Organic & Biomolecular Chemistry

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c6ob01160h

Horner–Wadsworth–Emmons approach to piperlongumine analogues with potent anti-cancer activity†

Li-Chen Han,[a] Paul A. Stanley,[a] Paul J. Wood,[b] Pallavi Sharma,[a] Anchala I. Kuruppu,[b] Tracey D. Bradshaw*[b] and John E. Moses*[a]

Received 27th May 2016,
Accepted 15th July 2016

DOI: 10.1039/c6ob01160h

www.rsc.org/obc

Natural products with anti-cancer activity play a vital role in lead and target discovery. We report here the synthesis and biological evaluation of the plant-derived alkaloid, piperlongumine and analogues. Using a Horner–Wadsworth–Emmons coupling approach, a selection of piperlongumine-like compounds were prepared in good overall yield from a novel phosphonoacetamide reagent. A number of the compounds displayed potent anti-cancer activity against colorectal (HCT 116) and ovarian (IGROV-1) carcinoma cell lines, *via* a mechanism of action which may involve ROS generation. Contrary to previous reports, no selective action in cancer cell (MRC-5) was observed for piperlongumine analogues.

Introduction

Natural products have made an enormous contribution in the development of molecular therapies for disease and, to this day, remain a key source of valuable drug leads.[1,2] A detailed analysis of new medicines approved by the US Food and Drug Administration (FDA) between 1981 and 2010 revealed that 34% of those medicines were based on small-molecule natural products or were direct derivatives of natural products.[3–5] The trend is even more pronounced in the field of anti-cancer chemotherapy, where a significant proportion of drugs in clinical use are natural products or their derivatives.[6]

The overwhelming majority of clinical anti-cancer agents target DNA synthesis and cell division; while these treatments are indeed effective, they often lack selectivity for cancerous cells, leading to severe side effects.[7] Thus, one of the greatest challenges in anti-cancer chemotherapy is the selective killing of cancer cells on the basis of cancer-specific features, rather than generic properties that are shared with normal cells.[8,9] In response, many successful approaches have been developed in recent times.[10] One particular strategy that has gained significant attention involves targeting of the stress response to reactive oxygen species (ROS), which has demonstrated specificity and broad effect in a variety of cancers.[11] Malignant transformation caused by gain-of-function activation in oncogenes, or loss-of-function mutations in tumour suppressor genes, are believed to induce enhanced cellular stress. Thus, adaptation to this enhanced stress is necessary for cancer cell survival, whilst it is not required in normal cells.[12]

Schreiber and co-workers recently identified piperlongumine (**1**)[13] (piplartine) as a lead compound that increased ROS levels and induced apoptosis in both cancer cells and normal cells engineered to have a cancer genotype, irrespective of p53 status. Piperlongumine (**1**) of the genus *Piper*, belongs to the *Piperaceae* family of natural products that are widely known for their medicinal properties (Fig. 1).

Although piperlongumine had been previously shown to exhibit cytotoxic activity,[14] the study by the Schreiber group revealed that **1** had little inhibitory effect on either rapidly or

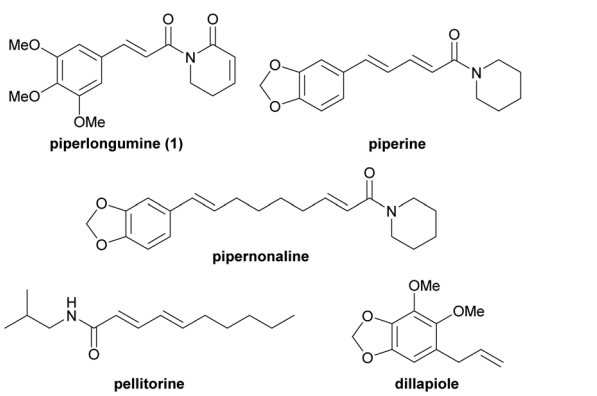


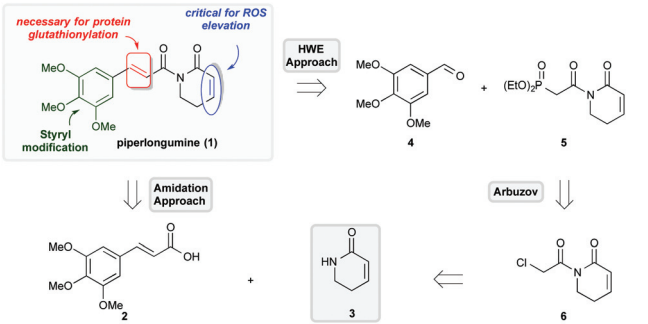
Fig. 1 Representative members of the *Piperaceae* family of natural products.

[a]School of Chemistry, University of Nottingham, University Park, Nottingham, NG7 2RD, UK. E-mail: John.Moses@nottingham.ac.uk
[b]School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK.
E-mail: Tracey.Bradshaw@nottingham.ac.uk

† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra of all newly synthesised compounds. See DOI: 10.1039/c6ob01160h

slowly dividing normal cells,[13] demonstrating impressive selectivity for neoplastic cells. A number of subsequent studies have further demonstrated **1** as a promising anti-cancer drug lead.[15]

The observed selectivity towards malignant cells; high efficacy and low systemic toxicity warrant further SAR investigation of piperlongumine (**1**) as a potential therapeutic candidate. We report here a new synthetic route to **1** and analogues thereof. We also demonstrate *in vitro* anti-cancer activity of a number of the synthetic compounds and identify a promising new lead with comparable activity to the parent piperlongumine. A Horner–Wadsworth–Emmons (HWE) olefination approach was designed to enable the direct diastereoselective construction of the focused library of piperlongumine analogues (**11**–**21**).



Scheme 1 Retrosynthetic Horner–Wadsworth–Emmons approach towards piperlongumine.

Results and discussion

Synthesis of piperlongumine and analogues

The structure of piperlongumine (**1**) was first assigned in 1968, suggesting a 5,6-dihydropyridin-2(1*H*)-one fused to a 3,4,5-trimethoxyphenyl group *via* an *E*-acryloyl group.[16] Corroboration of the structure of **1** through total synthesis and X-ray crystal structure determination was reported by Simonsen in 1984, who employed 3,4,5-trimethoxycinnamic anhydride in an amide bond formation strategy.[17] A number of piperlongumine analogues has since been reported by several research groups.[18]

A common synthetic approach to piperlongumine type structures involves the coupling of an α,β-unsaturated δ-lactam **3** with a variety of cinnamates.[13b,18] In each case the major synthetic challenge is the preparation of the key 5,6-dihydropyridin-2(1*H*)-one **3**, which has been achieved most frequently using alkene metathesis of the corresponding homoallyl crotonamide.[13b,18]

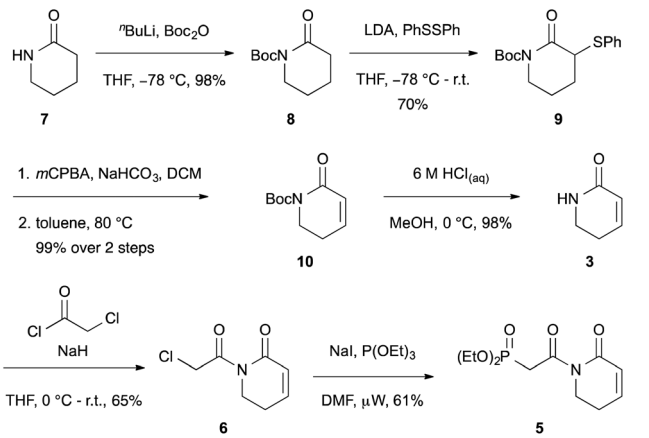
The vast majority of SAR studies on piperlongumine analogues have been focused on the modification of the heterocyclic dihydropyridinone region of the ligand, which itself has been implicated in attenuating ROS levels in cells; or on the glutathionyl binding–bridging alkene region. The effect of modification of the styryl ring system has been comparatively less extensively studied.

