Synthesis and Evaluation of Azetidinone Analogues of Combretastatin A-4 as Tubulin Targeting Agents

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Synthesis and Evaluation of Azetidinone Analogues of Combretastatin A-4 as Tubulin Targeting Agents

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Abstract

The synthesis and antiproliferative activity of a new series of rigid analogues of combretastatin A-4 are described which contain the 1,4-diaryl-2-azetidinone (β-lactam) ring system in place of the usual ethylene bridge present in the natural combretastatin stilbene products. These novel compounds are also substituted at position 3 of the β-lactam ring with an aryl ring. A number of analogues showed potent nanomolar activity in human MCF-7 and MDA-MB-231 breast cancer cell lines, displayed in vitro inhibition of tubulin polymerization and did not cause significant cytotoxicity in normal murine breast epithelial cells. 4-(4-Methoxyaryl)-substituted compound 32, 4-(3-hydroxy-4-methoxyaryl)-substituted compounds 35 and 41 and the 3-(4-aminoaryl)-substituted compounds 46 and 47 displayed the most potent anti-proliferative activity of the series. β-Lactam 41 in particular showed sub-nanomolar activity in MCF-7 breast cancer cells (IC₅₀ = 0.8nM) together with significant in vitro inhibition of tubulin polymerization and has been selected for further biochemical assessment. These novel β-lactam compounds are identified as potentially useful scaffolds for the further development of antitumour agents which target tubulin.

Key words: Combretastatin A-4 analogues, β-lactam, azetidinone, cytotoxicity, tubulin, colchicine, structure-activity.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CA-4</td>
<td>Combretastatin A-4</td>
</tr>
<tr>
<td>EI</td>
<td>Electron Impact</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanidine triphosphate</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Molecular Ion Determination</td>
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<tr>
<td>IC</td>
<td>Inhibitory concentration</td>
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<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NMR</td>
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<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>SAR</td>
<td>Structure-Activity Relationship</td>
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<td>TMCS</td>
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Introduction

Microtubules are cytoskeletal structures which formed by self assembly of $\alpha$ and $\beta$ tubulin heterodimers and are involved in many cellular functions\(^1\). Their most important role is in the formation of the mitotic spindle and they are essential to the mitotic division of cells. Tubulin is an $\alpha$–$\beta$ heterodimeric protein which is the main constituent of microtubules. Tubulin is the target of numerous small molecule antiproliferative ligands that act by interfering with microtubule dynamics\(^2\). These ligands can be broadly divided into two categories; those that inhibit the formation of the mitotic spindle such as colchicine (1, figure 1)\(^3\), \(^4\) and vinblastine\(^5\) and those that inhibit the disassembly of the mitotic spindle once it has formed, such as paclitaxel\(^6\). The three characterised binding sites of tubulin are the taxane domain, the vinca domain and the colchicine domain and many compounds interact with tubulin at these known sites. Many tubulin binding compounds, such as paclitaxel and vinblastine are in clinical use for various types of cancer.\(^2\) Antimitotic agents are one of the major classes of cytotoxic drug for cancer treatment and microtubules are a significant target for many natural product anticancer agents such as combretastatin A-4 (2a, figure 1)\(^7\) and podophyllotoxin (4, figure 1)\(^2\), \(^8\).

The combretastatins are a group of diaryl stilbenes isolated from the stem wood of the South African tree *Combretum Caffrum*\(^9\). 2a was found to have potent anticancer activity against a number of human cancer cell lines including multi-drug resistant cancer cell lines and binds to the colchicine-binding site of tubulin\(^10\). A water-soluble prodrug, combretastatin A-4-phosphate (2b, figure 1) is now in clinical trials for thyroid cancer\(^11\)-\(^13\) and in patients with advanced cancer\(^14\). 2b induces vascular shutdown within tumours at doses less than one-tenth of the maximum tolerated dose and without detectable morbidity, assuming a MTD of 1000mg/kg\(^7\). Hydrolysis *in vivo* by endogenous non-specific phosphatases under physiological conditions affords 2a\(^15\), \(^16\). The amino derivative of 2a (compound 3a) is also in clinical trials as a water soluble amino acid prodrug (3b, figure 1)\(^17\). In contrast to colchicine, the anti-vascular effects of 2a *in vivo* are apparent well below the maximum tolerated dose, offering a wide therapeutic window. 2a as well as being a potent inhibitor of colchicine binding is also shown to
inhibit the growth and development of blood vessels, angiogenesis\textsuperscript{5, 18-21}. The \textit{cis} configuration only of \textbf{2a} is biologically activity, with the \textit{trans} form showing little or no activity\textsuperscript{22}. The active \textit{cis} double bond in \textbf{2a} is readily converted to the more stable \textit{trans} isomer during storage or metabolism, resulting in a dramatic decrease in antitumour activity\textsuperscript{23, 24}.

Various structural modifications to \textbf{2a} have been reported including variation of the A and B-rings substituents\textsuperscript{25-27}. Many modifications of the B-ring result in decreased bioactivity; however, substitution of the 3'-OH with an amino group results in potent bioactivity and good water solubility\textsuperscript{28}. The 3,4,5-trimethoxy substituted pattern in ring A, resembling the trimethoxyaryl ring of colchicine is optimal for bioactivity of \textbf{2a}\textsuperscript{24}. Many conformationally restricted analogues of \textbf{2a} are known. The majority of these compounds replace the \textit{cis}-double bond in \textbf{2a} with a heterocyclic rigid ring scaffold structures which prevents isomerisation of the \textit{cis}-double bond. These analogues include the heteroarylcoumarin\textsuperscript{29}, aroylindole\textsuperscript{27, 27} imidazole\textsuperscript{30}, 1,3-dioxolane \textsuperscript{31}, pyrazole\textsuperscript{23}, furazan (1,2,5-oxadiazole)\textsuperscript{25} and benzoexepin\textsuperscript{32} ring systems. Such non-isomerisable compounds inhibit cell growth of several human cancer cell lines and many have been shown significant tubulin binding and depolymerising effects. We now describe the synthesis and tubulin targeting activity of non-isomerisable \(\beta\)-lactam-containing analogues of \textbf{2a}.

The anticancer activity of some \(\beta\)-lactam-containing compounds has been reported\textsuperscript{33}, including the non-isomerisable combretastatin analogues containing OH and OMe substituents at C-3\textsuperscript{34}. We have recently reported the potent antiproliferative activity of \(\beta\)-lactam-containing compounds which are unsubstituted at C-3 or contain methyl substituent(s) at C-3\textsuperscript{35}. We have also reported antiproliferative and antiestrogenic activity of compounds containing the \(\beta\)-lactam scaffold in MCF-7 cells\textsuperscript{36}. In continuation of our earlier work, a further panel of compounds containing the \(\beta\)-lactam ring were examined as potential tubulin targeting agents. These novel compounds contain an aryl-type substituent at C-3, while the rigid \(\beta\)-lactam ring scaffold structure allows a similar spatial arrangement between the two phenyl rings as observed in the \textit{cis} conformation of \textbf{2a}.
Chemistry

The synthesis of the target β-lactams is illustrated in Schemes 2-4. The compounds which were initially chosen for synthesis contained the 3,4,5-trimethoxyphenyl (Ring A) as the β-lactam N-1 and 4-methoxyphenyl as the β-lactam C-4 substituents, which are present in 2a. β-Lactam synthesis was achieved with a Staudinger cycloaddition reaction between a ketene (generated from an acid chloride) and an imine under basic conditions\textsuperscript{33, 37}. The required imines 5a-f were obtained by condensation reaction of the appropriately substituted benzaldehydes and anilines, (Scheme 1). In the case of 5e, the phenolic group present on the starting 3-hydroxy-4-methoxybenzaldehyde was first protected as the tert-butylidimethylchlorosilane (TBDMS) ether\textsuperscript{38} which can be removed later under mild conditions with tetrabutylammonium fluoride which is suitable for the stability of the β-lactam functional group\textsuperscript{39}. Acid chlorides 6a-6d were prepared by reaction of the appropriately substituted acetic acids with thionyl chloride. For the preparation of the acid chloride 6c, (3-hydroxyphenyl)acetic acid was first treated with benzyl bromide to afford (3-benzyloxyphenyl)acetic acid\textsuperscript{40}. The acid chloride 6d is prepared from (4-aminophenyl)acetic acid on first reaction with benzyl chloroformate to afford the (4-benzyloxy carbonylaminophenyl)acetic acid\textsuperscript{41}, which was then converted to the unstable acid chloride 6d on reaction with thionyl chloride, (Scheme 2, step a). The chlorination reactions were monitored by IR spectroscopy until acid chloride carbonyl absorption was observed (ν1790-1815 cm\textsuperscript{-1}).

