The enone motif of (+)-grandifloracin is not essential for ‘anti-austerity' antiproliferative activity

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\section*{A B S T R A C T}

We report the synthesis and biological evaluation of three analogues of the natural product (+)-grandifloracin (+)\textsuperscript{-1}. All three analogues exhibit enhanced antiproliferative activity against PANC-1 and HT-29 cells compared to the natural product. The retention of activity in an analogue lacking the enone functional group, 9, implies this structural element is not an essential part of the (+)-grandifloracin pharmacophore.

Pancreatic cancer has one of the lowest 5-year survival rates of any cancer, at just 3–5%; in contrast to many other cancers, this figure has not improved in the last 40 years.\textsuperscript{1} The reasons for this poor survival rate are numerous. It is one of the most aggressive of all human malignancies and as it is often asymptomatic in its early stages, the vast majority of patients already have metastatic tumours upon presentation.\textsuperscript{1} Therefore, surgical resection (the only effective treatment modality) is not appropriate in most cases; those patients who do undergo surgery have a 5-year survival rate which is improved only to 20%.\textsuperscript{1} Furthermore, there is a lack of effective chemotherapies for pancreatic cancer, with anti-cancer agents that are effective against other tumour types having negligible effect.\textsuperscript{2} Gemcitabine is typically administered in palliative chemotherapy for pancreatic cancer, but the survival benefit it imparts is marginal.\textsuperscript{2,3} On the basis of the above, it can be stated that an effective pancreatic cancer therapy constitutes a pressing unmet medical need. Accordingly, much effort is currently being directed towards the development of second-line therapies, adjuvant therapies and combination therapies. Recently, the combination of gemcitabine and erlotinib has been shown to lead to an enhanced 1-year survival rate of 23% as opposed to 17% for gemcitabine alone.\textsuperscript{4} Nevertheless, it is clear that a major breakthrough in the treatment of pancreatic cancer will likely require the use of wholly new therapeutic strategies exploiting emerging targets.\textsuperscript{5}

In this context, the ‘anti-austerity' strategy first described by Esumi and co-workers\textsuperscript{6} in 2000 is particularly promising. Pancreatic tumours are generally hypovascular, with the consequence that the tumour microenvironment is hypoxic and comparatively nutrient-deprived in comparison with normal tissues. Despite this, pancreatic cancer cells are nevertheless able to proliferate rapidly under these austere conditions. Their ability to tolerate nutrient deprivation is far greater than that of normal tissues and indeed of other cancer cell lines.\textsuperscript{6} An anti-austerity agent is defined as a drug that is able to remove the ability of cancer cells to survive under conditions of nutrient starvation, whilst cells with adequate nutrition remain unaffected.\textsuperscript{6} Such a drug would represent a novel means of selectively targeting pancreatic tumours in vivo. It should be noted that such behaviour would be in contrast to that of most chemotherapeutic agents, whose efficacy is typically reduced under conditions of nutrient deprivation.\textsuperscript{7} Esumi’s initial study identified two agents, troglitazone and LY294002, that possess such anti-austerity activity in PANC-1 cells. In the subsequent period, many more anti-austerity agents have been identified and the field has recently been reviewed.\textsuperscript{8} Most anti-austerity agents...
identified to date are natural products, due to screening campaigns on plant extracts enabled by the assay reported by Esumi. To date, three agents identified by this method have been evaluated in vivo as well as in vitro—the known anthelmintics, pyrvinium pamoate and the natural products kigamicin D, and (−)-arctigenin; all were found to suppress tumour growth in mouse models. The full details of the mode(s) of action of these anti-austerity agents have not yet been elucidated, but it has recently been disclosed that pyrvinium pamoate inhibits the NADH-fumarate reductase system.