We have devised a convergent approach to **1** (and analogues thereof), employing a late stage HWE olefination of the corresponding phosphonoacetamide **5**, which in principle would enable *E*-stereoselective installation of the central acryloyl group directly from the corresponding aldehyde (Scheme 1). We envisaged that this approach would be amenable to the synthesis of a number of modified aromatic/styryl analogues, circumventing the need for preparing bespoke cinnamates required by other approaches. The imidation and Arbuzov reaction of **3** would lead to the coupling reagent phosphonoacetamide **5** (Scheme 1).

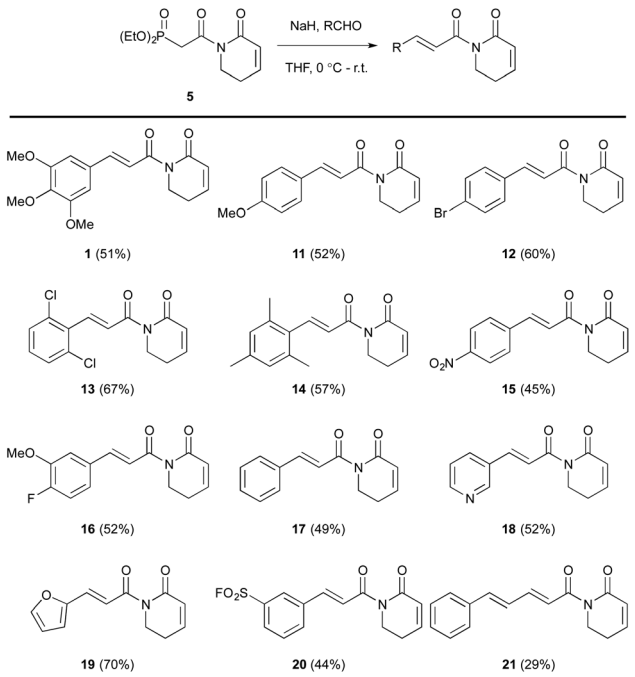
The synthesis of the key α,β-unsaturated δ-lactam **3** began with *N*-Boc protection of the commercially available δ-valerolactam (**7**), using di-*tert*-butyl dicarbonate in 98% yield. The protected lactam **8** was next converted into the corresponding 3-(phenylthio)piperidin-2-one **9** upon consecutive treatment with freshly prepared lithium diisopropylamide and diphenyl disulfide. The sulfide **9** was next oxidised using *m*CPBA, followed by a thermally induced *syn*-elimination of the resultant sulfoxide, yielding the protected α,β-unsaturated δ-lactam **10** in 99% yield. Finally, *N*-Boc deprotection under acidic conditions delivered the target lactam **3** in five linear steps and multigram quantities, with an overall yield of 67%.

The synthesis of the key HWE reagent **5** was achieved from the lactam **3** in two steps: first a base mediated addition of the lactam **3** to chloroacetyl chloride delivered α-chloro imide **6**, which smoothly underwent an Arbuzov reaction with triethyl phosphite under microwave irradiation to deliver the key phosphonoacetamide **5** in 61% yield (Scheme 2).

The sodium hydride mediated deprotonation of the phosphonoacetamide **5**, followed by subsequent reaction with 3,4,5-trimethoxybenzaldehyde (**4**) gave the target piperlongumine (**1**) in 51% yield (Scheme 3). Applying the same reaction conditions to a selection of aryl and heteroaryl aldehydes, enabled the synthesis of eleven structural analogues (**11**–**21**)[13b,18c] of the lead natural product (Scheme 3).



Scheme 2 Synthesis of Horner–Wadsworth–Emmons reagent **5**.



Scheme 3 Synthesis of piperlongumine and analogues.

Growth inhibitory effects of piperlongumine and piperlongumine analogues in colorectal (HCT 116) and ovarian (IGROV-1) carcinoma cell lines

To determine growth inhibitory effects, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed at the time of test agent addition (T zero) and following 72 h exposure of cells to test compounds.[19] Both ovarian and colorectal cancer cell lines showed dose-dependent sensitivity to each compound tested; increasing concentrations reduced growth/proliferation. The test agent potencies varied as illustrated in Table 1 (see ESI, Fig. S1†). While each compound assayed demonstrated promising activity, ligands **15** and **18** stood out as the most active. To test the hypothesis that activity was cancer cell-selective, we also examined growth inhibitory properties against a non-transformed foetal lung fibroblast cell line – MRC-5. Contrary to previous reports, selectivity was not observed; piperlongumine analogues appeared equi-active against MRC-5, as they were against HCT 116 and IGROV-1 cell lines (GI_{50} values <50 μM were obtained for all analogues).

Table 1 Growth inhibitory activities of **1** and related analogues in HCT 116 colorectal, IGROV-1 ovarian carcinoma cell lines and MRC-5. Data represent mean ± SD GI_{50} values from >3 independent MTT assays (n = 4 per concentration in each trial)

	Analogue	HCT 116	IGROV-1	MRC-5
GI_{50} (μM) mean ± SD	Piperlongumine (**1**)	17.3 ± 1.7	4.9 ± 1.7	3.1 ± 0.04
	11	23.0 ± 3.5	11.6 ± 3.5	5.5 ± 1.5
	12	39.9 ± 3.3	10.8 ± 3.3	6.7 ± 0.5
	13	26.1 ± 5.3	11.8 ± 5.3	5.5 ± 0.4
	14	64.4 ± 2.5	36.0 ± 2.5	25.7 ± 1.2
	15	7.78 ± 0.5	7.1 ± 0.5	2.8 ± 0.05
	16	9.4 ± 2.8	11.4 ± 2.8	3.1 ± 0.06
	17	97.0 ± 9.3	29.45 ± 9.3	6.3 ± 1.1
	18	7.4 ± 0.6	4.34 ± 0.6	5.5 ± 1.7
	19	17.2 ± 1.7	5.4 ± 1.7	14.1 ± 6.1
	20	43.5 ± 8.0	31.0 ± 8.0	7.8 ± 0.6
	21	52.8 ± 12.3	29.5 ± 12.3	17.5 ± 2.4

Effects of piperlongumine and piperlongumine analogues on HCT 116 and IGROV-1 colony formation

Clonogenic assays were next performed to determine the ability of single cells to survive brief challenge with the piperlongumine analogues (24 h) and recover proliferative potential to form progeny colonies.

Guided by the MTT assay results, we selected the parent compound **1** and four analogues: **15**, **16**, **18** and **19** for inclusion in the clonogenic assays as they were found to yield low GI_{50} values (most potent activity) against both cell lines. The non-substituted phenyl compound **17**, which yielded a high GI_{50} value against both cell lines and hence demonstrated poorest performance in the MTT assay was included as a negative control. Each compound was tested in duplicate on three separate occasions. Table 2 illustrates the mean IC_{50} values ± SDs. Dose-dependent inhibition of colony formation by the piperlongumine analogues was demonstrated (ESI, Fig. S2†).

Treatment of cells with the parent compound **1** and analogue **15** revealed IC_{50} values <4 μM in both the ovarian and colorectal carcinoma cell lines. As illustrated in Table 2 (also see ESI, Fig. S2†), significant inhibition of colony formation was detected at concentrations of ≥100 nM for both **1** and **15**; at 10 μM HCT 116 and IGROV-1 inhibition of colony formation was absolute. For both compounds **1** and **15** clonogenic IC_{50} values correlated with MTT GI_{50} values. In a similar manner, the GI_{50} and IC_{50} values for compound **16** were comparable against both cell lines; (IC_{50} values in clonogenic assays <10 μM).

