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Discovery of Novel Cinchona-Alkaloid-Inspired Oxazatwistane Autophagy Inhibitors

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Abstract

The cinchona alkaloids define a privileged natural product class endowed with diverse bioactivities. However, for compounds with the closely-related oxazatricyclo[4.4.0.0]decane ("oxazatwistane") scaffold, accessible from Cinchonidine and Quinidine by means of ring distortion and modification, biological activity has not been identified. We report the synthesis of an oxazatwistane compound collection employing state-of-the-art C-H functionalisation and metal-catalyzed cross coupling reactions as key late diversity generating steps. Exploration of oxazatwistane bioactivity in phenotypic assays monitoring different cellular processes revealed a novel class of autophagy inhibitors termed Oxautins that, as opposed to the guiding natural products, selectively inhibit autophagy by inhibiting both autophagosome biogenesis and autophagosome maturation.

Natural products (NPs) are a rich source of pharmaceutical agents and tool compounds for biology.^[1] Given their privileged scaffolds, structurally related compounds may possess potent but varying biological activities. Therefore synthetic strategies to access natural-product derived or inspired compound collections have

received significant attention.^[2] Hergenrother *et al.* have proposed a ring-distortion and modification strategy which employs readily accessible natural products to generate a range of different scaffolds^[3] and compound collections that possess a high degree of diversity, whilst retaining many favourable properties of NPs including high sp^3 content.^[4]

Cinchona alkaloids (Figure 1a) are endowed with multiple bioactivities.^[5] Therefore, application of the ring-distortion strategy to cinchona alkaloids might yield novel natural product-inspired compound classes bestowed with novel kinds of bioactivity. Notably, acid-catalysed cyclisation of cinchonine or quinidine can deliver non-natural analogues containing the oxazatricyclo[4.4.0.0.0]decane scaffold^[6] (oxazatwistanes; Figure 1b).^[7] Oxazatwistanes have been used as organocatalysts,^[8] however biological activity of oxazatwistanes has not been reported, and the chemistry surrounding this scaffold has hardly been explored.

Figure 1. Cinchona alkaloids and their derivatives. (a) Naturally occurring alkaloids from the *Cinchona* bark; (b) Oxazatwistane containing derivatives.

Herein we describe the synthesis of a cinchona-alkaloid derived, oxazatwistane-containing compound collection using step-economical C-H functionalisation reactions and metal-catalysed cross coupling reactions. Biological investigation of

the collection revealed new inhibitors of starvation-induced autophagic flux with a novel mode of action.

Oxazatwistanes **4a-b** were synthesized from cinchonine and quinidine following previously described procedures.^[8a, 8b] Several C2-substituted derivatives were synthesised in moderate yield by means of the borono-Minisci reaction (Scheme 1, **5a-d**).^[9] Radical zinc sulfinate chemistry was employed to synthesise derivatives with alkyl substituents at C2 (**6a-b**).^[10] Using very electron-deficient radicals, such as the trifluoromethyl radical, resulted mainly in the reaction adjacent to the methoxy group to deliver compound **7**, with only minimal reactivity at C2.^[11] The core scaffold, was varied by means of selective reduction using Adam's catalyst in TFA to deliver the 5,6,7,8-tetrahydroquinoline (**8**).

Further C2-substituted derivatives were synthesized by *N*-oxide formation followed by chlorination protocol to yield compound **10** which was subjected to Suzuki and Buchwald-Hartwig couplings yielding derivatives **11** and **12**. *N*-Boc-piperazine derivative **12c** was further functionalised through Boc-deprotection and acylation or reductive amination giving **13a-h**.