The racemic β-lactam products 7-14 were obtained in low yields on treatment of the imines 5a-f at reflux in dichloromethane with the appropriate acid chloride in the presence of triethylamine (Scheme 2, Route I; one enantiomer only shown). β-Lactam compounds 15-17 were prepared using a modified procedure at room temperature as they were not successfully obtained by the usual conditions of the Staudinger reaction (Scheme 2, Route II). Failure to form β-lactams with 4-nitrophenylacetyl chloride under standard Staudinger conditions has previously been reported\textsuperscript{42}. The stereochemistry of the β-lactam products obtained in Staudinger reactions depends on numerous factors, including the reaction conditions, the order of addition of the reagents and the substituents present on both the imine and on
the acid chloride$^{33, 43}$. In the present reactions, the \textit{trans} products were isolated exclusively in all cases, as evident from the $^1$H NMR spectrum of compound 7 where the H-3 and H-4 were identified at $\delta$4.23 and $\delta$4.79 respectively as a pair of coupled doublets, $J_{3,4} = 2.0$Hz.

The \(\beta\)-lactams 18-31 were obtained directly from the appropriate phenylacetic acid using an acid-activating agent in a one-step reaction, (Scheme 2, Route III), which was the reaction of choice for the majority of the products. Many acid-activating agents have been reported for this reaction e.g. Mukaiyama’s reagent (2-chloro-N-methylpyridinium iodide), ethyl chloroformate, trifluoroacetic anhydride, \(p\)-toluenesulfonyl chloride and various phosphorous derived reagents including triphosgene, which was successfully used in the present study.$^{44}$ \textit{Trans} stereochemistry was also observed for these products, e.g. for compound 18 where the H-3 and H-4 were identified at $\delta$4.20 and $\delta$4.89 respectively as a pair of coupled doublets, $J_{3,4} = 2.5$Hz. With selected phenylacetic acid derivatives, this reaction was unsuccessful and the acids were converted to acid chlorides 6a – 6d. \(\beta\)-Lactams were then obtained by the Staudinger route (Route I) as described above.

In some cases, a Reformatsky reaction was used for the synthesis of the required \(\beta\)-lactam compounds, (Scheme 3)$^{45, 46}$. We have recently reported the application of the Reformatsky reaction to obtain 1,3-diaryl azetidinones which are unsubstituted at C-3 or contain methyl substituent(s) at C-3$^{35}$. We now report the preparation of 1,3,4-triaryl azetidinones using a Reformatsky reaction which has been adapted for microwave conditions. Previous investigations found TMCS to be superior for zinc activation$^{47}$ in Reformatsky reactions than either iodine or zinc washed with 10% nitric acid. We examined the use of zinc powder which was pre-activated with trimethylchlorosilane in microwave conditions in the Reformatsky reactions, resulting in slightly increased yield of the desired \(\beta\)-lactam products 32-34 and a significant reduction in the reaction time from 8 hours to 30 minutes, (Scheme 3). In the present reactions, the \textit{trans} products were isolated exclusively in all cases, as evident from the $^1$H NMR spectrum of compound 32 where the H-3 and H-4 were identified at $\delta$4.29 and $\delta$4.87 ppm respectively as a pair of coupled doublets, $J_{3,4} = 2.5$Hz. The \textit{trans} configuration can be seen in the X-
ray crystal structure of β-lactam 32 in Figure 2. The enantiomeric composition of compound 32 was clearly demonstrated on analysis by chiral HPLC, (rt 11.93 min and 12.53 min).

The phenolic products 35-38 were obtained on treatment of the silyl ethers 13, 29, 30 and 34 respectively with tetrabutylammonium fluoride at 0°C (Scheme 4). Phenolic products 39-42 were obtained by careful hydrogenolysis of the corresponding benzyl ethers 12, 28, 37 and 31 respectively (Scheme 5). Reduction of the nitro group in compounds 15, 14 and 17 to the corresponding amines 43, 44 and 45 respectively was achieved by treatment with zinc dust and glacial acetic acid (Scheme 6). Treatment of the carbamate protected compounds 38 and 16 with hydrogen and palladium afforded the amines 46 and 47 respectively (Scheme 7).

To further investigate the contribution of the β-lactam carbonyl group to the activity of this compound class, a novel thione analogue 48 was prepared in good yield by reaction of the compound 32 with Lawesson’s reagent (2,4-bis-(4-methoxyphenyl)-1,2,3,4-dithiaphosphetane-2,4-disulfide), (Scheme 8). The characteristic C=S absorption band was observed at $\nu$ 1592.5 cm$^{-1}$ for this product. A broad variety of β-lactams have been subjected to reductive ring-opening with metal hydrides to yield the corresponding γ-aminoalcohols.$^{48}$ To investigate the contribution of the intact β-lactam ring structure to the activity of this series of compounds, 35 was subjected to treatment with LiAlH$_4$ to afford the aminoalcohol product 49 which was isolated by an acid-base extraction to give the desired γ-aminoalcohol, (Scheme 9). Cleavage of the β-lactam N1-C2 bond usually takes place with nucleophilic reagents such as alkoxide ion.$^{49-52}$ The ease of hydrolysis is apparently a function of increasing size and number of substituents at the 3-position; with increased size and number hydrolysis becomes increasingly difficult.$^{53}$ A methoxide ring opening reaction was carried out on 35 with 4 equivalents of sodium methoxide at room temperature over eight days, until reaction was complete on TLC. $^1$H NMR analysis of the product 50 indicated formation of a diastereomeric product mixture, (Scheme 9).$^{51,52}$ For methyl ester 50, the presence of two amino signals at $\delta$ 5.75 ppm and $\delta$ 6.10 ppm are indicative of two isomers. There are also two signals for each of the α and β protons (formerly at positions 3 and 4 of the
azetidinone ring) between δ 3 ppm and δ 5 ppm. In the $^{13}$C NMR spectrum of 50, there are two carbonyl signals at δ 171.55 ppm and δ 172.70 ppm.

The structures of the various β-lactams synthesized are illustrated in Tables 1, 2 and 3, together with their routes of synthesis. Table 1 lists the initial β-lactams with the methoxy functionalities at different positions of the β-lactam scaffold. The comprehensive series of β-lactams prepared with structural modification of aryl ring at the C-3 position of the β-lactam heterocycle are listed in Table 2. Finally, Table 3 shows further phenolic and amino modifications to the initial series of compounds with the aim of improving activity. Compounds 32 and 35 were selected for chemical stability analysis and further development based on the analysis of their drug-like (Lipinski) properties together with predictions of permeability, metabolic stability, Pgp substrate status, blood-brain barrier partition, plasma protein binding and human intestinal absorption properties. The stability of the target compounds 32 and 35 was evaluated in phosphate buffer at pH values 4, 7.4 and 9, and the half-life was determined to be greater than 24 hours for each compound at these pH values.

**Antiproliferative activity**

These analogues were first evaluated for their anti-proliferative activity in the MCF-7 (ER-positive) human breast cancer cell line, and the most potent compounds were then screened against the MDA-MB-231 cell line (ER-negative). The activities of the azetidin-2-ones are presented in Tables 4 and 5. In addition, compounds 32 and 35 were evaluated in the NCI-60 cell line screen (Table 9, Supporting information). Initially, the azetidin-2-ones prepared containing a 4-methoxy substituent on the C-4 phenyl ring were examined (compounds 7-12, 14, 15, 17-27, 32). From these early lead compounds, a more extensive series of active derivatives were synthesized containing the 3-hydroxy-4-methoxy and 3-amino-4-methoxy substitution pattern on the C-4 phenyl ring, (compounds 35, 36, 39-47). This latter series of compounds was designed to contain similar structural features to 1, 2a and the CA-4 amino analogue (3a).
The positions of the 3,4,5-trimethoxyphenyl and 4-methoxyphenyl ring substitution were initially examined at different location on the azetidinone ring (compounds 7, 18, 32, and 33), Table 4. 2a was used as the positive control. The optimal positioning was found to be in compound 32 (IC\textsubscript{50} MCF-7 = 34nM), where the 3,4,5-trimethoxyphenyl ring is located at the N-1 position and the 4-methoxyphenyl ring is at the C-4 position, as previously observed by Sun et al for other \(\beta\)-lactam compounds\textsuperscript{34}. Any other arrangement of these two rings was significantly less active than 32 by a factor of over 1,000. The effect of the introduction of substituents onto the C3-aryl ring of compound 32 was next investigated. In the 3-arylsubstituted series (compounds 7 to 31), larger substituents such as 3,4,5-trimethoxyphenyl (20), 4-trifluoromethylphenyl (27) and 4-benzyloxyphenyl (12) were not tolerated and lead to a significant decrease in activity against MCF-7 cells. The most active analogues in this series were found to be those with small, polar substituents including examples 24 (4-fluorophenyl), 39 (4-hydroxyphenyl), 40 (3-hydroxyphenyl) and 43 (4-aminophenyl) with IC\textsubscript{50} values in MCF-7 cells of 42nM, 59nM, 68nM and 50nM respectively.

