	0	-	98	0	0	-	-	-	-	-

[a] WGB, WLR and TBT results are given as percent control relative to the untreated control plants. Rates are given in ppm. Gray shading represents control in excess of 70%.

amides (entries 15–18). It remained true that heteroatoms were required to be proximal to the linkage to the core, as simple alkyl and phenyl groups were inactive.

2.3.3. Influence of substitution at the 5-positon of the pyrazinone on fungicidal activity

The least permissive portion of the molecule proved to be 5-chloro in our original hit compound **11a** (Table 3, entry 1). We found that it could be replaced with another halogen, such as bromine, or a methyl group (entries 2 and 3) without significant change in activity. Replacement of the chlorine with a proton resulted in a near-complete loss of activity. Substitution with larger groups was also detrimental. Due to the biological and synthetic benefits of the 5-chloro, we did not examine additional modifications.

2.3.4. Influence of substitution at the 6-positon of the pyrazinone on fungicidal activity

Due to analogy with related areas already present in the literature, we knew the clear importance of an *ortho* halogen on the 6-phenyl, and thus our initial hit compound **11a** possessed a 2,6-difluoro phenyl (Table 4, entry 1). Adding an extra fluorine in the *para* position of the phenyl clearly improved the potency of the molecule (entry 2), but it is debatable as to whether the addition of a third fluorine in the *meta*-position was as successful (entry 3). 2,4-Disubstituted phenyls were also quite successful, especially with a larger *ortho*-group such as chlorine (entry 4). ^{1}H NMR analysis of the 2-chloro-4-fluoro-phenyl analog (entry 4) revealed that the methylene protons on the *iso*-butyl were clearly diastereotopic, implying the presence of atropisomers due to restricted rotation about the pyrazinone-phenyl bond.[20] Subsequently, we were able to separate the two atropisomers by chiral HPLC and determined that all of the fungicidal activity resided with one atropisomer, while the other was completely inactive.

We also found that the phenyl ring could be replaced with larger cycloalkyl groups, such as cyclopentyl (entry 5), while smaller rings such as cyclopropyl (entry 6) resulted in a complete loss of activity. The cyclopropyl could be opened up to form an isopropyl (entry 7), which results in very similar activity as the larger cycloalkyl rings. These findings, plus the importance of 2-substitution on the phenyl ring, imply that this group needs to be out of plane from the pyrazinone core in order to achieve significant potency. Somewhat surprisingly, a *t*-butyl group was completely inactive.

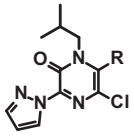
2.3.5. Elaboration of the 6-phenyl: influence on fungicidal and anti-proliferative activity

From the time that we had first confirmed the tubulin-based mode of action for the pyrazinone fungicides, we were interested in determining whether they would also exhibit anti-proliferative activity on cancer cell lines. We were pleased to find that against rhabdomyosarcoma cells (RMS) our leading antifungal molecules exhibited IC_{50} values in the 10–20 nM range (Table 5, entries 1 and 2), comparable to paclitaxel, which was run as a standard. Knowing the relationship between our pyrazinones and the clinical candidate cevipabulin (**5**), we chose to further elaborate the *para* position of the phenyl ring on our leading antifungal candidates with alkylamino-alkoxy chains.[21] While a two carbon linker proved to diminish the activity (entry 3), the analogs with a three-carbon linker improved potency in excess of 10 fold (entries 4 and 5). This excellent potency was ultimately confirmed and extended to a wide variety of cancer cell lines at an independent laboratory. When examined in our traditional whole plant antifungal assays, the most proficient compounds from the RMS assay (entries 4 and 5) exhibited diminished potency as compared to the parent antifungal compounds (entries 1 and 2). This is potentially due to less-than-optimal physical properties, for whole plant translation, imparted by the alkylamino-alkoxy side chain.