Scheme 1. Synthesis of an oxazatwistane collection **a**. Synthesis of C1-, C5- and C7-substituted oxazatwistanes. (a) ArB(OH)_2 , TFA, AgNO_3 (40 mol%), $\text{K}_2\text{S}_2\text{O}_8$, $\text{CHCl}_3\text{:H}_2\text{O}$ (2:1), rt, 16-48 h, 27-45%; (b) zinc sulfinate, TBHP, TFA, 50 °C, 16-48 h, $\text{CHCl}_3\text{:H}_2\text{O}$ (3:1), 29-44%; (c) sodium triflate, TBHP, TFA, 50 °C, 48 h, $\text{CHCl}_3\text{:H}_2\text{O}$ (3:1), 19% + 11% C1 regioisomer, (d) PtO_2 , TFA, H_2 (5 bar), rt, 16 h, 89%, (e) mCPBA, CHCl_3 , rt, 2 h, *then* NaHSO_3 , MeOH, rt, 18 h, 39% over two steps, (f) SOCl_2 , CH_2Cl_2 , reflux, 84%, (g) ArB(OH)_2 , PdCl_2dppf (20 mol%), Na_2CO_3 , toluene: H_2O (5:2), 80 °C, 15-19 h, 48-68%; (h) amine (6 eq), Pd(OAc)_2 (30 mol%), BINAP (65 mol%), NaO^tBu , toluene, 80 °C, 16 h, 52-71% (i) 4M HCl, dioxane, 50 °C, 3 h, *then* acyl chloride, DIPEA, CH_2Cl_2 , rt, 16 h, or aldehyde, AcOH, CH_2Cl_2 , NaBH(OAc)_3 , rt, 15 h, 29-59%; (j) HNO_3 , H_2SO_4 , -10 °C – rt, 39%; (k) Pd/C , H_2 (3 bar), MeOH, 5 h, *then* propylisocyanate or acetyl chloride, CH_2Cl_2 , 0 °C – rt, 24 h, 25-58%. **b**. Synthesis of C6-substituted oxazatwistanes. (a) PhNTf_2 , NEt_3 , CH_2Cl_2 , rt, 20h, 68%, (b) ArB(OH)_2 , PdCl_2dppf

(5 mol%), K₂CO₃, dioxane:H₂O (2:1), 130 °C, 1 h, (c) amine (6 eq), Pd(OAc)₂ (30 mol%), BINAP (65 mol%), NaOtBu, toluene, 80 °C, 16 h, (d) acetylene (1.5 eq), PdCl₂(PPh₃)₂ (30 mol%), CuI (30 mol%), toluene:NEt₃ (5:1), 80 °C, 16 h, (e) 2-chloroethyl ether, KOtBu, MeCN, 90 °C, 2 h, 19% + 15% dialkylated product (**20i**). **c.** Synthesis of lactone-containing twistane **25** (see SI Scheme 3 for full details).

Further derivatives were accessed by C5-nitration to yield **14** (Scheme 1)^[12] and subsequent reduction of the nitro group and acylation yielded ureas or amides (**15a-b**). For functionalisation at C6 (Scheme 1b) β -iso-cupreidine (**3**) was converted to triflate (**16**) to enable Suzuki couplings (**17a-c**), Buchwald-Hartwig aminations (**18a-b**) and Sonogashira couplings (**19**). Furthermore, etherification of **3** with chloroethyl ether and potassium *tert*-butoxide was successful to afford **20**. In addition, lactone analogue **21** was synthesized from quinidine (**2b**) (Scheme 1c and SI Scheme 1).