The activity of the series was further optimized by modification of the C-4 aryl substituent of the azetidinone ring to include either a hydroxy or an amino group as in compounds 35 – 38 and 41 – 47 (Schemes 4-7, Table 4). Analogues of the three most active compounds from Table 2 were synthesised with both 4-(3-hydroxy-4-methoxyphenyl) and 4-(3-amino-4-methoxyphenyl) substitution (Table 3). With one exception, the 3-hydroxy-4-methoxy substituted analogues were over 10-fold more active than their corresponding 3-amino-4-methoxy derivatives (compare 41 to 42, for example, IC\textsubscript{50} values of 0.8nM and 39.5nM respectively in MCF-7 cells). The exception was the fluorinated analogues, in which the introduction of an amino substituent to the C-4 phenyl ring of 24 resulted in a slight increase in potency (compound 45) when compared to the hydroxyl analogue 36, and both were less active than compound 24 (IC\textsubscript{50} values of 55nM, 66nM and 42nM respectively in MCF-7 cells). The activity of nitro-substituted compound 17 is included for comparison (IC\textsubscript{50} of 1.4\(\mu\)M), indicating the critical importance of a polar H-bonding substituent on the C-3 phenyl ring. The phenolic hydroxyl group is the optimal substituent at the 3-position of the 4-phenyl ring. The most potent compound in the series, 41,
displayed sub-nanomolar antiproliferative activity in the MCF-7 cell line with an IC$_{50}$ of 0.8nM. Figure 3 shows a dose-response result for the antiproliferative effect of β-lactam compounds 32 and 41 in MCF-7 cells. Compound 46, containing a 3-(4-aminophenyl) substituent also showed greater activity than the lead compound 32, with an IC$_{50}$ of 4nM in MCF-7 cell line.

The replacement of the β-lactam carbonyl group with the thione in compound 48 resulted in a reduction in the observed antiproliferative activity (IC$_{50}$ = 0.72μM for MCF-7 cells and IC$_{50}$ = 0.89μM for MDA-MB 231 breast cancer cells). The β-lactam ring is strained and can undergo a number of ring-opening reactions. In the present work, two ring-opened products were also synthesised and evaluated for antiproliferative activity to assess the lactam scaffold dependence of biological activity. Alcohol 49 is the result of treatment of 35 with lithium aluminium hydride while ester 50 is the product from the nucleophilic ring-opening reaction of 35 with sodium methoxide. These compounds are more flexible than the rigid β-lactam parent compound 35. They show over 2,000-fold decrease in antiproliferative activity, indicating the critical importance of the rigid β-lactam scaffold and the relative orientations of the two methoxy-containing aromatic rings for preservation of antiproliferative activity. The importance of the relative orientations of the two aromatic rings has also been noted previously for CA-422.

The more potent compounds in the MCF-7 cell line were next examined in an ER-negative MDA-MB-231 cell line and the results are displayed in Table 5. Compound 35 was found to be the most effective of the series with IC$_{50}$ value of 28nM; compounds 32 and 45 are also seen to be effective, with IC$_{50}$ values of 78nM and 80nM respectively. Compound 41 was also evaluated in the leukaemia cell lines HL-60 and K562 and was found to be extremely potent with IC$_{50}$ values of 0.34nM and 0.89nM respectively, which compared favourably with the control 2a [IC$_{50}$ values of 1.99nM(HL-60 ) and 3.68nM(K562)].

Based on their potency, compounds 9 and 35 were evaluated in the National Cancer Institute (NCI)/Division of Cancer Treatment and Diagnosis (DCTD)/Developmental Therapeutics Program (DTP)$^{54}$, in which the activity of each compound was determined using approximately 60 different cancer cell lines of diverse tumour origins. These studies were performed at the NCI as part of their
Compounds 9 and 35 were tested for inhibition of growth (GI₅₀) and for cytotoxicity (LC₅₀) in the NCI panel of 56 cell lines and showed broad-spectrum antiproliferative activity against tumour cell lines derived from leukaemia, breast cancer, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer and prostate cancer, (see Table 9, Supporting Information). β-Lactam 9 showed submicromolar GI₅₀ values in all but 5 cell lines (the GI₅₀ value is the concentration of drug required for 50% inhibition of cellular growth). The mean GI₅₀ value for compound 9 across all cell lines is 0.21μM [log GI₅₀=(-6.67M)]. For compound 35, the GI₅₀ values obtained were below 10nM for 38 of the cell lines investigated, including breast cancers MCF-7, MDA-MB-231 and the Adriamycin resistant NCI/ADR-RES cell line, with the mean GI₅₀ value across all cell lines = 23.99nM, [log GI₅₀=(-7.62M)]. LC₅₀ values are a measure of the cytotoxicity of these compounds and were greater than 100μM for compound 9 in all but three cell lines and greater than 100μM for compound 35 in all but ten cell lines (Table 9, Supporting Information). These results confirm the requirement for the 3-hydroxy-4-methoxy substitution pattern on Ring B for optimum antiproliferative activity in these analogues.

**COMPARE analysis of β-lactams 9 and 35**

Matrix COMPARE analysis⁵⁵, ⁵⁶ (measuring the correlation between two compounds with respect to their differential antiproliferative activity) demonstrated good correlation between 35 and 2a (r=0.62), and other tubulin binding drugs vincristine, vinblastine, paclitaxel, maytansine and rhizoxin (Table 10, Supporting Information). However, this algorithm does not distinguish between different tubulin-based mechanisms of action.⁵⁷ In vitro tubulin-binding studies confirmed that 35 is acting as a tubulin-binding agent. The antiproliferative activity observed for these compounds indicated that there is a significant therapeutic window between the concentration required for cancer cell growth inhibition and the concentration that is toxic to cells.

**Evaluation of toxicity in normal murine mammary epithelial cells**
Two of the most potent β-lactam compounds from antiproliferative studies in the MCF-7 cell line, 35 and 41, were evaluated further for cytotoxicity in murine mammary epithelial cells. 2a was used as the positive control. Mouse mammary epithelial cells were harvested from mid- to late- pregnant CD-1 mice and cultured as described previously58, 59. Two different cell concentrations were used in the toxicity assay; 25,000 cells/ml and 50,000 cells/ml. The results are presented in Figure 4 and in the Supplementary information (figures 10, 11, 12). The IC₅₀ value is greater than 10mM for the three compounds evaluated. This is further evidence that these β-lactam compounds are minimally toxic to non-proliferating cells.

**Tubulin binding studies**

The effects of representative β-lactam CA-4 analogues on the assembly of purified bovine tubulin were evaluated. Compounds 32, 35 and 41 which demonstrated potent antiproliferative effects in vitro were assessed. The ability of 2a to effectively inhibit the assembly of tubulin was assessed as a positive control. Tubulin polymerisation was determined by measuring the increase in absorbance over time at 340nm. The Vₘₐₓ value offers the most sensitive indicator of tubulin/ligand interactions and hence Vₘₐₓ values were calculated for each test compound. Fold changes in Vₘₐₓ values for polymerisation curves of each test compound with reference to ethanol control were calculated and are detailed in Table 6. As anticipated, the active β-lactam CA-4 analogues 32, 35 and 41 inhibited the polymerisation of tubulin (Table 9, Supporting information). In more detail, the active β-lactams when evaluated at 10 µM concentration, reduced the Vₘₐₓ value for the rate of tubulin polymerisation from 6-fold for 2a to 11.7-fold for compound 41, and to 8.3-fold for compound 35, while compound 32 showed a 6-fold reduction in the value for Vₘₐₓ. This value is comparable if not superior to the rate of inhibition of tubulin assembly (6-fold) observed with 2a. An IC₅₀ value of 3.65µM was calculated for compound 41 for the reduction in Vₘₐₓ, while IC₅₀ value of 4.89 µM was obtained for the effect in overall polymer mass (calculated from area under the polymerization curve). Inhibition of tubulin polymerisation for
compound 41 is illustrated in Figure 5. The tubulin binding studies clearly demonstrated that tubulin is the target of these new compounds; however, the specific binding site on tubulin was not investigated e.g. by use of a radiolabelled colchicine displacement assay. Based on the very close 3D structural similarity between the ligands 1, 2a and the beta-lactam analogues reported in this study (figures 6b and 7d), we propose that the binding site for these compounds is most likely to be the colchicine site, since it has been demonstrated that 2a and many reported examples of the structurally related conformationally constrained 2a analogues bind at the colchicine site27, 60, 61.

**Structural and Molecular modeling studies of selected azetidinones**

The molecular structure of a representative example of the active series, compound 32, was determined by single-crystal X-Ray crystallography. The ORTEP diagram is presented in Figure 2, where the *trans* configuration at positions 3 and 4 of the β-lactam ring clearly seen (note that in the X-ray numbering scheme in Figure 2, the carbon at the 3-position of the beta-lactam ring is denoted as C2 and the carbon the 4-position of the beta-lactam ring is denoted as C3). The structure revealed a conformation for the azetidinone 32 in which the two aromatic rings located at N-1 and C-4 are not coplanar. The rigid azetidinone ring provides a scaffold which can accommodate the steric and geometric requirements of the aromatic pharmacophores for tubulin binding. Ligands that bind at the colchicine-binding site of tubulin have the common feature of a trimethoxy-aromatic ring, noted for such ligands as 1, 2a and 4.