3. Conclusions

A new class of pyrazinone fungicides has been demonstrated to exhibit potent broad spectrum activity on a number of economi-

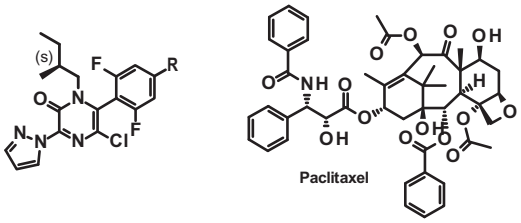
Table 4
Influence of substitution at the 6-positon of the pyrazinone on fungicidal activity[a]



		WGB				WLR				TBT			
Entry	R	200	40	10	2	200	40	10	2	200	40	10	2
1 (**11a**)	2,6-di-F-Ph	-	99	92	0	-	100	100	28	-	99	97	75
2 (**11d**)	2,4,6-tri-F-Ph	100	100	90	0	100	100	100	92	100	100	100	100
3	2,3,6-tri-F-Ph	-	100	87	0	-	100	99	32	-	100	98	94
4	2-Cl-4-F-Ph	100	100	99	0	100	100	100	83	100	100	100	100
5	*c*-pentyl	0	0	-	-	100	91	-	-	100	99	-	-
6	*c-pro*	0	0	-	-	0	0	-	-	0	0	-	-
7	*i*-pro	0	0	-	-	98	18	-	-	99	99	-	-
8	*t*-butyl	0	0	-	-	0	0	-	-	0	0	-	-

[a] WGB, WLR and TBT results are given as percent control relative to the untreated control plants. Gray shading represents control in excess of 70%.

Table 5
Elaboration of the 6-phenyl and the influence on fungicidal and anti-proliferative activity[a,b]



Entry	R	RMS	WGB				WLR				TBT			
			200	40	10	2	200	40	10	2	200	40	10	2
1	F	20	-	100	99	0	-	100	100	99	-	100	100	100
2 (**11c**)	-OMe	10	100	100	100	69	100	100	100	99	99	99	99	99
3	$-O(CH_2)_2NMe_2$	210	-	-	-	-	100	91	55	0	99	68	0	0
4 (**17a**)	$-O(CH_2)_3NMe_2$	1.3	94	0	0	0	100	99	89	68	-	99	94	0
5 (**18b**)	$-O(CH_2)_3NHMe$-HCl	0.6	100	88	33	0	100	100	99	68	100	99	99	21
Paclitaxel	-	8.4	91	-	-	-	95	-	-	-	0	-	-	-

[a] WGB, WLR and TBT results are given as percent control relative to the untreated control plants. Rates are given in ppm. Gray shading represents control in excess of 70%.
[b] RMS Cellular proliferation results are given as the IC_{50} (nM).

cally important plant pathogens including *Stagonospora nodorum*, *Puccinia recondita f.* sp. *tritici* and *Botryotinia fuckeliana*. The mode of action of these compounds was determined to be through interference with tubulin polymerization dynamics.

A highly-convergent synthesis allowed for the study of the structure-activity relationships in all four quadrants of the pyrazinone core. Ultimately, it was found that branched alkyl groups and an N-linked pyrazole in the 1 and 3 positions respectively, lead to optimal activity. 2,4,6-Trisubstituted phenyl rings in the 6 position of the pyrazinone core exhibited the best potency and spectrum, although fungicidal activity was observed with other non-aromatic and acyclic groups.

While the optimization program was driven by the control of agronomically-relevant plant pathogens, it was found that this class of chemistry shows promise in pharmaceutical applications. The best fungicides exhibited activity comparable to paclitaxel against rhabdomyosarcoma cells, while a small number of analogs optimized for pharmaceutical utility showed a ten-fold increase in potency. Given the need for novel mode of action antifungals in both agriculture and medicine, coupled with the potential for other applications,[22] this class of tubulin-modulating pyrazinones should be the subject of further investigation.

4. Experimental section

4.1. Chemistry

All new compounds were characterized by standard spectroscopic methods. ^{1}H NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 MHz using deuterated solvent and tetramethylsilane as an internal standard. Chemical shifts are reported in ppm downfield from the standard (TMS, $\delta = 0.00$). All reactions were carried out under anhydrous conditions under an inert atmosphere (nitrogen or argon) with commercially available dry solvents. Additional synthetic examples and compound characterization can be found in the patent literature.[13,21]

4.1.1. 2,6-Difluoro-α-[(2-methylpropyl)amino]benzeneacetonitrile (8a)