Biological activity of the > 45-member oxazatwistane collection was investigated in gene reporter assays monitoring inhibition of the Wnt and Hedgehog (Hh) pathways at 10 μ M, however no hits were identified (*data not shown*).^[13] In addition, the compound collection was tested for the ability to inhibit autophagy induced by amino acid starvation. Autophagy is required for the recycling of cellular nutrients, removal of damaged or unused proteins and organelles, as well as the clearance of pathogens. In autophagy, initially the double-membraned phagophore is formed that engulfs cargo (Figure 2a)^[14] and expands to the autophagosome that subsequently fuses with lysosomes, which contain degradative enzymes. During this process, the key autophagy regulator microtubule-associated light chain protein 3 (LC3), is initially produced in its non-lipidated form (LC3-I). Lipidation with phosphatidylethanolamine (PE) produces LC3-II which localises to the autophagosomes. Autophagy activators are expected to be potential therapies for neurodegenerative disorders.^[15] In cancer, autophagy may play a role in tumour initiation,^[16] and can promote tumor growth, particularly in nutrient-deprived and hypoxic environments.^[17] As a result, there is a need for new autophagy-modulating small molecules with novel modes of action and targets^[18] to further delineate the biological roles of autophagy in cancer and for the development of novel drugs.

To assay autophagy inhibition, MCF7 cells stably expressing eGFP-LC3 (eGFP-LC3 cells) were treated under starved conditions using Earle's balanced salt solution (EBSS) to induce autophagy. Chloroquine, an inhibitor of the autophagosome-

lysosome fusion, was used to increase the accumulation of eGFP-LC3 structures (Figure 2a). In this assay 16 compounds dose-dependently reduced the number of eGFP-LC3 puncta (Table 1 and Figure 2b). However, the original Cinchona alkaloids (Figure 1) did not inhibit autophagy at concentrations up to 30 μ M (SI Table 1).^[19]

Structure-activity relationships revealed that the unsubstituted β -iso-cupreidine, β -iso-quinidine, and β -iso-cinchonine (**3**, **4a** and **4b** respectively) were not active at a concentration of 10 μ M, suggesting that substitution of the quinoline is required for activity. Varying the substitution at C2 produced several compounds that were active in the low micromolar range. Large 4-substituted phenyls (biphenyl or biphenyl ethers) were less tolerated, however the 4-fluoro-phenyl substituent significantly increased potency to 0.65 μ M (**5b**). This compound, termed Oxautin-1, was almost ten times more potent than the simple phenyl derivative (**11a**), suggesting that electron withdrawing groups are beneficial for high levels of potency. Substituted piperazines **13g** and **13h** were moderately active, but highly toxic within a 3-hour timeframe in the concentration range at which they inhibited autophagy, as determined by measuring cellular confluency in the Incucyte™ Zoom experimental set-up (SI Figure 1). Oxautin-1 was not toxic at concentrations up to 10 μ M in the same timeframe. Substitution at C6 (**17c** and **19**) also weakly promoted autophagy inhibition. Interestingly, the C7-trifluoromethyl substituted derivative **7** was also able to weakly inhibit autophagy. When combining this with the 4-fluoro-phenyl at C2 using two sequential C-H activation reactions,^[10b] this boosted potency further (**26**, Oxautin-2).

To determine whether the bioactivity of the Oxautins is primarily determined by the tricyclic aliphatic or the heterocyclic aromatic part of the scaffold or whether a combination of two sub-scaffolds is required, and in light of the finding that both non-substituted quinolinyl oxazatwistanes **4a/b** and non-substituted quinolinyl natural products **1a/b** and **2a/b** do not inhibit autophagy, we subjected a range of 2-(4-fluorophenyl)-quinolines (SI Table 2) to the autophagy assay. Interestingly, none of these molecules inhibited autophagy at concentrations up to 30 μ M. Thus, the aromatic heterocyclic sub-scaffold alone is not sufficient for autophagy inhibition but a combination with a cinchona alkaloid-derived oxazatwistane subscaffold is required. Thus, the ring distortion strategy employed here led to the identification of a novel class of autophagy inhibitors starting from natural products that do not share this activity under the same conditions.

Table 1. Identification of cinchona-alkaloid inspired inhibitors of starvation-induced autophagy. All data is shown as mean $\pm$ SD of three independent experiments. All compounds were initially assayed at a concentration of 10 μ M. For hits reducing the number of LC3-II punctae by more than 50% IC_{50} -values were determined. n/a = inactive (no reduction of LC3-II punctae at 10 μ M).