The azetidinone 32 is structurally similar to 1 and 2a, with the common structural features of a trimethoxyphenyl ring and a second aromatic ring substituted with methoxy groups in a non co-planar diaryl system, with the observed tortional angle for Ring A–Ring B of 46.9° compared to 55° and 53° for the corresponding rings in 2a62 and 163 respectively. These structural similarities support the observed antiproliferative and tubulin polymerization inhibitory activity for these compounds as tubulin-targeting agents. The X-ray crystal structures of 2a62 and 2b22 suggest that the conformation of this stilbene is not planar. These crystal structures reveal that the planes of the two phenyl rings are inclined to each other,
suggesting a low-energy conformation that may be the one involved in binding at the tubulin receptor site\textsuperscript{22}.

To rationalize the potential binding modes of these β-lactam compounds in tubulin, docking studies were carried out with two of the most potent compounds in the 3-aryl β-lactam series, \textbf{32} and \textbf{41}. Using the reported X-ray structure of tubulin co-crystallised with a colchicine derivative, N-deacetyl-N-(2-mercaptoacetyl)-colchicine (DAMA-colchicine, PDB entry – 1SA0), possible binding orientations for \textbf{32} and \textbf{41} were probed with the docking program FREDv2.2.3 (Openeye Scientific Software)\textsuperscript{64}. Due to the structural similarity between the β-lactams and colchicine site ligands such as \textbf{2a}, it was proposed that \textbf{32} and \textbf{41} bind to tubulin at the colchicine-binding site. The colchicine-binding site in tubulin is mainly buried in the β-subunit, whilst maintaining few interactions with the α-subunit; there is one such site on each tubulin heterodimer. The H7 and H8 α-helices, the T7 loop and the S8 and S9 β-strands contribute to the binding site and interact with the ligand. Two important residues for binding of colchicine-type ligands to tubulin have been identified as Val β318 and Cys β241\textsuperscript{63}. Val β318 tubulin variants have reduced sensitivity to \textbf{1} and colchicine substituted with more reactive groups instead of the methoxys can be crosslinked with Cys β241\textsuperscript{63}.

Molecular modeling studies were carried out with the β-lactam compounds synthesised and evaluated to determine if these interactions were predicted to be present. Figure 6a illustrates β-lactam \textbf{32} docked in the colchicine-binding site of tubulin, while Figures 7a and 7b show similar graphics with β-lactam \textbf{41} docked. β-Lactam \textbf{41} was the most active compound in anti-proliferative assays and also in the tubulin polymerization inhibition assay. The two important residues for binding in the colchicine-binding site of tubulin are identified as Val β318 and Cys β241. Figures 6a, 7a and 7b show the trimethoxy ring of both \textbf{32} and \textbf{41} is well positioned in proximity to the Cys241 residue (residue numbers are those used by Ravelli et al\textsuperscript{63}), and the compounds adopt a very similar orientation to that of the trimethoxy ring of DAMA-colchicine in the co-crystallized structure. In addition, for compound \textbf{41}, Thr 179 is seen to establish a strong H-bond to the 3-OH substituent of ring B (Figure 7a, 7b). This
residue has previously been identified as interacting with a number of colchicine site ligands\textsuperscript{32, 65, 66}. This interaction may also explain the binding of the related compound in the series with an OH substituent in similar position (e.g. compounds 35, 36, 46), and also the related analogues with NH\textsubscript{2} substituent at C-3 of the aryl B-Ring. Activity is optimized by additional substituents on the C-3 aryl such as 4-F, 4-NH\textsubscript{2}, 4-OH, 3-OH (compounds 24, 39, 40, 43) which preserve the key interactions with active residues for Ring A and Ring B. The H-bonding interaction between the 4-hydroxyaryl group at the 3-position of β-lactam 41 and the Lys 254 residue (Figure 7a, 7b), in addition to hydrophobic contacts with the 3-phenyl ring (not shown for clarity) could contribute to the observed higher antiproliferative activity observed for this analogue. The hydrophobic interactions are illustrated in Figure 7c and include interactions of the 3-phenyl ring with Leu248, Ala250 and Leu255. Activity is significantly reduced for β-lactam analogues of 32 that have multiple methoxy substitutions on the C-3 aryl ring as in compound 20, or increased steric hindrance with the benzyloxy ether (compound 28).

Several other hydrophobic contacts are predicted to stabilize the binding of 32 and 41 to tubulin. Figures 6c and 7c depict the predicted binding interactions of docked β-lactams 32 and 41 in 2D format\textsuperscript{67}. From these results it can be seen that the important interactions discussed above are predicted to be present for these compounds. The docked pose of 32 (colored by atom) overlaid with colchicine (yellow) in the tubulin binding site is illustrated in Figure 6b. It can be seen that the methoxy substituent of Ring B is in close proximity in the binding site to the corresponding methoxy group on Ring C of colchicine. A similar binding orientation is observed for colchicine and 41 (Figure 7d). These binding parallels may rationalise the potency observed for 32 and 41 in their inhibition of tubulin polymerisation.
Conclusion

We have synthesized a comprehensive series of β-lactam compounds which show potent antiproliferative activity in a range of tumour cell lines, notably in human breast cancer MCF-7 and MDA-MB-231 cell lines. The most potent compound in the series 41 displayed sub-nanomolar activity in the MCF-7 cell line with an IC$_{50}$ of 0.8nM. Compound 46 containing a 3-(4-aminophenyl) substituent also showed greater activity than the lead compound 32, with an IC$_{50}$ of 4nM in MCF-7 cell line, with the 3-phenylsubstituted β-lactam 32 having an IC$_{50}$ of 34nM. The compounds did not cause significant cytotoxicity in normal murine breast epithelial cells. Compounds 32, 35 and 41 were shown to inhibit the polymerization of tubulin with improved efficacy when compared with 2a.

X-ray crystal structure of 32 revealed that the compound provides a scaffold structure with a similar spatial arrangement between the two phenyl rings as observed in cis configuration of 2a. Preliminary modeling studies indicate a possible binding mode for these potent inhibitors of tubulin polymerization. These conformationally restricted β-lactam structures are not easily isomerised, unlike the cis-stilbene 2a, and are identified as promising lead compounds in the development of new anticancer agents. Further studies are in progress to further rationalize SAR for this series of azetidinones and to determine the antiangiogenic effects of these compounds.
Experimental section

All reagents were commercially available and were used without further purification unless otherwise indicated. Tetrahydrofuran (THF) was distilled immediately prior to use from Na/Benzophenone under a slight positive pressure of nitrogen, toluene was dried by distillation from sodium and stored on activated molecular sieves (4Å) and dichloromethane was dried by distillation from calcium hydride prior to use. IR spectra were recorded as thin films on NaCl plates or as KBr discs on a Perkin-Elmer Paragon 100 FT-IR spectrometer. ¹H and ¹³C NMR spectra were obtained on a Bruker Avance DPX 400 instrument at 20°C, 400.13MHz for ¹H spectra, 100.61MHz for ¹³C spectra, in either CDCl₃, CD₂COCD₃ or CD₃OD (internal standard tetramethylsilane). Low resolution mass spectra were run on a Hewlett-Packard 5973 MSD GC–MS system in an electron impact mode, while high resolution accurate mass determinations for all final target compounds were obtained on a Micromass Time of Flight mass spectrometer (TOF) equipped with electrospray ionization (ES) interface operated in the positive ion mode at the High Resolution Mass Spectrometry Laboratory by Dr. Martin Feeney in the School of Chemistry, Trinity College Dublin. Thin layer chromatography was performed using Merck Silica gel 60 TLC aluminium sheets with fluorescent indicator visualizing with UV light at 254nm. Flash chromatography was carried out using standard silica gel 60 (230-400 mesh) obtained from Merck. All products isolated were homogenous on TLC. The purity of the tested compounds was determined by HPLC or combustion analysis and unless otherwise stated, the purity level was ≥95%. Elemental analyses were performed on an Exetor Analytical CE4400 CHN analyser in the microanalysis laboratory, Department of Chemistry, University College Dublin. Analytical high-performance liquid chromatography (HPLC) was performed using a Waters 2487 Dual Wavelength Absorbance detector, a Waters 1525 binary HPLC pump and a Waters 717plus Autosampler. The column used was a Varian Pursuit XRs C18 reverse phase 150 x 4.6mm chromatography column. Samples were detected using a wavelength of 254 nm. All samples were analyzed using acetonitrile (70%): water (30%) over 10 min and a flow rate of 1 mL/min. Chiral liquid chromatography was carried out on selected compounds using a Chiral-AGF™ 150x4.0mm column supplied by ChromTech Ltd. (now supplied by Chiral
Technologies Europe) with a Chiral- AGP™ guard column. The HPLC system consisted of the following components: a Waters 1525 binary HPLC pump, a Waters 2487 Dual Wavelength Absorbance Detector, a Waters In-Line Degasser AF and a Waters 717 plus Autosampler. Gradient elution was used beginning with 10% of organic phase and finishing with 90% of organic phase over a period of 20 minutes. The organic mobile phase was 2-propanol and the aqueous phase was a sodium phosphate buffer. The sodium phosphate buffer, consisting of 10mM sodium dihydrogen orthophosphate dihydrate (NaH₂PO₄) in HPLC-grade water, was made up to pH 7.0 using sodium hydroxide. The flow rate was 0.5ml/minute and detection was carried out at 225 nm. Details of the synthesis of intermediate compounds and characterizations of target β-lactams are contained in the supplementary information.