To a solution of isobutylamine (2.92 g, 40 mmol) and sodium cyanide (1.94 g, 40 mmol) in water (40 mL) was added a solution of 2,6-difluorobenzaldehyde (5.7 g, 40 mmol) in methanol (40 mL). The addition was done at such a rate so that the temperature remained below 35 °C. The reaction mixture was stirred at room temperature for 18 h. The mixture was partitioned between water (150 mL) and dichloromethane (150 mL). The organic layer was washed with water (2 × 50 mL). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure to give an oil. Flash chromatographic purification on silica gel with hexanes as eluant and pooling of appropriate fractions gave 2,6-difluoro-α-[(2-methylpropyl)amino]benzeneacetonitrile (**8a**, 4.92 g, 21 mmol, 55%) of the title compound as an oil. ^{1}H NMR ($CDCl_3$): δ 7.36 (m, 1 H), 6.98 (m, 2 H), 4.94 (bs, 1H), 2.75 (m, 1H), 2.52 (bs, 1 H), 1.84 (bs, 1 H), 1.78 (m, 1H), 0.95 (m, 6H).

4.1.2. 3,5-Dichloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-2(1*H*)-pyrazinone (10a)

A solution of oxalyl chloride (3.34 g, 26 mmol) in chlorobenzene (35 mL) was stirred at 25 °C and 2,6-difluoro-α-[(2-methylpropyl)amino]-benzeneacetonitrile (**6a**, 2.5 g, 9.0 mmol) was added via an addition funnel. The resulting reaction mixture was heated at 70 °C for 18 h and at 90 °C for 24 h. The solvent was evaporated under reduced pressure to leave an oil. This residue was subjected to silica gel chromatographic purification using a gradient of ethyl acetate/hexanes (1:9 to 2:3), and the appropriate fractions were pooled to afford 3,5-dichloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-2(1*H*)-pyrazinone (10a, 1.2 g, 3.6 mmol, 33%) as an oil which solidified on standing. ^{1}H NMR ($CDCl_3$): δ 7.6 (m, 1H), 7.1 (m, 1H), 7.0 (m, 1H), 3.7 (m, 2H), 1.9 (m, 1H), 0.9 (m, 3H), 0.7 (d, 3H).

4.1.3. 5-Chloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (11a)

A mixture of 3,5-dichloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-2(1*H*)-pyrazinone (10a, 0.20 g, 0.60 mmol), pyrazole (45 mg, 0.66 mmol) and potassium carbonate (166 mg, 1.2 mmol) dissolved in *N*,*N*-dimethylformamide (2 mL) was heated at 60 °C for 18 h. The mixture was partitioned between ethyl acetate (20 mL) and water (10 mL). The organic layer was washed with water (3 × 10 mL). The residue after evaporation was subjected to silica gel chromatographic purification using a gradient of hexanes/ethyl acetate (1:9 to 2:3) as eluant to give 5-chloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (**11a**, 0.060 g, 0.16 mmol, 27%). ^{1}H NMR ($CDCl_3$): δ 9.1 (m, 1H), 7.9 (m, 1H), 7.5 (m, 1H), 7.1 (m, 2H), 6.5 (m, 1H), 3.8 (d, 2H), 2.0 (m, 1H), 0.8 (d, 6H). Mp = 118–119 °C.

4.1.4. 5-Chloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-3-(2-pyridinyl)-2(1*H*)-pyrazinone (12a)

A mixture of 3,5-dichloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-2(1*H*)-pyrazinone (**10a**, 200 mg, 0.6 mmol), tributylstannylpyridine (240 mg, 0.63 mmol) and *bis*(triphenylphosphino)palladium(II) chloride (20 mg, 0.03 mmol) was heated in toluene at 110 °C for 18 h. The mixture was filtered through a pad of Celite® and rinsed with ethyl acetate. The solvent was evaporated under reduced pressure. The residue after evaporation was subjected to silica gel chromatographic purification using a gradient of ethyl acetate/hexanes (1:9 to 2:3) to give 5-chloro-6-(2,6-difluorophenyl)-1-(2-methylpropyl)-3-(2-pyridinyl)-2(1*H*)-pyrazinone (**12a**, 0.056 g, 0.15 mmol, 25%) as an oil. ^{1}H NMR ($CDCl_3$): δ 8.86 (m, 1H), 8.43 (m, 1H), 7.83 (m, 1H), 7.59 (m, 1H), 7.38 (m, 1H), 7.12 (m, 2H), 3.79 (d, 2H), 2.00 (m, 1H), 0.79 (d, 6H).