No.	R ¹	R ²	R ³	Starvation-induced autophagy, IC_{50} [μ M]
3	-H	-H	-OH	n/a
4a	-H	-H	-OMe	n/a
4b	-H	-H	-H	n/a
5a	2-Nap	-H	-OMe	2.7 ± 0.9
5b (Oxautin-1)	4-F-C₆H₄	-H	-OMe	0.65 ± 0.22
5c	3,4-(OMe) ₂ -C ₆ H ₄	-H	-OMe	4.7 ± 2.7
6a	-Bn	-H	-OMe	2.8 ± 1.9
6b	- ⁱ Pr	-H	-OMe	4.5 ± 1.7
7	-H	-CF ₃	-OMe	8.6 ± 0.8
7i	-CF ₃	-H	-OMe	4.6 ± 2.5
10	-Cl	-H	-OMe	7.6 ± 1.9
11a	-Ph	-H	-OMe	5.2 ± 1.0
11b	3,5-(OMe) ₂ -C ₆ H ₄	-H	-OMe	6.0 ± 0.7
11c	4-Ac-C ₆ H ₄	-H	-OMe	11.0 ± 4.0
12c		-H	-OMe	19.0 ± 5.0
13g		-H	-OMe	3.2 ± 0.5
13h		-H	-OMe	5.0 ± 1.0
17c	-H	-H	3,5-(OMe) ₂ -C ₆ H ₄	6.1 ± 0.8
19	-H	-H	-Ph	6.2 ± 0.4
26 (Oxautin-2)	4-F-C₆H₄	-CF₃	-OMe	0.48 ± 0.13

Subsequently, Oxautin-1 and -2 were tested for their ability to modulate LC3 lipidation. Typically the induction of autophagy enhances the lipidation of LC3 to form LC3-II (lipidated LC3).^[20] Inhibition of autophagy should thus inhibit LC3 lipidation (Figure 2a). However inhibitors of autophagosome-lysosome fusion would induce an increase in LC3-II levels, while still inhibiting autophagy. Surprisingly, Oxautin-1 and -2 both significantly increased LC3-II levels in a dose-dependent manner under starved conditions (Figure 2d) and, to a lower extent, under fed conditions (SI Figure 2). In agreement with this finding, a significant increase in eGFP-LC3 puncta was seen in both starved and, to a lesser extent, in fed cells treated with Oxautin-1 in the absence of Chloroquine (SI Figure 4). Since Oxautins inhibited the formation of eGFP-LC3 puncta in the presence of Chloroquine, we assumed that they might act at steps upstream of autophagosome formation rather than at the downstream step of autophagosome maturation (i.e. autophagosome-lysosome fusion) (Figure 2b). To address this question, we utilised a tandem mCherry-eGFP-LC3 construct stably expressed in MCF7 cells. This allows the visualisation of both autophagosome (red and green) and autolysosome (red only, resulting from the quenching of the green fluorescence in the acidic autolysosomes) populations, thereby permitting quantitation of autophagy flux.^[21] Under starved conditions, Oxautin-1 led to a dose dependent increase in autophagosomes, with a concomitant decrease in autolysosomes (SI Figure 3). This result suggests that Oxautin-1 is an inhibitor of autophagosome maturation. Consistent with inhibition of autophagic flux, Oxautins led to dose-dependent accumulation of the autophagy substrate p62 under starved conditions (Figure 2d).