**General method for imine preparation**

The appropriate amine (10 mmol) was refluxed with of the appropriate aldehyde (10 mmol), in ethanol (50 mL) for 3 hours. The reaction mixture was reduced *in vacuo* and the resulting solution was allowed to stand until solid product crystallised from solution. The resulting imine was recrystallised from ethanol.

[3-(tert-Butyldimethylsilyloxy)-4-methoxybenzylidene]-(3,4,5-trimethoxyphenyl)amine  (5e) was prepared by reacting 3-(tert-butyldimethylsilyloxy)-4-methoxybenzaldehyde with 3,4,5-trimethoxybenzenamine following the general method above. The product was obtained as a yellow solid. Yield 64.3%, M.p.105°C. IR (KBr) ν 1619.8 cm⁻¹, 1579.73 cm⁻¹ (-C=N-); ¹H NMR (400 MHz, CDCl₃) δ 0.20 (s, 6H, 2xCH₃), 1.03 (s, 9H, C(CH₃)₃), 3.87 – 3.91 (m, 12H, 4xOCH₃), 6.48 (s, 2H, ArH), 6.93 (d, 2H, J=8.04 Hz, ArH), 7.43 – 7.47 (m, 1H, ArH), 8.35 (s, 1H, CH=N); ¹³C NMR (100 MHz, CDCl₃) δ −5.04(CH₃-Si-CH₃), 18.03(CH₃-C-CH₃), 25.27(C(CH₃)₃), 54.98(- OCH₃), 55.63(-OCH₃), 97.62, 110.94, 119.71, 123.48, 128.95, 135.53, 144.87, 147.94, 153.05, 153.59 (ArC), 158.84(-C=N-). Elemental analysis: Found: C, 63.99; H, 7.65; N, 3.21; C₂₃H₃₃NO₅Si requires C, 64.01; H, 7.71; N, 3.25%.
General method for β-lactam synthesis I: Staudinger reaction (compounds 7 – 14): The appropriate imine (5 mmol) and triethylamine (15 mmol) were added to dry CH₂Cl₂ (50 mL) and the mixture was brought to reflux at 60°C. The appropriate acid chloride (7.5 mmol) was then added dropwise to the mixture via a septum. The reaction mixture was then refluxed for 3 h, then cooled and washed firstly with distilled water (2x50 mL) and then with saturated aqueous sodium bicarbonate solution (50 mL). The organic layer was dried by filtration through anhydrous sodium sulfate. The organic layer containing the product was collected and reduced in vacuo. To afford the crude product which was purified by flash column chromatography over silica gel (eluent: hexane:ethyl acetate gradient).

General method for β-lactam synthesis II: Staudinger reaction modified (compounds 15 – 17): A solution of the appropriate imine (10 mmol) and acid chloride (10 mmol) in CH₂Cl₂ (50 mL) under nitrogen was stirred for 2 hours. Triethylamine (10 mmol) was added dropwise and the mixture was left to stir overnight. The mixture was washed firstly with distilled water (2x50 mL) and then with saturated aqueous sodium bicarbonate solution (50 mL). The organic layer was dried by filtration through anhydrous sodium sulfate. The organic layer containing the product was collected and reduced in vacuo to afford the crude product which was purified by flash column chromatography over silica gel (eluent: hexane:ethyl acetate gradient 10:1 to 2:1).

General method for β-lactam formation III: Acid activation with triphosgene (compounds 18 – 31): Mixture of the appropriate acetic acid (15 mmol) and triphosgene [bis(trichloromethyl) carbonate] (5 mmol) in dry CH₂Cl₂ (50 mL) was heated at reflux for 30 minutes. A solution of the appropriately substituted imine (10 mmol) in dry CH₂Cl₂ (10 mL) was added dropwise to the refluxing solution followed by triethylamine (30 mmol). The reaction mixture was heated at reflux for 5 hours and stirred at room temperature overnight. The mixture was washed firstly with distilled water (2x50 mL) and then with saturated aqueous sodium bicarbonate solution (50 mL). The organic layer was dried over anhydrous sodium sulphate to afford the crude product, which was purified by flash column chromatography over silica gel (eluent: hexane : ethyl acetate gradient).
3-(4-(Benzyloxy)phenyl)-4-(3-((tert-butyldimethylsilyl)oxy)-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one 29 was obtained from (4-benzyloxyphenyl)acetic acid and imine 5e following general method III as a brown oil, yield 6.8% and was deprotected immediately without further purification to afford 37 (see below).

**Benzyl(4-(2-(3-((tert-butyldimethylsilyl)oxy)-4-methoxyphenyl)-4-oxo-1-(3,4,5-trimethoxyphenyl)azetidin-3-yl)phenyl) carbamate 30** was obtained from (4-benzyloxycarbonylaminophenyl)acetic acid and imine 5e following general method III as an oil, yield 10.6% and was deprotected immediately to afford 38 without further purification.

**General method for β-lactam preparation IV: Reformatsky reaction using microwave (compounds 32 – 34):** Zinc powder (0.927g, 15 mmol) was activated using trimethylchlorosilane (0.65 mL, 5 mmol) in anhydrous benzene (5 mL) by heating for 15 minutes at 40°C and subsequently for 2 minutes at 100°C in a microwave. After cooling, the appropriately substituted imine (10 mmol) and substituted ethylbromoacetate (12 mmol) were added to the reaction vessel and the mixture was placed in the microwave for 30 minutes at 100°C. The reaction mixture was filtered through Celite to remove the zinc catalyst and then diluted with dichloromethane. This solution was washed with saturated ammonium chloride solution (20 mL) and 25% ammonium hydroxide (20 mL), and then with dilute HCl (40 mL), followed by water (40 mL). The organic phase was dried over anhydrous sodium sulfate and the solvent was removed in vacuo to afford the crude product which was purified by flash column chromatography over silica gel (eluent: hexane, ethyl acetate gradient) to afford the required β-lactam product

**4-(4-Methoxyphenyl)-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one 32** was obtained from ethyl 2-bromo-2-phenylacetate and imine 5a following general method IV as a white crystalline solid, yield 6.8%; m.p. 108°C; IR (NaCl) ν: 1753.3 cm⁻¹ (C=O, β-lactam); ¹H NMR (400 MHz, CDCl₃) δ 3.72 (s, 6H, 2x-OCH₃), 3.77 (s, 3H, -OCH₃), 3.83 (s, 3H, -OCH₃), 4.29 (d, 1H, J=2.5 Hz, H-3), 4.87 (d, 1H, J=2.5 Hz, H-4), 6.60 (s, 2H, ArH), 6.95 (d, 2H, J=8.5 Hz, ArH), 7.32 – 7.40 (m, 7H, ArH); ¹³C NMR (100 MHz, CDCl₃) δ 54.92(-OCH₃), 55.57(-OCH₃), 60.52(-OCH₃), 63.40(C-3), 64.59(C-4), 94.38,
4-(3-((tert-butyldimethylsilyl)oxy)-4-methoxyphenyl)-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one 34 was obtained from ethyl 2-bromo-2-phenylacetate and imine 5e following general method IV as a yellow oil, yield 70%, and was immediately deprotected to form 35 without further purification.

Desilylation of β-lactams (General method V) (compounds 35 – 38): To a solution of the appropriately protected phenol (10 mmol) in THF (50 mL) was added 1M tetrabutylammonium fluoride (1.5 equivalents). The solution was stirred in an ice-bath for 15 minutes. The reaction mixture was diluted with EtOAc (100 mL) and quenched with HCl (10%, 100mL). The layers were separated and the aqueous layer was extracted with EtOAc (2 x 50 mL). The organic layer was then washed with water (100 mL) and brine (100 mL), followed by drying over anhydrous sodium sulphate. The solvent was removed by evaporation under reduced pressure and the crude product was purified by flash column chromatography over silica gel (eluent: hexane and ethyl acetate gradient) to afford the required phenol product.