This is a mitochondrial energy-generating system that shows increased activity in PANC-1 cells cultured under austere conditions and is also employed by parasitic helminths for survival in the hypoxic conditions of their hosts' intestines. The therapeutic promise of anti-austerity agents has attracted a high and sustained level of interest in anti-austerity agents in recent years; a key report, from Awale et al.,11 has reported the synthesis of a library of angelmarin analogues, one of which exhibited enhanced potency with respect to the natural product.19 Additionally, Carrico–Moniz has reported a novel geranylgeranylated hydroxycoumarin having some structural homology with angelmarin, that displays anti-austerity activity against PANC-1 cells also.20 No total syntheses of the kigamicins have been disclosed to date, but Whatmore, Shipman and co-workers have synthesised and evaluated truncated analogues in an attempt to establish the kigamycin pharmacophore; they report that 7-phenyltetrahydroxanthone displays anti-austerity activity, being 100-fold less potent than kigamicin C.21 Elsewhere, several total syntheses have been reported of (−)-arctigenin22–25 (and also of (+)-arctigenin26 and (+)-arctigenin, which is also a natural product,28 although it has only very weak anti-austerity activity29,30). It should be noted that in fact (−)-arctigenin is readily accessible from natural sources.31,32 Significantly, a recent report from Tooyooka, Tezuka and co-workers25 describes two analogues with enhanced potency compared to (−)-arctigenin.

There has been a high and sustained level of interest in anti-austerity agents in recent years; a key report, from Awale et al.,33 disclosed that (+)-grandifloracin, (+)-1, isolated from Uvaria dac, is a potent anti-austerity agent in four pancreatic cancer cell lines: PANC-1 (PC50 14.5 μM), PSN-1 (PC50 32.6 μM), MIA PaCa-2 (PC50 17.5 μM), and KLM-1 (PC50 32.7 μM). Awale’s report also represented the first time that the (+)-enantiomer of 1 had been isolated from nature (the antipodal (−)-1 had been isolated previously from several other species of the same genus,34–37 but its antiproliferative activity has not been evaluated). Most recently, a 2014 report from Awale details studies on the mode of action of (+)-grandifloracin, which reveal that it induces the autophagic cell death of PANC-1 cells under nutrient deprivation.38 Furthermore, (+)-grandifloracin was found to inhibit strongly both the phosphorylation of Akt at Ser473 and the phosphorylation of mTOR at Ser2448. In most pancreatic cancer cell lines the serine/threonine kinase Akt/mTOR pathway is constitutively activated and under conditions of nutrient deprivation, Akt has been shown to be overexpressed.

We recently reported39 the first enantioselective total synthesis of (+)-1, employing a starting material 2 whose chemistry we have extensively developed.40–49 Homochiral diol acid 2 is derived from a deaeromatising biooxidation of benzoic acid 3 (Scheme 1). A synthesis of (+)-1 has also been reported.50 The small quantities of (+)-1 available from the natural source (100 g of plant material yielded 7 mg of (+)-1) have precluded analogue semisynthesis to date. Our route is sufficiently concise to provide larger quantities of (+)-1 and also sufficiently flexible to allow analogue total synthesis. In this Letter we describe the synthesis of analogues of (+)-1 (by both semisynthesis and total synthesis) and their evaluation as anti-austerity agents.

In the first instance we targeted the ester motifs of (+)-grandifloracin as sites for diversification. In our original synthesis of (+)-1,39 the benzoate group was introduced selectively on the benzoate moiety, that pre-mixing the aroyl chloride and 2,4,6-collidine minimised the formation of by-products. This is a dearomatising biooxidation of benzoic acid

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competing overacylation at the secondary alcohol. Esters 5a and 5b were characterised by X-ray crystallography (Figs. 1 and 2).

The secondary alcohols in esters 5a and 5b were oxidised to ketones 6a and 6b with manganese dioxide. Cyclohexadienones are known to undergo spontaneous Diels–Alder dimerisation, but when complexed as $\eta^4$ ligands to iron(0), this process is suppressed and 6a/6b were found to be stable and characterisable. Upon treatment with cerium ammonium nitrate, 6a and 6b underwent smooth decomplexation to free cyclohexadienones 7a and 7b. These were not isolated, but instead underwent spontaneous dimerisation to give grandifloracin analogues 8a and 8b (Scheme 3).

A second approach to analogue preparation was undertaken, this time effecting semisynthetic functional group deletion by means of reduction of (+)-1 to give tetrahydrograndifloracin 9 (Scheme 4). We envisaged that this analogue would provide a means of assessing the importance or otherwise of the (nonaromatic) unsaturation in (+)-1 in terms of its anti-austerity activity.