4.1.5. 2,2,3,3,3-Pentafluoro-*N*-[(2,4,6-trifluorophenyl)methylene]-1-propanamine (9b)

A mixture of 2,4,6-trifluorobenzaldehyde (4.5 g, 28 mmol) and 2,2,3,3,3-pentafluoropropylamine (4.2 g, 28 mmol) in toluene (30 mL) was heated at reflux overnight using a Dean-Stark apparatus. The reaction mixture was allowed to cool to room temperature and concentrated in vacuo to provide 2,2,3,3,3-pentafluoro-*N*-[(2,4,6-trifluorophenyl)methylene]-1-propanamine (**9b**, 6.6 g, 23 mmol, 81%). This material was used directly in subsequent reactions without further purification. ^{1}H NMR ($CDCl_3$): δ 8.50 (s, 1H), 6.80–6.72 (m, 2H), 4.23–4.16 (m, 2H).

4.1.6. 2,4,6-Trifluoro-α-[(2,2,3,3,3-pentafluoropropyl)-amino] benzeneacetonitrile (8b)

A mixture of 2,2,3,3,3-pentafluoro-*N*-[(2,4,6-trifluorophenyl) methylene]-1-propanamine (**9b**, 6.6 g, 23 mmol), zinc iodide (7.2 g, 23 mmol), and 5 Å molecular sieves (23 g) in dichloromethane (25 mL) was treated with trimethylsilyl cyanide (18 mL, 135 mmol) and the reaction mixture was heated at reflux overnight. After cooling to room temperature, the reaction mixture was filtered through Celite® and concentrated in vacuo. The reaction residue was treated with methanol (100 mL) and 10% aqueous sodium bicarbonate solution (20 mL), and the resulting mixture was extracted with diethyl ether (2 × 50 mL). The ether phase was separated, dried over $MgSO_4$, and concentrated in vacuo. The resulting crude residue was purified via silica gel flash chromatography (5–10% gradient of ethyl acetate in hexane as eluant) to provide 2,4,6-trifluoro-α-[(2,2,3,3,3-pentafluoropropyl)-amino]benzeneacetonitrile (**8b**, 1.0 g, 3.1 mmol, 14%). ^{1}H NMR ($CDCl_3$): δ 6.86–6.74 (m, 2H), 5.04 (d, 1H), 3.55–3.30 (m, 2H), 2.27–2.21 (m, 1H).

4.1.7. 3,5-Dichloro-1-(2,2,3,3,3-pentafluoropropyl)-6-(2,4,6-trifluorophenyl)-2(1*H*)-pyrazinone (10b)

Oxalyl chloride (4.3 mL, 50 mmol) was added dropwise to a mixture of 2,4,6-trifluoro-α-[(2,2,3,3,3-pentafluoropropyl)amino] benzeneacetonitrile (**8b**, 3.2 g, 9.9 mmol) in chlorobenzene (20 mL) at room temperature. The resulting mixture was heated to 100 °C for 3 h, and then allowed to cool to room temperature. One drop of *N,N*-dimethylformamide was then added. The reaction mixture was reheated to 100 °C overnight. Then the reaction mixture was again allowed to cool to room temperature and concentrated in vacuo to provide a crude residue, which was purified via silica gel flash chromatography (10% ethyl acetate in hexane as eluant) to provide 3,5-dichloro-1-(2,2,3,3,3-pentafluoropropyl)-6-(2,4,6-trifluorophenyl)-2(1*H*)-pyrazinone (**10b**, 0.47 g, 1.1 mmol, 11%).

^{1}H NMR ($CDCl_3$): δ 6.94–6.89 (m, 2H), 4.65–4.45 (m, 2H).

4.1.8. 5-Chloro-1-(2,2,3,3,3-pentafluoropropyl)-3-(1*H*-pyrazol-1-yl)-6-(2,4,6-trifluorophenyl)-2(1*H*)-pyrazinone (11b)

A mixture of 3,5-dichloro-1-(2,2,3,3,3-pentafluoropropyl)-6-(2,4,6-trifluorophenyl)-2(1*H*)-pyrazinone (**10b**, 0.47 g, 1.1 mmol) and pyrazole (0.15 g, 2.2 mmol) in *N,N*-dimethylformamide (5 mL) was heated to 60 °C overnight. The reaction mixture was allowed to cool to room temperature and concentrated *in vacuo*. The resulting residue was subjected to silica gel flash chromatography (10% to 20% gradient of ethyl acetate in hexane as eluant) to provide partially purified material. Trituration of this material with a mixture of hexane and *n*-butyl chloride provided 5-chloro-1-(2,2,3,3,3-pentafluoropropyl)-3-(1*H*-pyrazol-1-yl)-6-(2,4,6-trifluorophenyl)-2(1*H*)-pyrazinone (**11b**, 0.30 g, 0.65 mmol, 59%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 9.05 (d, 1H), 7.93 (d, 1H), 6.94–6.88 (m, 2H), 6.55 (s, 1H), 4.75–4.50 (m, 2H). Mp = 147–149 °C.