4-(3-Hydroxy-4-methoxyphenyl)-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one 35 was obtained from azetidinone 34 following general method V as a white solid, yield 97.3%, m.p. 110°C; IR (KBr) ν: 1718.2 cm⁻¹ (C=O, β-lactam); ¹H NMR (400 MHz, CDCl₃) δ 3.73 (s, 6H, 2x-OCH₃), 3.78 (s, 3H, OCH₃), 3.91 (s, 3H, OCH₃), 4.27 (d, 1H, J=2.5 Hz, H-3), 4.81 (d, 1H, J=2.5 Hz, H-4), 5.75 (s, 1H, OH), 6.63 (s, 2H, ArH), 6.86 – 6.93 (m, 2H, ArH), 7.00 (d, 1H, J=2.0 Hz, ArH), 7.31 – 7.39 (m, 5H, ArH); ¹³C NMR (100 MHz, CDCl₃) δ 55.58(OCH₃), 55.60(OCH₃), 56.50(OCH₃), 60.51(OCH₃), 63.36(H-3), 64.49(H-4), 94.42, 110.59, 111.56, 117.40, 126.97, 127.43, 128.57, 130.10, 133.27, 134.02, 134.31, 145.91, 146.43, 153.05(ArC), 165.14(C=O); HRMS: Calculated for C₂₅H₅₅NO₆Na:458.1580; Found: 458.1575 (M⁺+Na).
3-(4-(Benzyloxy)phenyl)-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one 37 was obtained from azetidinone 29 following general method V as a brown oil, yield 96.8%, which was further deprotected to form 41 without purification.

[4-[2-(3-Hydroxy-4-methoxyphenyl)-4-oxo-1-(3,4,5-trimethoxyphenyl)-azetidin-3-yl]phenyl] carbamic acid benzyl ester 38 was obtained from carbamate 30 following general method V, yield 33.7%, and was further deprotected to 46 without purification.

Debenzylation of β-lactams (General method VI) (compounds 39 – 42): The appropriate benzyl ether compound (2 mmol) was dissolved in ethanol: ethyl acetate (50mL; 1:1 mixture) and hydrogenated over palladium on carbon (1.2g, 10 %) at atmospheric pressure for 2 h. The catalyst was filtered, the solvent was removed under vacuum and the product was purified by flash column chromatography over silica gel (eluent: hexane: ethyl acetate gradient) to afford the phenolic product. 4-(3-Hydroxy-4-methoxyphenyl)-3-(4-hydroxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one 41 was obtained from azetidinone 37 following general method VI as a white powder, yield 2.9%, m.p. 152°C; IR (NaCl film) v: 1720.6 cm⁻¹ (C=O, β-lactam); ¹H NMR (400 MHz, CDCl₃) δ 3.65 (s, 3H, -OCH₃), 3.70 (s, 6H, 2x-OCH₃), 3.86 (s, 3H, -OCH₃), 4.26 (d, 1H, J=2.4 Hz, H-3), 4.98 (d, 1H, J=2.4 Hz, H-4), 6.71 (s, 2H, ArH), 6.87 (d, 2H, J=8.8 Hz, ArH), 7.00 (s, 3H, ArH), 7.22 (d, 2H, J=8.8 Hz, ArH); ¹³C NMR (100 MHz, CDCl₃) δ 54.93(-OCH₃), 59.23(-OCH₃), 63.05(C-3), 63.76(C-4), 94.66, 111.31, 112.22, 115.13, 117.48, 125.72, 128.25, 130.39, 133.49, 134.12, 146.70, 147.32, 153.25, 156.50(ArC), 165.27(C=O); HRMS: Calculated for C₂₅H₂₅NO₇Na: 474.1529; Found 474.1548; (M⁺+Na).

General method for reduction of nitro-substituted azetidinones (General method VII) (compounds 43 – 45): To the appropriate nitro substituted β-lactam (10 mmol), dissolved in the minimum amount of glacial AcOH (2-3 mL) was added metallic zinc dust (10 equiv). The mixture was stirred for 6 days at room temperature under nitrogen. The residue was filtered through Celite and was extracted with dichloromethane. The solvent was evaporated and the residue purified by flash column chromatography over silica gel (eluent: hexane and ethyl acetate gradient) to afford the required amine product.
Deprotection of the Cbz protected azetidinones (General method VIII) (compounds 46 and 47):
The Cbz-protected compound (2 mmol) was dissolved in ethanol: ethyl acetate (50mL; 1:1 mixture) and hydrogenated over palladium on carbon (1.2g, 10 %) for 2 hours. The catalyst was filtered, the solvent was removed under vacuum and the product was purified by flash column chromatography over silica gel (eluent: hexane : ethyl acetate gradient) to afford the amine product.

3-(4-Aminophenyl)-4-(3-hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)azetidin-2-one

46 was obtained from carbamate 38 following general method VIII, as an orange oil, yield 39.4%; IR: (NaCl film) v: 1737.4 cm\(^{-1}\) (C=O, β-lactam); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 3.75 (s, 6H, 2x-OCH\(_3\)), 3.80 (s, 3H, -OCH\(_3\)), 3.93 (s, 3H, -OCH\(_3\)), 4.18 (d, 1H, J=2.5 Hz, H-3), 4.74 (d, 1H, J=2.5 Hz, H-4), 6.63 (s, 2H, ArH), 6.70 (d, 2H, J=8.3 Hz, ArH), 6.89 (m, 2H, ArH), 6.99 (s, 1H, ArH), 7.11 (d, 2H, J=8.3 Hz, ArH); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 55.56 (-OCH\(_3\)), 55.59 (-OCH\(_3\)), 60.51 (-OCH\(_3\)), 63.85(C-3), 64.19(C-4), 94.39, 110.54, 111.53, 115.15, 117.33, 124.24, 128.03, 130.32, 133.39, 145.39, 145.8, 146.32, 153.02(ArC), 165.60 (-C=O); HRMS: Calculated for C\(_{25}\)H\(_{27}\)N\(_2\)O\(_6\): 451.1869; Found: 451.1859 (M\(^+\)).
**Antiproliferative MTT assay:** All assays were performed in triplicate for the determination of mean values reported. Compounds were assayed as the free bases isolated from reaction. The human breast tumour cell line MCF-7 was cultured in Eagles minimum essential medium in a 95%O₂/5% CO₂ atmosphere with 10% fetal bovine serum, 2mM L-glutamine and 100 µg/mL penicillin/streptomycin. The medium was supplemented with 1% non-essential amino acids. MDA-MB-231 cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM), supplemented with 10% (v/v) Fetal bovine serum, 2mM L-glutamine and 100 µg/mL penicillin/streptomycin (complete medium). Cells were trypsinised and seeded at a density of 2.5 x 10⁴ cells/mL in a 96-well plate and incubated at 37°C, 95%O₂/5% CO₂ atmosphere for 24 h. After this time they were treated with 2 µL volumes of test compound which had been pre-prepared as stock solutions in ethanol to furnish the concentration range of study, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The culture medium was then removed and the cells washed with 100 µL phosphate buffered saline (PBS) and 50 µL MTT added, to reach a final concentration of 1 mg/mL MTT added. Cells were incubated for 2 h in darkness at 37°C. At this point solubilization was begun through the addition of 200 µL DMSO and the cells maintained at room temperature in darkness for 20 min to ensure thorough colour diffusion before reading the absorbance. The absorbance value of control cells (no added compound) was set to 100 % cell viability and from this graphs of absorbance versus cell density per well were prepared to assess cell viability using GraphPad Prism software⁴⁸.

**Cytotoxicity assay:** Mammary glands from 14-18 day pregnant CD-1 mice were used as source and primary mammary epithelial cell cultures were prepared from these. 1st to 3rd thoracic glands were exposed by pulling the skin back from the rib cage to the forelimb and extracted in a similar way to the inguinal glands. Mid-pregnant glands are large, soft and are pink in colour. Mammary glands that were spongy and pale in colour were not used as these glands had matured and were producing milk. The harvested glands were then subjected to mechanical and enzymatic digestion to release immature
alveolar structures and isolate mammary epithelial cells. The dissected glands were weighed and fresh collagenase digestion mixture prepared. It contained 480mg of F10 powdered medium (Sigma), 70mg of trypsin (GibcoBrl, Life Technologies™), 150mg collagenase A (GibcoBrl, Life Technologies™) and 2.5ml of foetal calf serum (GibcoBrl, Life Technologies™) in a final volume of 50ml (with sterile water). The collagenase digestion mixture was subsequently adjusted to pH 7.4, filtered through a 0.2μM sterile filter and stored at 4°C until required. The dissected glands were minced criss-cross using two sterile scalpel blades. The minced glands were placed in an autoclaved, sterile 250ml glass conical flask with the collagenase digestion mixture (~4ml of digestion mixture per gram of tissue) and digested for 90 minutes on a shaking table, 250rpm at 37°C. After this step, a stringent washing/centrifugation protocol was used to isolate epithelial cells from fibroblasts. Selective centrifugation was carried out as follows: the digested cell suspension was removed to a 50ml tube and centrifuged for 30 seconds at 100rpm. The supernatant was transferred to a fresh tube and centrifuged at 800rpm for 3 minutes. The pellet was then subjected to a DNase treatment in order to achieve separation of single epithelial cells rather than cell clumps. The DNase mixture contained 480mg of F10 powdered medium (Sigma), 250μl of 10mg/ml DNase (Roche Diagnostics) and 250μl of 1M MgCl₂ brought up to a final volume of 50ml (with water) and passed through a 0.2μM sterile filter. The pellet was resuspended in the DNase mixture and incubated at 37°C for 30 minutes, on a shaking table at 150rpm. The cell suspension was then transferred to a fresh 50ml tube and centrifuged at 800rpm for 3 minutes. The supernatant was discarded. The pellet was resuspended in 30-50ml of Ham's F-12 medium (with gentamycin, Biowhittaker), depending on the size of the pellet. Finally, the cell suspension was added to F-12 medium containing gentamycin (50μg/ml) with the following hormones; hydrocortisone (H) 1μg/ml (Sigma, stock solution: 1mg/ml in 100% ethanol), insulin (I) 5μg/ml (Sigma, stock solution: 5mg/ml in 5mM HCl) and epidermal growth factor (EGF) 5ng/ml (Promega, stock solution: 5μg/ml in F-12 medium). The isolated mammary epithelial cells were seeded at two concentrations (25,000 cells/ml and 50,000 cells/ml). Initially a third concentration of 100,000 cells/ml was also used, but this proved to be
too high to give meaningful results. After 24 hours, they were treated with 2 µL volumes of test compound which had been pre-prepared as stock solutions in ethanol to furnish the concentration range of study, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The cytotoxicity was assessed using alamar blue dye.