With three novel analogues of (+)-grandifloracin in hand, we undertook the evaluation of their anti-austerity properties in a variety of cell lines. In the first instance, we determined the antiproliferative effects of (+)-1, 8a, 8b and 9 in the PANC-1 pancreatic cancer cell line. Each agent was evaluated both under comparatively nutrient rich (10% fetal bovine serum) and nutrient deprived (0.5% fetal bovine serum) culture conditions. Cells were exposed to the test agent for 72 h and gemcitabine and 5-fluorouracil were employed as positive controls (see Supplementary information).

The cell culture conditions were not identical to those employed by Awale et al. and as such the data presented here are not directly comparable with those in the previous Letter. Rather, we adopted conditions of less extreme nutrient deprivation (see Supplementary information) analogous to those that are likely present in the actual tumour microenvironment, fully expecting that prospective anti-austerity agents might be less potent under such conditions. The calculated IC$_{50}$ values are shown in Table 1.

The data are noteworthy in several respects. Contrary to our expectations, (+)-1 proved inactive up to 500 $\mu$M in 0.5% serum, yet exhibited an IC$_{50}$ of 123 $\mu$M in 10% serum. The anomalous nat-
ure of this result is underlined by the fact that analogues varying only in the nature of the ester side chains, 8a and 8b, are active. Indeed, they are more potent than (+)-1 under both cell culture conditions. It is possible, however, that (+)-1, 8a and 8b may in fact be prodrugs, all affording the same tetraol upon the action of intracellular esterases; the enhanced activity of 8a and 8b with respect to (+)-1 may be due to differences in lipophilicity and cell permeability. Arguably the most significant result presented in Table 1 is the increase in antiproliferative activity upon hydrogenation of (+)-1 to 9. Inspection of the structure of (+)-1 had initially led us to speculate that the enone motif might serve as a Michael acceptor and that (+)-1 (or its metabolite) might exert its effects by covalent modification of its target(s) [Scheme 5]. That tetrahydrograndifloracin 9 retains its potency in the absence of the enone motif leads us to conclude that in fact neither the enone motif, nor the electron-rich alkene, are required for antiproliferative activity in this cell line. Both (+)-1 and 9 were modelled using Maestro and it was determined that hydrogenation of (+)-1 to 9 induces only very subtle conformational changes. The rigid nature of the bicyclo[2.2.2]octene skeleton in the western hemisphere of (+)-1 means it has very limited scope to undergo conformational change upon reduction to 9. The eastern hemisphere of (+)-1 is less constrained, but even here the only appreciable change upon reduction to 9 is in the relative positions of the two carbons that have rehybridised from sp² to sp³; more broadly other functional groups do not move significantly. Thus, all functionality which might constitute part of the grandifloracin pharmacophore is highly conserved between the two structures. For example, in (+)-1, the distance between two representative hydrogen bond acceptors, the two ketone oxygens (labelled as A7 and A8 in Fig. 3b), is 7.12 Å, and this distance is unchanged in 9. Similarly, the distance between the two hydrogen bond donors, the tertiary hydroxyl oxygens (labelled D9 and D10 in Fig. 3b) is 4.50 Å in (+)-1 and 4.54 Å in 9.

We next sought to evaluate the effects of (+)-1, 8a, 8b and 9 on other (non-pancreatic) tumour cell lines. IC₅₀ values obtained for these agents in HT-29 human colon cancer cells are shown in Table 2. Three trends are evident in the data: (a) all three novel analogues are more active than (+)-1, under both cell culture conditions (as was the case for PANC-1 also), (b) all three novel analogues are more active than in PANC-1 cells. (c) All three novel analogues exhibited lower IC₅₀ values under the comparatively nutrient deprived conditions (0.5% serum), that is, an anti-austerity effect. To our knowledge, this is the first time such an anti-austerity effect has been demonstrated for HT-29 cells.

In summary, we have prepared three novel analogues of (+)-grandifloracin 1, all of which show enhanced antiproliferative activity towards PANC-1 and HT-29 cells with respect to the parent compound. We have also determined antiproliferative activities in other cell lines. Current work in our laboratory is focused on the preparation and evaluation of further analogues of (+)-1; results will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.111.

References and notes


Figure 3. Molecular modelling: (a) minimised structure of (+)-1; (b) schematic representation of the potential pharmacophore of (+)-1; (c) minimised structure of 9; (d) schematic representation of the potential pharmacophore of 9. (A = hydrogen bond acceptor, D = hydrogen bond donor, H = hydrophobic region, R = aromatic ring).