The piperlongumine analogues **18** and **19** inhibited colony formation to an almost identical extent in both cell lines (IC_{50} values ~8 μM). Intriguingly, although the activity of **17** was poor in the MTT assay against HCT 116 cells (GI_{50} 97 μM), clonogenic survival did not reflect this lack of activity, indicating that single cells were less able to survive challenge by **17** to form progeny colonies (IC_{50} 5.05 μM). Against IGROV-1 cells, similar inhibition of colony formation was evident (IC_{50} 7.2 μM); again greater potency was observed in the clonogenic

Table 2 Inhibition of colony formation by piperlongumine analogues. Mean ± SD IC_{50} values (n = 6) are shown

	Analogue	HCT 116	IGROV-1
IC_{50} (μM) mean ± SD	Piperlongumine (**1**)	3.7 ± 0.1	3.55 ± 0.3
	15	3.9 ± 0.5	3.75 ± 0.1
	16	9.75 ± 0.3	6.85 ± 0.7
	17	5.05 ± 0.4	7.2 ± 0.7
	18	7.9 ± 0.3	7.8 ± 1.7
	19	7.6 ± 0.2	7.35 ± 1.9

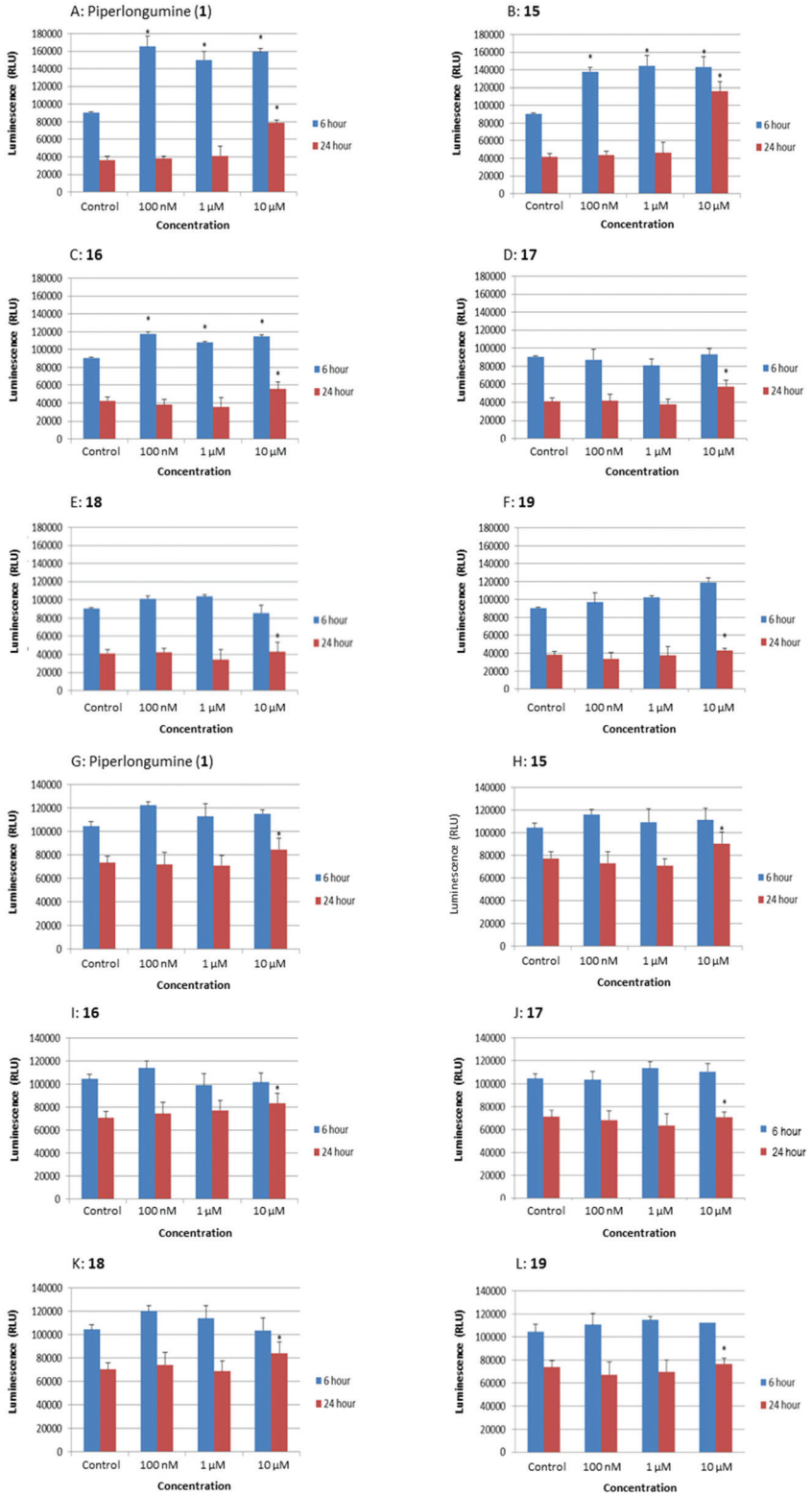


Fig. 2 The effects of selected piperlongumine analogues on ROS level at 6 h and 24 h. (A–F) HCT-116 cells treated with selected piperlongumine analogues. (G–L) IGROV-1 cells treated with selected piperlongumine analogues. Dose dependant increase of selected piperlongumine analogues causes an increase in ROS level after 6 h exposure in HCT 116 cells for (A) **1**, (B) **15** and (C) **16**. (A–L) 24 h exposure ROS level diminishes in both cell lines at all piperlongumine doses. (A–L) ROS increases at 10 μM – 24 h in both cell lines for all selected piperlongumine analogues. Graphs are representative of the mean + SEM taken from 2 independent repeats carried out in duplicate for both the 6 h and 24 h data points. One way ANOVA was used to calculate significant differences with (*$p < -0.05$).

assay compared with the MTT assay (GI_{50} 29.45 μM) following cellular challenge with compound **17**.

In clonogenic assays, HCT 116 and IGROV-1 cells showed similar sensitivity to all piperlongumine analogues tested, (IC_{50} < 10 μM). However, in the MTT assay, distinction between analogues and between cell lines was evident, *e.g.* HCT 116 cells were >13× more sensitive to **18** than **17**. In general, IGROV-1 cells appeared more sensitive to the growth inhibitory properties of piperlongumine analogues than HCT 116 cells (with the exception of **16**), as exemplified by parent compound **1**, ~3.5-fold greater sensitivity in IGROV-1 cells.

The effects of piperlongumine and selected piperlongumine analogues on ROS elevation in HCT 116 and IGROV-1 cell lines

The roles of cellular ROS are manifold, including, for example, as critical components in signal transduction cascades or apoptotic trigger. Generation of ROS is an important mechanism by which many anticancer agents exert cytotoxicity and it is recognised that piperlongumine (**1**) elevates cellular levels of ROS in cancer cells.[13] However, ROS generation may not be essential to the mechanism of action of the piperlongumine class of agents as Adams *et al.*[13b] synthesised piperlongumine analogues that retained ROS activity but were markedly less cytotoxic, associating irreversible protein glutathionylation as an additional mechanism contributing to piperlongumine's cellular toxicity. To begin to understand the mechanisms of action of the piperlongumine analogues, assays were performed to measure cellular generation of ROS following treatment of cells with piperlongumine (**1**) and analogue compounds **15–19**. The assays were conducted following 6 h and 24 h exposure of cells to the test agents, and results presented in Fig. 2.