**Tubulin polymerization:** Tubulin polymerisation was carried out using a kit supplied by Cytoskeleton. It is based on the principal that light is scattered by microtubules to an extent that is proportional to the concentration of the microtubule polymer. Compounds that interact with tubulin will alter the polymerisation of tubulin, and this can be detected using a spectrophotometer. The absorbance at 340nm at 37°C is monitored. The experimental procedure of the assay was performed as described in version 8.2 of the tubulin polymerisation assay kit manual68.

**Stability study for compounds 32 and 35:** Analytical high-performance liquid chromatography (HPLC) stability studies were performed using a Symmetry® column (C18, 5 µm, 4.6×150 mm), a Waters 2487 Dual Wavelength Absorbance detector, a Waters 1525 binary HPLC pump and a Waters 717plus Autosampler. Samples were detected at wavelength of 254 nm. All samples were analysed using acetonitrile (80%): water (20%) as the mobile phase over 10 min and a flow rate of 1 mL/min. Stock solutions are prepared by dissolving 5mg of compound in 10 mL of mobile phase. Phosphate buffers at the desired pH values (4, 7.4, and 9) were prepared in accordance with the British Pharmacopoeia monograph 2010. 30 µL of stock solution was diluted with 1 mL of appropriate buffer, shaken and injected immediately. Samples were withdrawn and analysed at time intervals of t=0 min, 5 min, 30 min, 60 min, 90 min, 120 min and 21 hours.

**Computational Procedure:** For ligand preparation, all compounds were built using ACD/Chemsketch v10 to generate SMILES. A single conformer from each string was generated using Corina v3.4 and ensuring Omega v2.2.1 was subsequently employed to generate a maximum of 1000 conformations of each compound. For the receptor preparation, the PDB entries 1SA0 were downloaded from the Protein Data Bank (PDB). All waters were retained in both isoforms. Addition and optimisation of hydrogen
positions for these waters was carried out using MOE 2007.09 ensuring all other atom positions remained fixed. Using the reported X-ray structure of tubulin co-crystallised with a colchicine derivative, DAMA-colchicine (PDB entry – 1SA0)\(^6^3\), possible binding orientations ligands were probed with the docking program FREDv2.2.3 (Openeye Scientific Software)\(^6^4\). Docking was carried out using FREDv2.2.3 in conjunction with Chemgauss3, PLP Scoring function. 3-D ligand conformations were enumerated using CORINAv3.4 (Molecular Networks GMBH)\(^6^9\) for ligands followed by generation of multiple conformations using OMEGAv2.2.1 (Openeye Scientific Software)\(^7^0\). Each conformation was subsequently docked and scored with Chemgauss3 PLP as outlined previously. The top binding poses were refined using the LigX procedure (MOE - Chemical Computing Group)\(^7^1\) together with Postdock analysis (SVL script; MOE) of the docked ligand poses.

**X-ray crystallography**

The X-ray crystallography data for crystal \(^3^2\) was collected on a Rigaku Saturn 724 CCD Diffractometer. A suitable crystal was selected and mounted on a glass fiber tip and placed on the goniometer head in a 123K N2 gas stream. The data set was collected using Crystalclear-SM 1.4.0 software and 1680 diffraction images, of 0.5° per image, were recorded. Data integration, reduction and correction for absorption and polarization effects were all performed using Crystalclear-SM 1.4.0 software. Space group determination, structure solution and refinement were obtained using Crystalstructure ver. 3.8 and Bruker Shelxtl Ver. 6.14 software.\(^7^2\) Crystal Data for \(^3^2\); C\(_{25}\)H\(_{25}\)NO\(_5\), MW 419.46, Orthorhombic, Space group Pbca; a = 18.876(9), b = 9.608(5), c = 23.662(12)Å, α, β, γ = 90°; U = 4291(4) Å\(^3\); Z = 8; Dc = 1.298 Mg m\(^{-3}\); m = 0.090 mm\(^{-1}\); Range for data collection = 1.12–25.00; Reflections collected 17904, Unique Reflections 3777 [Rint=0.1746]; Data/restraints/parameters 3777/0/284; Goodness-of-fit on F\(^2\) 1.357; R indices (all data) = R1 = 0.1908, wR2 = 0.2609; Final R indices [I > 2σ(I)] = R1 = 0.2366, wR2 = 0.2809. CCDC deposition No. 775568.
Figure 1: Colchicine (1), Combretastatin A-4 (2a), related compounds 2b, 3a, 3b and Podophyllotoxin (4)
Figure 2: Ortep representation of the X-ray crystal structure of azetidinone 32 with 50% thermal ellipsoids.
Figure 3: Antiproliferative effect of β-lactam compounds 32 and 41 alongside 2a in MCF-7 human breast cancer cells

MCF-7 cells were seeded at a density of $2.5 \times 10^4$ cells per well in 96 well plates. The plates were left for 24 hours to allow the cells to adhere to the surface of the wells. A range of concentrations (0.01 nM-50 µM) of the compound were added in triplicate and the cells left for another 72 hours. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). An MTT assay was performed to determine the level of anti-proliferation. The values represent the mean ± S.E.M (error values) for three experiments performed in triplicate.
Mouse mammary epithelial cells were harvested from mid- to late-pregnant CD-1 mice and cultured. The isolated mammary epithelial cells were seeded at two concentrations. After 24 hours, they were treated with 2 μL volumes of test compound which had been pre-prepared as stock solutions in ethanol to furnish the concentration range of study, 1 nM–100 μM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The cytotoxicity was assessed using alamar blue dye.

**Figure 4:** Dose-response curve for β-lactams 35, 41 and 2a in murine mammary epithelial cells at 25,000 cells/ml
Figure 5: Inhibition of tubulin polymerisation for compound 41

Effects of compound 41 on *in vitro* tubulin polymerisation. Purified bovine tubulin and GTP were mixed in a 96-well plate. The reaction was started by warming the solution from 4 °C to 37°C. 2a (10μM) was used as a reference, while ethanol (1%v/v) was used as a vehicle control. The effect on tubulin assembly was monitored in a Spectramax 340PC spectrophotometer at 340nm at 30 second intervals for 60 minutes at 37 °C. Fold inhibition of tubulin polymerization was calculated using the Vmax value for each reaction. The results represent the mean ± standard error of the mean for three separate experiments.
**Figure 6a:** Docked pose of β–lactam 32 in the colchicine binding site of tubulin

Docked pose of β–lactam 32 in the colchicine binding site of tubulin (PDB entry 1SA0). Significant binding residues Cys 241 and Val 318 are indicated. Hydrogens are not shown for clarity. Coloured by atom: Grey (carbon); red (oxygen); blue (nitrogen); yellow (sulfur). Residue numbers are those used by Ravelli et al.\textsuperscript{63}.
**Figure 6b:** Docked pose of 32 (coloured by atom) overlayed with N-deacetyl-N-(2-mercaptopacetyl)-colchicine (DAMA-colchicine) (yellow) in the tubulin binding site (PDB entry 1SA0). Residues are not shown for clarity.
**Figure 6c:** 2D representation of the binding of β-lactam 32 in the colchicine-binding site of tubulin.

2-D rendering of ligand–protein interactions using LigX module of MOE used to create docked structures of 32 in the colchicine-binding site of tubulin.
Figure 7a: Proposed binding of β-lactam 41 in the colchicine binding site of tubulin (PDB entry 1SA0).

Docked pose of β-lactam 41 (green) in colchicine binding site of tubulin (PDB entry 1SA0). Hydrogens are not shown for clarity. Significant binding residues Thr 179, Cys 241, Val 318 and Lys 254 are indicated. Residue numbers are those used by Ravelli et al.63.
**Figure 7b:** Docked pose of β-lactam 41 in the colchicine binding site of tubulin (PDB entry 1SA0).