4.1.9. 6-Chloro-3-oxo-4-(2,2,3,3,3-pentafluoropropyl)-5-(2,4,6-trifluorophenyl)-pyrazine-2-carboxamide (13b)

To a solution of 3,5-dichloro-1-(2,2,3,3,3-pentafluoropropyl)-6-(2,4,6-trifluorophenyl)-2(1*H*)-pyrazinone (**10b**, 0.85 g, 2.0 mmol) in tetrahydrofuran (10 mL) was added 1*H*-benzotriazole-1-acetonitrile (0.48 g, 3.0 mmol) and lithium-bis(trimethylsilyl)amide (1.0 M solution in tetrahydrofuran, 5.0 mL, 5 mmol). The resulting mixture was stirred at room temperature for 1.5 h. A solution of ammonia in dioxane (0.5 M, 12 mL, 6 mmol) was then added, and the reaction mixture was stirred for an additional 10 min. Peracetic acid (32 wt % solution in acetic acid), 1.68 mL) was added dropwise to the reaction mixture, and the resulting mixture stirred at room temperature for 3 h. Saturated aqueous sodium hydrogensulfite was then added (50 mL) and the reaction mixture was extracted with ethyl acetate (2 × 50 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel chromatography (50–80% ethyl acetate in hexanes as eluant) to afford 6-chloro-3-oxo-4-(2,2,3,3,3-pentafluoropropyl)-5-(2,4,6-trifluorophenyl)-pyrazine-2-carboxamide (13b, 0.150 g, 0.34 mmol, 17%). ^{1}H NMR ($CDCl_3$): δ 877 (br s, 1H), 6.94 (t, 2H), 6.17 (br s, 1H), 4.65 (m, 2H), Mp = 242–243 °C.

4.1.10. 2-(2,6-Difluoro-4-methoxy-phenyl)-2-((2*S*)-2-methylbutylamino)-acetonitrile (8c)

To a solution of sodium bisulfite (1.71 g, 17 mmol) in a mixture of deionized water (32 mL) and methanol (3.0 mL) at room temperature was added 2,6-difluoro-4-methoxybenzaldehyde (2.7 g, 16 mmol). The reaction mixture was stirred for 15 min, and sodium cyanide (0.81 g, 17 mmol) was added. The reaction mixture was stirred for an additional 20 min and cooled using an ice water bath. A solution of (*S*)-2-methylbutylamine (Sigma–Aldrich, 1.5 g, 17 mmol) in methanol (6.0 mL) was added over approximately 2 min, and the resulting reaction mixture was stirred at 0 °C for 15 min and then heated to 35 °C for 2 h. The resulting mixture was then extracted with ethyl acetate (2 × 40 mL), and the combined organic layers were washed with brine, dried ($MgSO_4$) and concentrated to give 2-(2,6-difluoro-4-methoxy-phenyl)-2-((2*S*)-2-methylbutylamino)-acetonitrile (**8c**, 3.9 g, 15 mmol, 93%) as an oil. ^{1}H NMR ($CDCl_3$): δ 6.51 (m, 2H), 4.83 (br s, 1H), 3.80 (s, 3H), 2.76 (m, 1H), 2.52 (m, 1H), 1.49 (m, 2H), 1.17 (m, 1H), 0.90 (m, 6H).