Figure 2. Oxautins potently inhibit autophagy after LC3 lipidation but before autophagosome formation. (a) Outline of autophagy and assay principle: when autophagy is induced, LC3-I is lipidated with phosphatidylethanolamine to form LC3-II, which localises to the autophagosomal membrane. Chloroquine inhibits the autophagosome-lysosome fusion which causes an accumulation of LC3-II, visible as green punctae when tagged with eGFP. Autophagy inhibitors will reverse this accumulation. (b) Fed cells (MEM) show a diffuse GFP-LC3 localisation (left). Cells starved with EBSS and treated with Chloroquine (CQ) show GFP-LC3 accumulation at autophagosomes (middle). This phenotype is reversed with Oxautin-2 treatment (right). Blue = Hoechst, green = eGFP-LC3; scale bar = 50 μ m; (c) Dose-dependency of Oxautin-2 autophagy inhibition; (d) Oxautin-1 and -2 induce the accumulation of LC3-II and inhibit the degradation of p62 in MCF7-LC3 cells upon amino acid starvation; (e) Oxautin-1 inhibits the growth of starved MCF7-LC3 cells more potently than fed cells.

Experiments performed using the WST-1 reagent, (n = 3, representative graph shown); (f) Oxautin-1 induces apoptosis dose-dependently in amino acid starved MCF7-LC3 cells. Experiments performed on the Incucyte Zoom, using a Caspase 3/7 selective probe; (n = 3, representative graph shown).

To gain further insights into the opposing effects of Oxautin-1 on the formation of eGFP-LC3 puncta in the presence and the absence of Chloroquine and to confirm that Oxautin-1 indeed acts at steps upstream of autophagosome formation, we utilised HEK293 cells stably expressing eGFP- WIPI2 that serves as a specific sensor for autophagosomal phosphatidylinositol 3-phosphate (PI3P), a phospholipid that plays a key role in the biogenesis of early autophagic structures like the phagophore.^[22] Oxautin-1 significantly reduced the number of WIPI2 puncta in starved cells, which phenocopies the effect of the phosphatidylinositol 3-kinase inhibitor Wortmannin, in contrast to Chloroquine that led to opposite effect (Figure 3). Therefore, Oxautin-1 inhibits both autophagosome biogenesis and autophagosome maturation. This mode of action is unprecedented.

Potent induction of autophagy can lead to non-apoptotic or necrotic cell death (autosis).^[23] Conversely, autophagy inhibitors potently induce cell death upon nutrient deprivation.^[24] Consistently, Oxautin-1 selectively inhibited the growth of starved MCF7-LC3 cells as compared to fed cells, assessed by a WST-1 proliferation assay (Figure 2e, GI_{50} starved = 2.3 μ M, GI_{50} fed = 4.6 μ M). Use of a caspase 3/7 selective probe showed that Oxautin-1 causes cell death by apoptosis (Figure 2f and supplementary methods). This result is consistent with previous reports that autophagy inhibitors including Chloroquine induce cell death by apoptosis.^[25]

In summary, we have synthesised a Cinchona alkaloid inspired oxazastwistane compound collection employing C-H functionalisation and metal catalysed cross-coupling reactions as key transformations. This compound collection yielded novel, potent autophagy inhibitors termed Oxautins which appear to inhibit both autophagosome biogenesis and autophagosome maturation. Identification of the targets and mechanism of action of the Oxautins will provide new insights into the processes regulating autophagy.

Figure 3 – Oxautin 1 inhibits WIPI-2b puncta formation. (a) HEK293 cells stably expressing eGFP-WIPI2b were treated with Oxautin 1 and control compounds in fed and starved conditions. (b) Quantification of puncta showed a dose-dependent decrease in starved cells treated with Oxautin 1 (5 μ M, $p = 0.009$ and 2.5 μ M, $p = 0.02$). This is in contrast with Chloroquine (CQ), which resulted in a marked increase in puncta. Data shown as mean $\pm$ s.e.m, $N > 34$.

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Natural Product-Inspired Synthesis

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Discovery of Novel Cinchona-Alkaloid-Inspired Oxazatwistane Autophagy Inhibitors

A twist to inhibit Autophagy: Synthesis of a Cinchona-alkaloid inspired collection of small molecules by ring-distortion and modification of natural products delivers novel *Oxazatwistanes* that, as opposed to the guiding natural products, inhibit autophagy by an unprecedented mode of action.