Docked pose of β-lactam 41 in colchicine binding site of tubulin (PDB entry 1SA0) with isolated important residues. Hydrogens are not shown for clarity. Significant binding residues Cys 241, Val 318 and Lys 254 are indicated. Coloured by atom: Grey (carbon); red (oxygen); blue (nitrogen); yellow (sulfur). Residue numbers are those used by Ravelli et al\textsuperscript{63}.  

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**Figure 7c:** 2D representation of the binding of β-lactam 41 in the colchicine-binding site of tubulin

2-D rendering of ligand–protein interactions using LigX module of MOE used to create docked structures of 41 in the colchicine-binding site of tubulin.
**Figure 7d:** Docked pose of 41 (coloured by atom) overlayed with N-deacetyl-N-(2-mercaptoacetyl)-colchicine (DAMA-colchicine) (yellow) in the tubulin binding site (PDB entry 1SA0). Residues are not shown for clarity.
Scheme 1. Synthesis of imines 5a-f

Reagents and conditions: (a) EtOH, reflux, 3 h

5a: R₁=R₂=R₃=R₅=OCH₃; R₄=R₆=H
5b: R₁=R₂=R₃=R₄=R₅=H; R₆= OCH₃
5c: R₁=R₃=H; R₂=R₄=R₅=R₆= OCH₃
5d: R₁=R₂=R₃=H; R₄=R₅=R₆= OCH₃
5e: R₁=R₂=R₃=R₅= OCH₃; R₄=H; R₆= OSi(CH₃)₂C(CH₃)₃
5f: R₁=R₂=R₃=R₅= OCH₃; R₄=H; R₆= NO₂
Scheme 2: Synthesis of azetidinones 7 - 31, Routes I, II and III

Reagents and conditions: (a) SOCl₂, CHCl₃, reflux, 3 h; (b) (Route I) triethylamine, anhydrous CH₂Cl₂, reflux, 3 h; (c) (Route II) triethylamine, anhydrous CH₂Cl₂, 20°C, 18 h; (d) (Route III) triphosgene, triethylamine, anhydrous CH₂Cl₂, reflux, 5 h; 20°C, stirred 18 h.
Scheme 3: Synthesis of azetidinones 32 - 34; Route IV

\[ \text{BrOCOCH}_3 + 5a, 5c, 5e \xrightarrow{(a)} \text{Product} \]

R=H unless otherwise indicated

32: \( R_1=R_2=R_3=R_5=\text{OCH}_3 \)
33: \( R_2=R_4=R_6=\text{OCH}_3 \)
34: \( R_1=R_2=R_3=R_5=\text{OCH}_3, \quad R_4=\text{OSi(C}_3\text{H}_3)_2\text{C(CH}_3)_3 \)

\( ^a \)Reagents and conditions: (a) Zinc, trimethylchlorosilane, benzene, microwave
Scheme 4. Synthesis of azetidinones 35 - 38; Route V<sup>a</sup>

Reagents and conditions: TBAF, THF, 0°C, 15 min
Scheme 5. Synthesis of azetidinones 39 - 42; Route VI

Reagents and conditions: (a) H₂, Pd/C, EtOH:EtOAc (1:1).
Scheme 6: Synthesis of amino substituted azetidinones 43 – 45; Route VII$	extsuperscript{a}$

Reagents and conditions: (a) Zn, CH$_3$CO$_2$H, 7 days
Scheme 7. Synthesis of azetidinones 46 and 47; Route VIII

Reagents and conditions: (a) H₂, Pd/C, EtOH:EtOAc (1:1)
Scheme 8. Synthesis of azetidinethione 48

Reagents and conditions: (a) Lawesson's reagent, toluene, reflux, 3h
Scheme 9: Azetidinone reduction and methanolysis$^a$

Reagents and conditions: (a) LiAlH₄, dry THF, reflux 3h (b) NaOCH₃, MeOH, 20°C, 8 days.
Table 1. Azetidinones with methoxy substitution at N-1, C-3 and C-4 aryl rings

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<th>R₅</th>
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Table 2. Azetidinones with C-3 aryl substitution

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Table 3. Azetidinones with hydroxyl and amine-type substituents on C-3 and C-4 aryl rings and related compounds

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Table 4: Antiproliferative effects of azetidinones in MCF-7 human breast cancer cells

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<th>Antiproliferative activity a,b MCF-7 cells IC&lt;sub&gt;50&lt;/sub&gt; value (µM)</th>
<th>Compound number</th>
<th>Antiproliferative activity a,b MCF-7 cells IC&lt;sub&gt;50&lt;/sub&gt; value (µM)</th>
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<td>37.75 ± 23.35</td>
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<td>1.6577 ± 0.4283</td>
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<td>8</td>
<td>0.3263 ± 0.1565</td>
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<td>0.0344 ± 0.0198</td>
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<td>0.1820 ± 0.0262</td>
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<td>43.12 ± 16.47</td>
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<td>&gt;100</td>
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<td>2a</td>
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^IC<sub>50</sub> values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 or MDA-MB-231 cells. Values represent the mean ± S.E.M (error values x 10<sup>-6</sup>) for three experiments performed in triplicate.

<sup>b</sup>The IC<sub>50</sub> values obtained for 2a in this assay are 0.0344 µM for MCF-7 and 0.043 µM for MDA-MB 231 and are in good agreement with the reported values for 2a using the MTT assay on human MCF-7 and MDA-MB 231 breast cancer cell lines, (see references 7, 73, 74).
Table 5. Antiproliferative effects of selected β-lactam compounds in MDA-MB-231 human breast cancer cells

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<tr>
<th>Compound number</th>
<th>Antiproliferative activity (^{a,b}) MDA-MB-231 cells IC(_{50}) value (µM)</th>
<th>Compound number</th>
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<td>24</td>
<td>0.2284 ± 0.1755</td>
<td>41</td>
<td>0.1764 ± 0.1578</td>
</tr>
<tr>
<td>32</td>
<td>0.0782 ± 0.0348</td>
<td>42</td>
<td>1.6839 ± 1.9756</td>
</tr>
<tr>
<td>35</td>
<td>0.0288 ± 0.0199</td>
<td>43</td>
<td>0.2245 ± 0.1778</td>
</tr>
<tr>
<td>36</td>
<td>0.1072 ± 0.0558</td>
<td>44</td>
<td>0.4288 ± 0.0670</td>
</tr>
<tr>
<td>39</td>
<td>0.1577 ± 0.0352</td>
<td>45</td>
<td>0.0808 ± 0.0738</td>
</tr>
<tr>
<td>2a</td>
<td></td>
<td></td>
<td>0.043</td>
</tr>
</tbody>
</table>

\(^{a}\)IC\(_{50}\) values are half maximal inhibitory concentrations required to block the growth stimulation of MCF-7 or MDA-MB-231 cells. Values represent the mean ± S.E.M (error values x 10\(^{-6}\)) for three experiments performed in triplicate.

\(^{b}\)The IC\(_{50}\) values obtained for 2a in this assay are 0.043 µM for MDA-MB 231 and is in good agreement with the reported values for 2a using the MTT assay on MDA-MB 231 breast cancer cell lines, (see references \(^7, 73, 74\)).
Table 6: Inhibition of tubulin polymerization for compounds 32, 35, 41 and 2a

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fold-reduction in $V_{\text{max}}$ at 10μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>6.0 ± 1.4</td>
</tr>
<tr>
<td>32</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>35</td>
<td>8.3 ± 2.6</td>
</tr>
<tr>
<td>41</td>
<td>11.76 ± 3.85</td>
</tr>
</tbody>
</table>

*Effects of 2a, 32, 35 and 41 on *in vitro* tubulin polymerisation. Purified bovine tubulin and GTP were mixed in a 96-well plate. The reaction was started by warming the solution from 4 °C to 37°C. 2a (10μM) was used as a reference, while ethanol (1%v/v) was used as a vehicle control. The effect on tubulin assembly was monitored in a Spectramax 340PC spectrophotometer at 340nm at 30 second intervals for 60 minutes at 37 °C. Fold inhibition of tubulin polymerization was calculated using the Vmax value for each reaction. The results represent the mean ± standard error of the mean for three separate experiments. Nd: not determined.
Acknowledgements

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Supporting Information Available: Experimental procedures and spectroscopic data for intermediate compounds are presented. Further cytotoxicity data in normal murine epithelial cells for compounds 35 and 41, together with the results of Comparative Antitumour Evaluations of compounds 9 and 35 in the NCI60 cell line in vitro primary screen and Standard COMPARE Analysis of 35 are available in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.
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   Wilcox, E.; Hamel, E.; Artico, M.; Silvestri, R., Arylthioindoles, potent inhibitors of tubulin 
Docked pose of β-lactam 41 (coloured in green) in the colchicine binding site of tubulin