Following 6 h exposure, significantly elevated ROS were generated in HCT 116 cells treated with piperlongumine analogues **1**, **15** and **16** at all concentrations tested (1, 5, and 10 μM). Following 24 h exposure, changes in ROS levels were much reduced with significantly raised levels being detected after treatment of cells with 10 μM compound only. In IGROV-1 cells, exposure (6 h; 24 h) to piperlongumine analogues resulted in detection of only minor increases in ROS levels.

Following 24 h exposure, ROS levels were significantly raised in HCT 116 cells by each analogue tested at 10 μM. Similarly, in IGROV-1 cells, 24 h exposure to 10 μM of the compounds resulted in significantly raised ROS; however, following 6 h exposure, only insignificant fluctuations in ROS levels were detected following exposure of IGROV-1 cells to piperlongumine analogues.

These data strongly support the hypothesis that ROS generation may not be a critical factor to the mechanism of action of piperlongumine analogues, indicating a decoupling of ROS and cell death.[13b] Nevertheless, it is noteworthy that the piperlongumine analogues possessing most potent HCT 116 growth inhibitory activity (as assessed by MTT assay; compounds **15**, **16** and **1**; GI_{50} values 7.8, 9.4 and 17.3 μM respectively) significantly elevate ROS in HCT 116 cells (detection 6 h post-treatment). Therefore, in this cell line at least, the generation of ROS may contribute to the observed growth inhibitory activity.

Conclusions

The synthesis of piperlongumine (**1**) and analogues (**11–21**) was achieved through a Horner–Wadsworth–Emmons coupling strategy. The development of the key phosphonoacetamide (**5**) reagent demonstrates a new and versatile approach to the piperine type alkaloids.

We have further demonstrated that piperlongumine and several of its analogues cause growth inhibition and cytotoxicity in HCT 116 and IGROV-1 cells. In MTT assays, several compounds showed approximately equi-activity in comparison to the parent piperlongumine, and all analogues tested revealed pot ent anticlonogenic activity. In addition contrary to previous reports, piperlongumine analogues appeared equi-active against MRC-5, as they were against HCT 116 and IGROV-1 cell lines.

When examined 6 h post-treatment, the compounds **1** and **15** had generated significantly enhanced ROS levels in HCT 116 cells. Cancer cell-specific elevation of ROS has been shown to cause dose-dependent cell death through application of **1**. Further mechanistic deconvolution studies are necessary, but it is anticipated that piperlongumine could be used in conjunction with other chemotherapeutics such as 5-FU or with ROS scavenging enzyme inhibitors to promote the damaging effects caused by ROS to induce cancer cell death.

For parent piperlongumine, systemic toxicity has been determined to be low *in vivo* but other analogues must be tested before safety can be assumed. Further pre-clinical investigation of piperlongumine analogues is warranted, including examination of activity and mechanisms of action against other cancer cell lines; additional *in vivo* studies of tolerability, stability and bioavailability require consideration.

Experimental

All reactions were performed in flame-dried glassware under an atmosphere of argon. All reagents were used as received unless otherwise stated. IR spectra were recorded using an ATR attachment; only significant absorptions (ν_{max}) are reported in wavenumbers (cm^{-1}). Proton magnetic resonance spectra (^{1}H-NMR) were recorded at either 300 or 400 MHz. Carbon magnetic resonance spectra (^{13}C-NMR) were recorded at 100 MHz. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced to the residual solvent peak; coupling constants (J) are quoted in hertz to the nearest 0.1 Hz. High resolution mass spectra were obtained by electrospray ionisation (ESI). Flash chromatography was performed using silica gel (40–63 micron) as the stationary phase.

General procedure for the synthesis of piperlongumine and analogues *via* Horner–Wadsworth–Emmons olefination

Phosphonoacetamide **5** (1.0 eq.) was dissolved in anhydrous THF (0.05 M) under an atmosphere of argon and the solution cooled to 0 °C. NaH (1.1 eq.) was added and the resulting mixture stirred until no further effervescence was observed.

The aldehyde (2.0 eq.) was then added and the reaction warmed to room temperature and stirred overnight. Saturated $NH_4Cl_{(aq.)}$ was added and the aqueous phase was extracted with EtOAc. The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography.

Piperlongumine (1). Piperlongumine **1** (14.0 mg, 51%) was prepared *via* the general procedure as a white solid. M.p.: 120–121 °C [lit. 122–123 °C];[18a] ν_{max}/cm^{-1} 3692, 3606, 1686, 1603, 1583; δ_H (400 MHz, $CDCl_3$) 2.47 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 3.86 (3H, s), 3.87 (6H, s), 4.02 (2H, t, J = 6.4 Hz), 6.04 (1H, dt, J = 9.7, 1.9 Hz), 6.79 (2H, s), 6.93 (1H, dt, J = 9.7, 4.2 Hz), 7.41 (1H, d, J = 15.6 Hz), 7.87 (1H, d, J = 15.6 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 56.3, 61.0, 105.6, 121.2, 125.9, 128.3, 139.9, 130.7, 143.9, 145.7, 166.0, 169.0; Found (ESI): 318.1327 $[MH]^+$, (required $C_{17}H_{20}NO_5$ 318.1336); Found (ESI): 340.1153 $[MNa]^+$, (required $C_{17}H_{19}NO_5Na$ 340.1155). Spectroscopic data in accord with the literature.[18c]

(*E*)-1-(3-(4 Methoxyphenyl)prop-2-enoyl)-5,6-dihydropyridin-2(1*H*)-one (11). Compound **11** (23.0 mg, 52%) was prepared *via* the general procedure as a white solid. M.p.: 76–77 °C; ν_{max}/cm^{-1} 3691, 3606, 3011, 2937, 2840, 1686, 1601, 1513; δ_H (400 MHz, $CDCl_3$) 2.46 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 3.83 (3H, s), 4.03 (2H, t, J = 6.4 Hz), 6.03 (1H, dt, J = 9.7, 1.9 Hz), 6.87–6.90 (2H, m), 6.92 (1H, dt, J = 9.7, 4.2 Hz), 7.41 (1H, d, J = 15.6 Hz), 7.49–7.57 (2H, m), 7.73 (1H, d, J = 15.6 Hz); δ_C (100 MHz, $CDCl_3$) 24.8, 41.6, 55.4, 114.2, 119.4, 126.0, 127.9, 130.0, 143.6, 145.3, 161.3, 165.8, 169.2; Found (ESI): 258.1124 $[MH]^+$, (required $C_{15}H_{16}NO_3$ 258.1125); Found (ESI): 280.0943 $[MNa]^+$, (required $C_{15}H_{15}NO_3Na$ 280.0944). Spectroscopic data in accord with the literature.[13b]

(*E*)-1-(3-(4-Bromophenyl)acryloyl)-5,6-dihydropyridin-2(1*H*)-one (12). Compound **12** (23.0 mg, 60%) was prepared *via* the general procedure as a white solid. M.p.: 93–94 °C [lit. 93 °C];[18c] ν_{max}/cm^{-1} 3692, 3607, 1686, 1618, 1602; δ_H (400 MHz, $CDCl_3$) 2.48 (2H, tdd, J = 6.5, 4.2, 1.9 Hz), 4.03 (2H, t, J = 6.5 Hz), 6.04 (1H, dt, J = 9.7, 1.9 Hz), 6.95 (1H, dt, J = 9.7, 4.2 Hz), 7.42–7.46 and 7.48–7.52 (4H, m), 7.49 (1H, d, J = 15.6 Hz), 7.65 (1H, d, J = 15.6 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 122.7, 124.4, 125.9, 129.8–132.1, 134.2, 142.2, 145.8, 165.9, 169.0; Found (ESI): 306.0123 $[MH]^+$, (required $C_{14}H_{13}BrNO_2$ 306.0124); Found (ESI): 327.9939 $[MNa]^+$, (required $C_{14}H_{12}BrNO_2Na$ 327.9944). Spectroscopic data in accord with the literature.[18c]