4.1.11. 3,5-Dichloro-6-(2,6-difluoro-4-methoxyphenyl)-1-((2*S*)-2-methylbutyl)-2(1*H*)-pyrazinone (10c)

A solution of 2-(2,6-difluoro-4-methoxy-phenyl)-2-((2*S*)-2-methylbutylamino)-acetonitrile (**8c**, 3.9 g, 15 mmol) in chlorobenzene (16 mL) was added dropwise over 20 min to a solution of oxalyl chloride (9.3 g, 73 mmol) in chlorobenzene (39 mL) at room temperature. The reaction mixture was then heated to 100 °C overnight. *N,N*-Dimethylformamide (0.5 mL) was then added, and the reaction

mixture was heated for an additional 2 h. The reaction mixture was then concentrated under reduced pressure and the resulting residue was purified by MPLC (0–100% gradient of ethyl acetate in hexanes as eluant) to give 2.9 g of 3,5-dichloro-6-(2,6-difluoro-4-methoxyphenyl)-1-((2*S*)-2-methylbutyl)-2(1*H*)-pyrazinone (**10c**, 3.8 g, 10 mmol, 69%) as an oil. ^{1}H NMR ($CDCl_3$): δ 6.61 (m, 2H), 3.90 (s, 3H), 3.76 (m, 2H), 1.70 (m, 1H), 1.20 (m, 1H), 1.03 (m, 1H), 0.74 (m, 6H).

4.1.12. 5-Chloro-6-(2,6-difluoro-4-methoxyphenyl)-1-((2*S*)-2-methylbutyl)-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (11c)

A mixture of 3,5-dichloro-6-(2,6-difluoro-4-methoxyphenyl)-1-((2*S*)-2-methylbutyl)-2(1*H*)-pyrazinone (**10c**, 3.8 g, 9.4 mmol), pyrazole (0.69 g, 10 mmol) and potassium carbonate (2.6 g, 19 mmol) in *N,N*-dimethylformamide (40 mL) was heated to 60 °C overnight. The reaction mixture was concentrated under reduced pressure and the residue was purified by MPLC (0–100% gradient of ethyl acetate in hexanes as eluant) to give 5-chloro-6-(2,6-difluoro-4-methoxyphenyl)-1-((2*S*)-2-methylbutyl)-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (**11c**, 2.5 g, 6.1 mmol, 67%). ^{1}H NMR ($CDCl_3$): δ 9.09 (m, 1H), 7.88 (m, 1H), 6.63 (m, 2H), 6.50 (m, 1H), 3.87 (m, 5H), 1.75 (m, 1H), 1.25 (m, 1H), 1.05 (m, 1H), 0.74 (m, 6H).

4.1.13. 5-Chloro-6-(2,6-difluoro-4-hydroxyphenyl)-1-((2*S*)-2-methylbutyl)-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (15)

To a solution of 5-chloro-6-(2,6-difluoro-4-methoxyphenyl)-1-((2*S*)-2-methylbutyl)-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (**11c**, 1.0 g, 2.4 mmol) in dichloromethane (24 mL) at −78 °C was slowly added a solution of boron tribromide (1 M solution in dichloromethane, 9.8 mL, 9.8 mmol). The reaction mixture was allowed to warm to room temperature overnight. Then the reaction mixture was cooled to 0 °C and quenched with saturated aqueous ammonium chloride solution. The reaction mixture was extracted with dichloromethane (2 × 40 mL) and ethyl acetate (2 × 30 mL). The organic layers were combined, dried over $MgSO_4$ and concentrated. The crude residue was purified by MPLC (0–100% gradient of ethyl acetate in hexanes as eluant) to yield 5-Chloro-6-(2,6-difluoro-4-hydroxyphenyl)-1-((2*S*)-2-methylbutyl)-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (**15**, 0.51 g, 1.3 mmol, 53%).

^{1}H NMR ($CDCl_3$): δ 10.53 (br s, 1H), 9.24 (d, 1H), 7.94 (d, 1H), 6.74 (d, 2H), 6.57 (m, 1H), 3.90 (d, 2H), 1.76 (m, 1H), 1.26 (m, 1H), 1.05 (m, 1H), 0.77 (m, 6H).

4.1.14. Phenylmethyl *N*-[3-[4-[3-chloro-1,6-dihydro-1-[(2*S*)-2-methylbutyl]-6-oxo-5-(1*H*-pyrazol-1-yl)-2-pyrazinyl]-3,5-difluorophenoxy]propyl]-*N*-methylcarbamate (17b)