(*E*)-1-(3-(2,6-Dichlorophenyl)acryloyl)-5,6-dihydropyridin-2(1*H*)-one (13). Compound **13** (15.0 mg, 67%) was prepared *via* the general procedure as a white solid. M.p.: 126–127 °C; ν_{max}/cm^{-1} 3692, 3606, 3083, 1688, 1624, 1602; δ_H (400 MHz, $CDCl_3$) 2.49 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 4.06 (2H, t, J = 6.4 Hz), 6.03 (1H, dt, J = 9.7, 1.9 Hz), 6.94 (1H, dt, J = 9.7, 4.2 Hz), 7.17 (1H, t, J = 8.1 Hz), 7.34 (2H, d, J = 8.1 Hz), 7.59 (1H, d, J = 16.0 Hz), 7.82 (1H, d, J = 16.0 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 125.8, 128.9, 129.7, 130.1, 132.8, 135.3, 136.7, 145.8, 165.7, 168.5; Found (ESI): 296.0240 $[MH]^+$, (required $C_{14}H_{12}Cl_2NO_2$ 296.0240); Found (ESI): 318.0057 $[MNa]^+$, (required $C_{14}H_{11}Cl_2NO_2Na$ 318.0059).

(*E*)-1-(3-Mesitylacryloyl)-5,6-dihydropyridin-2(1*H*)-one (14). Compound **14** (20.0 mg, 57%) was prepared *via* the general procedure as a pale yellow solid. M.p.: 104–105 °C [lit. 104 °C];[18c] ν_{max}/cm^{-1} 3691, 3067, 3011, 2926, 2857, 1687, 1608; δ_H (400 MHz, $CDCl_3$) 2.28 (3H, s), 2.38 (6H, s), 2.48 (2H, tdd, J = 6.4, 4.2, 1.8 Hz), 4.05 (2H, t, J = 6.4 Hz), 6.02 (1H, dt, J = 9.7, 1.8 Hz), 6.89 (2H, s), 6.93 (1H, dt, J = 9.7, 4.2 Hz), 7.09 (1H, d, J = 16.0 Hz), 7.91 (1H, d, J = 16.0 Hz); δ_C (100 MHz, $CDCl_3$) 21.2, 21.3, 24.9, 41.8, 126.0, 126.8, 129.3, 131.7, 138.3, 137.4, 142.0, 145.5, 165.8, 169.2; Found (ESI): 270.1494 $[MH]^+$, (required $C_{17}H_{20}NO_2$ 270.1489); Found (ESI): 292.1314 $[MNa]^+$, (required $C_{17}H_{19}NO_2Na$ 292.1308). Spectroscopic data in accord with the literature.[18c]

(*E*)-1-(3-(4-Nitrophenyl)acryloyl)-5,6-dihydropyridin-2(1*H*)-one (15). Compound **15** (10.0 mg, 45%) was prepared *via* the general procedure as a white solid. M.p.: 178–180 °C; ν_{max}/cm^{-1} 3691, 3606, 1688, 1602, 1522; δ_H (400 MHz, $CDCl_3$) 2.51 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 4.05 (2H, t, J = 6.4 Hz), 6.06 (1H, dt, J = 9.7, 1.9 Hz), 6.98 (1H, dt, J = 9.7, 4.2 Hz), 7.58 (1H, d, J = 15.7 Hz), 7.70 (1H, d, J = 15.7 Hz), 7.69–7.72 (2H, m), 8.21–8.24 (2H, m); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 124.2, 128.9, 125.7, 126.3, 140.0, 141.5, 146.2, 148.4, 165.9, 168.3; Found (ESI): 273.0877 $[MH]^+$, (required $C_{14}H_{13}N_2O_4$ 273.0870); Found (ESI): 295.0690 $[MNa]^+$, (required $C_{14}H_{12}N_2O_4Na$ 295.0689).

(*E*)-1-(3-(4-Fluoro-3 methoxyphenyl)acryloyl)-5,6-dihydropyridin-2(1*H*)-one (16). Compound **16** (13.0 mg, 52%) was prepared *via* the general procedure as a white solid. M.p.: 112–114 °C; ν_{max}/cm^{-1} 3692, 3607, 3012, 2928, 2855, 1687, 1602, 1515; δ_H (400 MHz, $CDCl_3$) 2.48 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 3.92 (3H, s), 4.04 (2H, t, J = 6.4 Hz), 6.05 (1H, dt, J = 9.7, 1.9 Hz), 6.95 (1H, dt, J = 9.7, 4.2 Hz), 7.04–7.17 (3H, m), 7.43 (1H, dd, J = 15.6, 0.6 Hz), 7.68 (1H, d, J = 15.6 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 56.4, 112.6 (d, J = 2.3 Hz), 116.5 (d, J = 18.5 Hz), 121.8 (d, J = 2.3 Hz), 122.1 (d, J = 7.5 Hz), 126.0, 131.9 (d, J = 3.9 Hz), 142.9, 145.7, 148.1 (d, J = 10.8 Hz), 153.8 (d, J = 251.5 Hz), 166.0, 168.9; δ_F (376 MHz, $CDCl_3$) −131.6; Found (ESI): 276.1023 $[MH]^+$, (required $C_{15}H_{15}FNO_3$ 276.1030); Found (ESI): 298.0842 $[MNa]^+$, (required $C_{15}H_{14}FNO_3Na$ 298.0850).

(*E*)-1-Cinnamoyl-5,6-dihydropyridin-2(1*H*)-one (17). Compound **17** (15.0 mg, 49%) was prepared *via* the general procedure as a yellow oil. δ_H (400 MHz, $CDCl_3$) 2.42 (2H, tdd, J = 6.4, 4.2, 1.8 Hz), 4.02 (2H, t, J = 6.4 Hz), 6.01 (1H, dt, J = 9.7, 1.8 Hz), 6.26–7.39 (2H, m), 6.93 (1H, dt, J = 9.7, 4.2 Hz), 7.09 (1H, d, J = 16.0 Hz), 7.44–7.61 (3H, m), 7.91 (1H, d, J = 16.0 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 121.8, 126.0, 126.8, 128.3, 128.7, 130.0, 143.7, 145.0, 166.2, 168.5; Found (ESI): 250.0847 $[MNa]^+$, (required $C_{14}H_{13}NO_2Na$ 250.0838). Spectroscopic data in accord with the literature.[18c]

(*E*)-1-(3-(Pyridin-3-yl)acryloyl)-5,6-dihydropyridin-2(1*H*)-one (18). Compound **18** (13.0 mg, 52%) was prepared *via* the general procedure as a white solid. M.p.: 113–114 °C; ν_{max}/cm^{-1} 3691, 3606, 3091, 1686, 1623, 1602; δ_H (400 MHz, $CDCl_3$) 2.49 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 4.04 (2H, t, J = 6.4 Hz), 6.04 (1H, dt, J = 9.7, 1.9 Hz), 6.96 (1H, dt, J = 9.7, 4.2

Hz), 7.31 (1H, dd, J = 7.8, 4.6 Hz), 7.55 (1H, d, J = 15.6 Hz), 7.69 (1H, d, J = 15.6 Hz), 7.90 (1H, dt, J = 7.7, 1.9 Hz), 8.58 (1H, d, J = 4.6 Hz), 8.76 (1H, s); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 123.8, 124.1, 125.8, 134.4, 139.6, 145.9, 150.2, 150.8, 165.9, 168.5; Found (ESI): 229.0966 $[MH]^+$, (required $C_{13}H_{13}N_2O_2$ 229.0972); Found (ESI): 251.0779 $[MNa]^+$, (required $C_{13}H_{12}N_2O_2Na$ 251.0791).

(*E*)-1-(3-Furan-2-yl)acryloyl)-5,6-dihydropyridin-2(1*H*)-one (19). Compound **19** (28.0 mg, 70%) was prepared *via* the general procedure as a yellow solid. M.p.: 51–53 °C; ν_{max}/cm^{-1} 3691, 3606, 3117, 3009, 2943, 2902, 1688, 1615; δ_H (400 MHz, $CDCl_3$) 2.45 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 4.01 (2H, t, J = 6.4 Hz), 6.03 (1H, dt, J = 9.7, 1.9 Hz), 6.45 (1H, dd, J = 3.4, 1.8 Hz), 6.62 (1H, d, J = 3.4 Hz), 6.91 (1H, dd, J = 9.7, 4.2 Hz), 7.36 (1H, d, J = 15.4 Hz), 7.46 (1H, d, J = 1.8 Hz), 7.51 (1H, d, J = 15.4 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 41.8, 112.4, 114.9, 119.5, 126.0, 130.3, 144.7, 145.5, 151.8, 165.8, 168.9; Found (ESI): 218.0813 $[MH]^+$, (required $C_{12}H_{12}NO_3$ 218.0812); Found (ESI): 240.0630 $[MNa]^+$, (required $C_{12}H_{11}NO_3Na$ 240.0631). Spectroscopic data in accord with the literature.[18c]

1-((2*E*,4*E*)-5-Phenylpenta-2,4-dienoyl)-5,6-dihydropyridin-2(1*H*)-one (21). Compound **21** (8.00 mg, 29%) was prepared *via* the general procedure as a colourless oil. ν_{max}/cm^{-1} 3691, 3606, 3080, 1686, 1602; δ_H (400 MHz, $CDCl_3$) 2.46 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 4.02 (2H, t, J = 6.4 Hz), 6.03 (1H, dt, J = 9.7, 1.9 Hz), 6.88–7.03 (3H, m), 7.09 (1H, d, J = 15.2 Hz), 7.27–7.39 (3H, m), 7.45–7.52 (2H, m), 7.55 (1H, dd, J = 14.8, 9.5 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 41.7, 125.1, 126.0, 127.3, 128.9, 129.1, 136.4, 140.5, 144.1, 145.5, 165.9, 169.0; Found (ESI): 254.1182 $[MH]^+$, (required $C_{16}H_{16}NO_2$ 254.1176); Found (ESI): 276.0989 $[MNa]^+$, (required $C_{16}H_{15}NO_2Na$ 276.0995).

(1′*E*)-3-(3′-Oxo-3′-(5′-oxo-5′,8′-dihydropyridin-4′(2*H*)-yl)prop-1′-enyl)benzenesulfonyl fluoride (20). Step 1: synthesis of 3-(hydroxymethyl)benzenesulfonyl fluoride (**22**). Commercially available 3-(fluorosulfonyl)benzoic acid (53.0 mg, 0.26 mmol) was dissolved in anhydrous THF (0.58 mL) under an atmosphere of argon and cooled to 0 °C. $BH_3{\cdot}SMe_2$ (2.0 M in THF, 0.26 mL, 0.52 mmol) was added dropwise and the reaction mixture stirred at room temperature overnight. H_2O (3 mL) was added dropwise at 0 °C and the aqueous phase extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, 20% EtOAc in petroleum ether 40–60 °C) to give alcohol **22** (49.0 mg, quant.) as a colourless oil. ν_{max}/cm^{-1} 3691, 3608, 1602, 1481; δ_H (400 MHz, $CDCl_3$) 2.35 (1H, br s), 4.81 (2H, s), 7.61 (1H, t, J = 7.8 Hz), 7.75 (1H, d, J = 7.8 Hz), 7.91 (1H, d, J = 7.8 Hz), 8.01 (1H, s); δ_C (100 MHz, $CDCl_3$) 64.0, 126.3, 127.4, 129.9, 133.3 (d, J = 24.2 Hz), 133.7, 143.3. δ_F (376 MHz, $CDCl_3$) 65.8; Found (EI^+): 190.0105 $[M]^+$, (required $C_7H_7O_3FS$ 190.0100).

Step 2: alcohol **22** (74.0 mg, 0.39 mmol) was dissolved in DCM (2.3 mL) and MnO_2 (678 mg, 7.80 mmol) was added. The mixture was vigorously stirred overnight and then filtered through a plug of Celite. The filtrate was concentrated *in vacuo* and the crude product was purified by flash column chromatography (SiO_2, 10% EtOAc in petroleum ether 40–60 °C) to give 3-formylbenzenesulfonyl fluoride, which was used immediately in the next step (46.0 mg, 63%) as a colourless oil. δ_H (300 MHz, $CDCl_3$) 7.86 (1H, t, J = 7.8 Hz), 8.24–8.31 (2H, m), 8.51 (1H, t, J = 1.7 Hz), 10.1 (1H, s); δ_F (282 MHz, $CDCl_3$) 66.0.

The phosphonoacetamide **5** (67.0 mg, 0.245 mmol) was dissolved in anhydrous THF (3 mL) under an atmosphere of argon and cooled to 0 °C. NaH (60%, 11.0 mg, 0.270 mmol) was added and the reaction stirred for 1 h at 0 °C. A solution of freshly prepared 3-formylbenzenesulfonyl fluoride (46.0 mg, 0.245 mmol) in anhydrous THF (1.9 mL) was added dropwise and the reaction stirred at room temperature for 8 h. Saturated $NH_4Cl_{(aq.)}$ (2 mL) was added and the aqueous phase extracted with EtOAc (3 × 20 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, 20% EtOAc in petroleum ether 40–60 °C) to give imide **20** (33.0 mg, 44%) as a white solid. M.p. 145–146 °C; ν_{max}/cm^{-1} 3692, 3606, 3012, 2947, 2902, 1686, 1626, 1471; δ_H (400 MHz, $CDCl_3$) 2.51 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 4.05 (2H, t, J = 6.4 Hz), 6.06 (1H, dt, J = 9.7, 1.9 Hz), 6.98 (1H, dt, J = 9.7, 4.2 Hz), 7.56 (1H, d, J = 15.7 Hz), 7.65 (1H, t, J = 7.9 Hz), 7.69 (1H, d, J = 15.7 Hz), 7.94 (1H, d, J = 7.9 Hz), 7.98 (1H, d, J = 7.9 Hz), 8.15 (1H, s); 24.9, 41.8, 125.6, 125.7, 127.7, 129.1, 134.7, 134.1 (d, J = 24.6 Hz), 137.3, 139.6, 146.2, 165.9, 168.2; δ_F (376 MHz, $CDCl_3$) 65.9; Found (EI^+): 309.0461 $[M]^+$, (required $C_{14}H_{12}NO_4FS$ 309.0471).

Synthesis of Horner–Wadsworth–Emmons reagent (5)

***N*-(*tert*-Butyloxycarbonyl)piperidine-2-one (8).**[20] δ-Lactam (1.00 g, 10.1 mmol) was dissolved in anhydrous THF (120 mL) and cooled to −78 °C under an atmosphere of argon. *n*-BuLi (2.43 M, 4.15 mL, 10.1 mmol) was added dropwise and the solution was stirred for 0.5 h. A solution of Boc_2O (3.30 g, 15.1 mmol) in anhydrous THF (30 mL) was added dropwise and then the reaction was allowed to stir at room temperature for 16 h. Saturated $NH_4Cl_{(aq.)}$ (20 mL) was added and the aqueous phase extracted with Et_2O (3 × 40 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, 20–30% EtOAc in petroleum ether 40–60 °C) to give lactam **8** (1.96 g, 98%) as a colourless oil. δ_H (400 MHz, $CDCl_3$) 1.52 (9H, s), 1.81–1.83 (4H, m), 2.48–2.52 (2H, m), 3.63–3.66 (2H, m); δ_C (100 MHz, $CDCl_3$) 20.7, 22.9, 28.2, 35.0, 46.4, 83.0, 152.9, 171.5.