To a solution of 5-chloro-6-(2,6-difluoro-4-hydroxyphenyl)-1-[(2*S*)-2-methylbutyl]-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (**15**, 0.35 g, 0.89 mmol) in *N,N*-dimethylformamide (4 mL) was added dry activated 4 Å molecular sieves (3.0 g). The reaction mixture was stirred for 3 h at room temperature. Tetrabutylammonium iodide (0.065 g, 0.18 mmol) and phenylmethyl *N*-(3-chloropropyl)-*N*-methylcarbamate (**16b**, 0.64 g, 2.7 mmol) in *N,N*-dimethylformamide (1 mL), were added and the reaction mixture was stirred for 15 min at room temperature. Cesium carbonate (0.87 g, 2.7 mmol) was added and stirring was continued for another 15 min. The reaction mixture was then heated to 75 °C for 2 h and then cooled to room temperature. After the molecular sieves and cesium carbonate were removed by filtering through Celite® the reaction mixture was concentrated under reduced pressure. The crude oil was purified by MPLC (0–100% gradient of ethyl acetate in hexanes as eluant) to provide phenylmethyl *N*-[3-[4-[3-chloro-1,6-dihydro-1-[(2*S*)-2-methylbutyl]-6-oxo-5-(1*H*-pyrazol-1-yl)-2-pyrazinyl]-3,5-difluorophenoxy]propyl]-*N*-methylcarbamate (**17b**, 0.44 g, 0.73 mmol, 82%). ^{1}H NMR ($CDCl_3$): δ 9.09 (d, 1H), 7.88 (d, 1H), 7.33 (m, 5H), 6.59 (m, 1H), 6.50 (m, 2H), 5.11 (s, 2H), 4.00 (m, 2H), 3.85 (m, 2H), 3.51 (t, 2H), 2.98 (s, 3H), 2.10 (m, 2H), 1.72 (m, 1H), 1.20 (m, 1H), 1.03 (m, 1H), 0.74 (m, 6H).

4.1.15. 5-Chloro-6-[2,6-difluoro-4-[3-(methylamino)propoxy]-phenyl]-1-[(2*S*)-2-methylbutyl]-3-pyrazol-1-yl-pyrazin-2-one hydrochloride (18b)

Phenylmethyl-*N*-[3-[4-[3-chloro-1,6-dihydro-1-[(2*S*)-2-methylbutyl]-6-oxo-5-(1*H*-pyrazol-1-yl)-2-pyrazinyl]-3,5-difluorophenoxy]propyl]-*N*-methylcarbamate (**17b**, 0.44 g, 0.73 mmol) was dissolved in methanol (50 mL) and flushed with nitrogen. Hydrogen chloride (1 M solution in diethyl ether, 4 mL) was added followed by palladium on carbon (10% wt/wt, 0.12 g, 0.11 mmol) and flushing with nitrogen was continued. A balloon containing hydrogen gas was attached to the reaction mixture and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was filtered through Celite®, diatomaceous filter aid, and concentrated under reduced pressure. The reaction mixture was redissolved in methanol, filtered, and then concentrated to give 5-chloro-6-[2,6-difluoro-4-[3-(methylamino)propoxy]-phenyl]-1-[(2*S*)-2-methylbutyl]-3-pyrazol-1-yl-pyrazin-2-one hydrochloride (18b, 0.34 g, 0.73 mmol, 99%). ^{1}H NMR (*methanol-d*$_4$) δ 9.08 (d, 1H), 8.23 (m, 1H), 7.89 (d, 1H), 6.91 (m, 2H), 6.59 (s, 1H), 4.22 (t, 2H), 3.87 (m, 2H), 3.23 (m, 2H), 2.75 (s, 3H), 2.21 (m, 2H), 1.73 (m, 1H), 1.24 (m, 1H), 1.06 (m, 1H), 0.73 (m, 6H).

4.1.16. 5-Chloro-6-[4-[3-(dimethylamino)propoxy]-2,6-difluoro-phenyl]-1-[(2*S*)-2-methylbutyl]-3-pyrazol-1-yl-pyrazin-2-one (17a)