***N*-(*tert*-Butyloxycarbonyl)-3-(phenylthio)piperidine-2-one (9).** Diisopropylamine (4.97 mL, 35.5 mmol) was dissolved in anhydrous THF (32.3 mL) and cooled to −78 °C under an atmosphere of argon. *n*-BuLi (2.5 M, 12.9 mL, 32.2 mmol) was added dropwise and the reaction stirred at −78 °C for 0.5 h. A solution of **8** (6.42 g, 32.2 mmol) in anhydrous THF (32.3 mL) was added dropwise and the resulting mixture stirred at −78 °C for 0.5 h. The reaction mixture was then added to a solution of PhSSPh (7.04 g, 32.2 mmol) in anhydrous THF (64.5 mL) and stirred at −78 °C for another 1.5 h before quenching by the addition of saturated $NH_4Cl_{(aq.)}$ (50 mL). The

aqueous phase was extracted with Et_2O (3 × 100 mL) and the organic layers combined, dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, 10–15% EtOAc in petroleum ether 40–60 °C) to give lactam **9** (6.93 g, 70%) as a colourless oil. δ_H (400 MHz, $CDCl_3$) 1.52 (9H, s), 1.79 (1H, m), 1.97–2.09 (2H, m), 2.22 (1H, m), 3.83 (1H, m), 3.74 (1H, m), 3.85 (1H, t, J = 6.0 Hz), 7.28–7.32 (3H, m), 7.52–7.55 (2H, m); δ_C (100 MHz, $CDCl_3$) 20.8, 28.2, 28.3, 45.9, 51.6, 83.2, 128.3, 129.1, 133.5, 133.7, 153.3, 169.9.

***tert*-Butyl 5,6-dihydro-2-oxopyridine-1(2*H*)-carboxylate (10).**[21] Lactam **9** (3.24 g, 10.5 mmol) was dissolved in DCM (116 mL) and saturated $NaHCO_{3(aq.)}$ (22 mL) was added. The solution was cooled to 0 °C and mCPBA (1.82 g, 10.5 mmol) added portion wise and the reaction stirred at room temperature for 2 h. The aqueous phase was extracted with DCM (3 × 100 mL) and the combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The residue was then dissolved in toluene (34 mL) and heated to 80 °C. The reaction was stirred for 3 h before concentrating *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, 20–40% EtOAc in petroleum ether 40–60 °C) to give α,β-unsaturated δ-lactam **10** (2.06 g, 99%) as a colourless oil. ν_{max}/cm^{-1} 3010, 2984, 2093, 1759, 1709; δ_H (400 MHz, $CDCl_3$) 1.54 (9H, s), 2.40 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 3.85 (2H, t, J = 6.4 Hz), 5.95 (1H, dt, J = 9.8, 1.9 Hz), 6.77 (1H, dt, J = 9.8, 4.2 Hz); δ_C (100 MHz, $CDCl_3$) 24.9, 28.2, 43.7, 83.1, 126.5, 143.6, 152.8, 163.9; Found (ESI): 198.1118 $[MH]^+$ (required $C_{10}H_{16}NO_3$ 198.1125); Found (ESI): 220.0946 $[MNa]^+$ (required $C_{10}H_{15}NO_3Na$ 220.0944).

5,6-Dihydropyridin-2(1*H*)-one (3).[18c] Lactam **10** (2.08 g, 10.5 mmol) was dissolved in MeOH (51.5 mL) and cooled to 0 °C. A solution of $HCl_{(aq.)}$ (6 M, 15.4 mL) was added dropwise and the reaction stirred at 0 °C for 1 h before concentrating *in vacuo*. The residue was treated with Et_3N (5 mL) and then concentrated *in vacuo*. The resulting crude product was purified by flash column chromatography (SiO_2, 5–10% MeOH in DCM) to give lactam **3** (1.00 g, 98%) as a white solid. M.p. 61–62 °C [lit. 61–65 °C];[21] ν_{max}/cm^{-1} 3691, 3424, 3009, 1677, 1609; δ_H (400 MHz, $CDCl_3$) 2.32 (2H, tdd, J = 7.2, 4.2, 1.9 Hz), 3.40 (2H, td, J = 7.2, 2.7 Hz), 5.87 (1H, dq, J = 9.9, 1.9 Hz), 6.62 (1H, dt, J = 9.9, 4.2 Hz), 6.65 (1H, br s); δ_C (100 MHz, $CDCl_3$) 23.9, 39.6, 124.9, 141.6, 166.7.

1-(2-Chloroacetyl)-5,6-dihydropyridin-2(1*H*)-one (6). Lactam **3** (2.80 g, 28.8 mmol) was dissolved in anhydrous THF (144 mL) under an atmosphere of argon and cooled to 0 °C. NaH (60%, 1.27 g, 31.7 mmol) was added in portions and the reaction stirred at 0 °C for 1 h. Chloroacetyl chloride (2.52 mL, 31.7 mmol) was then added dropwise and the reaction stirred at room temperature overnight. Saturated $NH_4Cl_{(aq.)}$ (200 mL) was added and the aqueous phase extracted with EtOAc (5 × 200 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, 20–30% EtOAc in petroleum ether 40–60 °C) to give lactam **6** (3.25 g, 65%) as a yellow oil. ν_{max}/cm^{-1} 3691, 3504, 3007, 2928, 1723, 1677, 1601; δ_H (400 MHz, $CDCl_3$) 2.48 (2H, tdd, J = 6.6, 4.2, 1.9 Hz), 4.04 (2H, t, J = 6.6 Hz), 4.77 (2H, s), 6.03 (1H, dt, J = 9.8, 1.9 Hz), 6.97 (1H, dt, J = 9.8, 4.2 Hz); δ_C (100 MHz, $CDCl_3$) 24.6, 41.8, 47.5, 125.2, 146.4; Found (ESI): 174.0318 $[MH]^+$ (required $C_7H_9ClNO_2$ 174.0316); Found (ESI): 196.0143 $[MNa]^+$ (required $C_7H_8ClNaNO_2$ 196.0136).

Diethyl(4-oxo-4,7-dihydropyridin-3(2*H*)-yl))phosphonacetate (5). Lactam **6** (233 mg, 1.34 mmol) was dissolved in DMF (2.7 mL), and NaI (20.0 mg, 0.134 mmol) and $P(OEt)_3$ (920 μL, 5.36 mmol) were added. The reaction mixture was heated by microwave (300 W, 110 °C) for 10 min. The DMF was removed by reduced pressure distillation and the resulting crude product purified by flash chromatography (SiO_2, 1% MeOH in DCM) to give phosphonoacetamide **5** (225 mg, 61%) as a pale yellow oil. ν_{max}/cm^{-1} 3691, 2999, 1695; δ_H (400 MHz, $CDCl_3$) 1.33 (6H, t, J = 7.1 Hz), 2.44 (2H, tdd, J = 6.4, 4.2, 1.9 Hz), 3.83 (2H, d, J = 21.2 Hz), 4.00 (2H, t, J = 6.4 Hz), 4.16 (4H, dq, J = 8.1, 7.1 Hz), 6.01 (1H, dt, J = 9.7, 1.7 Hz), 6.93 (1H, dt, J = 9.7, 4.2 Hz); δ_C (100 MHz, $CDCl_3$) 16.3, 24.5, 37.6, 41.4, 62.4, 125.4, 146.1, 165.4, 167.7; Found (ESI): 298.0799 $[MNa]^+$, (required $C_{11}H_{18}NO_5PNa$ 298.0815).

MTT assay

Piperlongumine analogues were prepared as 10 mM top stocks in DMSO. HCT 116 and IGROV-1, human-derived colorectal and ovarian adenocarcinoma cells respectively, were cultivated at 37 °C in RPMI 1640 medium supplemented with 10% FBS. Cells were seeded at a density of 3×10^3 (HCT 116) or 5×10^3 (IGROV-1) per well into 96-well microtiter plates and allowed to adhere for 24 h before test agent was introduced (final concentrations 1 μM–100 μM, n = 4). Dilutions were prepared in medium prior to each assay. At the time of agent addition and following 72 h exposure, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (final concentration 400 μg mL^{-1}). Incubation at 37 °C for 2.5 h allowed reduction of MTT by viable cells (mitochondrial dehydrogenases) to insoluble formazan crystals. Well supernatants were removed, and formazan solubilised by addition of DMSO (150 μL). Absorbance was read at 550 nm using a Perkin Elmer plate reader. Non-linear regression analysis was used to calculate compound concentrations required to inhibit cell growth by 50% (GI_{50}).

Clonogenic assay

Clonogenic-survival assays were used to determine the ability of single cells to survive brief challenge with piperlongumine analogues and retain proliferative capacity to form progeny colonies. HCT 116 and IGROV-1 cells were seeded at 250 per well in 2 mL complete medium in 6-well plates. After 24 h incubation at 37 °C, 5% CO_2, test agents were added (final concentrations 100 nM, 1 μM, 10 μM). After 24 h exposure medium containing piperlongumine were removed, cells washed (2 × 1 mL PBS) and medium alone added to wells (3 mL). When colonies in control wells contained >50 cells (8–10 days), colonies were washed, fixed (100% methanol,

10 min), stained (0.5% methylene blue; 1 : 1 methanol : water; 10 min), washed, air dried and counted.

Determination of reactive oxygen species

The ROS-Glo™H_2O_2 (Promega) assay allows detection of ROS that are generated in cell culture. Cells were seeded at 5×10^3 in 96-well opaque white plates in 80 μL RPMI-1640 supplemented with 10% FBS. After 24 h, test agents were added to the wells (final concentrations 1 μM, 5 μM and 10 μM, $n = 3$). Immediately, or following 18 h incubation, 20 μL H_2O_2 substrate solution was added to wells. ROS-Glo™ detection solution was added to each well (100 μL) then incubated at room temperature for 20 min in the dark. Relative luminescence at wavelength 700 nm was recorded using a multilabel plate reader.

Statistical analysis

All assays were performed ≥3 independent occasions: MTT, $n = 4$; clonogenic, $n = 2$; ROS, $n = 3$ per trial. Statistical significance was assessed using two-way analysis ANOVA and Dunnett's multiple comparison; significance was assigned where $p < 0.05$.

Acknowledgements

We thank the University of Nottingham for providing a generous PhD scholarship (PAS).

Notes and references

1 (*a*) M. S. Butler, A. A. B. Robertson and M. A. Cooper, *Nat. Prod. Rep.*, 2014, **31**, 1612–1661; (*b*) C. Basmadjian, Q. Zhao, E. Bentouhami, A. Djehal, C. G. Nebigil, R. A. Johnson, M. Serova, A. de Gramont, S. Faivre, E. Raymond and L. G. Désaubry, *Front. Chem.*, 2014, **2**, 20.

2 A. L. Harvey, R. Edrada-Ebel and R. J. Quinn, *Nat. Rev. Drug Discovery*, 2015, **14**, 111–129.

3 D. J. Newman and G. M. Cragg, *J. Nat. Prod.*, 2012, **75**, 311–335.

4 B. B. Mishra and V. K. Tiwari, *Eur. J. Med. Chem.*, 2011, **46**, 4769–4807.

5 G. T. Carter, *Nat. Prod. Rep.*, 2011, **28**, 1783–1789.

6 G. M. Cragg, D. G. I. Kingston and D. J. Newman, *Anticancer Agents from Natural Products*, CRV Press, 2011.

7 Z. H. Siddik, in *The Cancer Handbook*, John Wiley & Sons, Ltd, 2005.

8 D. J. Burgess, *Nat. Rev. Drug Discovery*, 2011, **10**, 658–659.

9 R. Pagliarini, W. Shao and W. R. Sellers, *EMBO Rep.*, 2015, **16**, 280–296.

10 For recent review see: (*a*) T. A. Baudino, *Curr. Drug Discovery Technol.*, 2015, **12**, 3–20; (*b*) A. M. Scott, J. D. Wolchok and L. J. Old, *Nat. Rev. Cancer*, 2012, **12**, 278–287.

11 D. Trachootham, J. Alexandre and P. Huang, *Nat. Rev. Drug Discovery*, 2009, **8**, 579–591.

12 P. T. Schumacker, *Cancer Cell*, 2006, **10**, 175–176.

13 (*a*) L. Raj, T. Ide, A. U. Gurkar, M. Foley, M. Schenone, X. Li, N. J. Tolliday, T. R. Golub, S. A. Carr, A. F. Shamji, A. M. Stern, A. Mandinova, S. L. Schreiber and S. W. Lee, *Nature*, 2011, **475**, 231–234; (*b*) D. J. Adams, M. Daia, G. Pellegrinoa, B. K. Wagnera, A. M. Sterna, A. F. Shamjia and S. L. Schreiber, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 15115–15120.

14 E.-H. Kong, Y.-J. Kim, Y.-J. Kim, H.-J. Cho, S.-N. Yu, K.-Y. Kim, J.-H. Chang and S.-C. Ahn, *Oncol. Rep.*, 2008, **20**, 785–792.

15 (*a*) Y. Wu, X. Min, C. Zhuang, J. Li, Z. Yu, G. Dong, J. Yao, S. Wang, Y. Liu, S. Wu, S. Zhu, C. Sheng, Y. Wei, H. Zhang, W. Zhang and Z. Miao, *Eur. J. Med. Chem.*, 2014, **82**, 545–551; (*b*) H. Dhillon, S. Chikara and K. M. Reindl, *Toxicol. Rep.*, 2014, **1**, 309–318; (*c*) J. L. Roh, E. H. Kim, J. Y. Park, J. W. Kim, M. Kwon and B. H. Lee, *Oncotarget*, 2014, **5**, 9227–9238.

16 B. S. Joshi, Y. N. Kamat and A. K. Saksena, *Tetrahedron Lett.*, 1968, **20**, 2395–2400.

17 P. M. Boll, J. Hansen and O. Simonsen, *Tetrahedron*, 1984, **40**, 171–175.

18 Other syntheses of piperlongumine (piplartine) and analogues: (*a*) Y. H. Seo, J.-K. Kim and J.-G. Jun, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 5727–5730; (*b*) Y. Wang, J. Wang, J. Li, Y. Zhang, W. Huang, J. Zuo, H. Liu, D. Xie and P. Zhu, *Chem. Biol. Drug Des.*, 2016, **87**, 833–840; (*c*) V. R. Rao, P. Muthenna, G. Shankaraiah, C. Akileshwari, K. H. Babu, G. Suresh, K. S. Babu, R. S. C. Kumar, K. R. Prasad, P. A. Yadav, J. M. Petrash, G. B. Reddy and J. M. Rao, *Eur. J. Med. Chem.*, 2012, **57**, 344–361.

19 L. Zhao, J. Su, L. Li, J. Chen, S. Hu, X. Zhang and T. Chen, *Food Res. Int.*, 2014, **66**, 186–196.

20 E. C. Garnier and L. S. Liebeskind, *J. Am. Chem. Soc.*, 2008, **130**, 7449–7458.

21 D. K. Winter, A. Drouin, J. Lessard and C. Spino, *J. Org. Chem.*, 2010, **75**, 2610–2618.