A solution of 5-chloro-6-(2,6-difluoro-4-hydroxyphenyl)-1-[(2*S*)-2-methylbutyl]-3-(1*H*-pyrazol-1-yl)-2(1*H*)-pyrazinone (**15**, 0.2 g, 0.51 mmol) and Cs_2CO_3 (0.83 g, 2.5 mmol) in *N,N*-dimethylformamide (10 mL) was heated to 70° C for 15 min. 3-Chloro-*N*,*N*-dimethyl-1-propaneamine hydrochloride (**16a**, 0.24 g, 1.5 mmol) was then added and the reaction mixture was then heated for an additional 1.5 h, and then cooled to room temperature. After the cesium carbonate was removed by filtering through Celite®, diatomaceous filter aid, the reaction mixture was concentrated under reduced pressure. The crude oil was purified by MPLC (0–30% gradient of methanol in methylene chloride as eluant) to provide 5-chloro-6-[4-[3-(dimethylamino)propoxy]-2,6-difluoro-phenyl]-1-[(2*S*)-2-methylbutyl]-3-pyrazol-1-yl-pyrazin-2-one (**17a**, 0.019 g, 0.040 mmol, 8%). ^{1}H NMR ($CDCl_3$) δ 9.09 (m, 1H), 7.88 (m, 1H), 6.63 (m, 2H), 6.50 (m, 1H), 4.08 (m, 2H), 3.85 (m, 2H), 2.48 (m, 2H), 2.28 (s, 6H), 2.00 (m, 2H), 1.74 (m, 1H), 1.25 (m, 1H), 1.04 (m, 1H), 0.75 (m, 6H).

4.2. Biochemistry

Purified bovine tubulin was purchased from Cytoskeleton Inc., Denver, USA and the polymerization assay performed as recommended by the manufacturer. The polymerization reaction contained 100 µl volume of 4 mg/ml tubulin in 80 mM PIPES pH 6.9, 0.5 mM EGTA, 2 mM $MgCl_2$ and 1 mM GTP in individual wells of a 96-well microtiter plate. Polymerization was started by incubation at 37 °C and followed over time at 340 nm (Spectramax plate reader). Paclitaxel (10 µM) and DMSO (0.5%) were used as positive and negative controls, respectively. Experimental compounds were added to different wells of the plate at the beginning of the reaction over a range of concentrations in constant concentrations of DMSO solution.

4.3. Biology

Compounds were first dissolved in acetone in an amount equal to 3% of the final volume and then suspended at the desired

concentration (in ppm) in acetone and purified water (50/50 mix) containing 250 ppm of the surfactant Trem® 014 (polyhydric alcohol esters). Spraying a 200 ppm test suspension to the point of run-off on the test plants was the equivalent of a field rate of 500 g/hectare. The biological evaluation for additional analogs can be found in the patent literature.[13,21]

4.3.1. *Stagonospora nodorum* (glume blotch on wheat)

The test suspension was sprayed to the point of run-off on Stevens wheat seedlings. The following day the seedlings were inoculated with a spore suspension of *Stagonospora nodorum*. (the causal agent of wheat glume blotch) and incubated in a saturated atmosphere at 20 °C for 48 h, and then moved to a growth chamber at 22 °C for 5 days, after which disease ratings were made.

4.3.2. *Puccinia recondita f.* sp. *Tritici* (brown rust on wheat)

The test suspension was sprayed to the point of run-off on Stevens wheat seedlings. The following day the seedlings were inoculated with a spore suspension of *Puccinia recondita f.* sp. *tritici*. (the causal agent of wheat leaf/brown rust) and incubated in a saturated atmosphere at 20 °C for 24 h, and then moved to a growth chamber at 20 °C for 7 days, after which disease ratings were made.

4.3.3. *Botryotinia fuckeliana* (gray mold on tomato)

The test suspension was sprayed to the point of run-off on Orange Pixie tomato seedlings. The following day the seedlings were inoculated with a spore suspension of *Botryotinia fuckeliana* (the causal agent of gray mould on tomatoes) and incubated in saturated atmosphere at 20 °C for 48 h, and then moved to a growth chamber at 24 °C for 1 additional day, after which disease ratings were made.

4.3.4. Rhabdomyosarcoma cell line (ATCC CCL-136)

Cells were dispensed into wells of an untreated 96-well microtiter plate at a density of 10,000 cells/well in growth media and allowed to adhere for 1–3 h before the media was removed and replaced with media containing the test compound over a range of concentrations. The plates were maintained at 37 °C in 5% CO_2 atmosphere for 96 h to allow for cell growth and proliferation. For determination of the proliferation IC_{50} value, a MTS tetrazolium compound was added as recommended by the manufacturer (Promega Celltiter 96 Aqueous Assay kit MTS/PMS solution) to indicate the number of viable cells remaining in each well using the colored formazan adduct that forms after 3–4 h at 37 °C by measurement at 490 nm. The data were normalized to control wells containing solvent alone.

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