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The synthesis of neurotensin antagonist SR 48692 for prostate cancer research

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ABSTRACT

An improved synthesis of the molecule SR 48692 is presented and its use as a neurotensin antagonist biological probe for use in cancer research is described. The preparation includes an number of enhanced chemical conversions and strategies to overcome some of the limiting synthetic transformations in the original chemical route.

1. Introduction

Since its original isolation in 1973,[1] the endogenously expressed tridecapeptide neurotensin (NT, 1, Fig. 1) has attracted considerable interest. Subsequent investigations have shown NT to be present within specific regions of the central nervous system,[2] as well as having peripheral expression in the gut.[3] Physiologically, NT has been shown to play important roles in a variety of biological processes including temperature control,[4] pain sensation,[5] and pituitary hormone secretion.[6] As a modulator of the bodies dopaminergic systems,[7] disruption of NT's binding has been proposed as a possible treatment of schizophrenia and Parkinson's disease.[8] In addition, recent research has demonstrated that NT has increasing relevance in several human cancers. Up regulation of NT receptor (NTR) expression has been observed in cancers of the Lung,[9] Breast,[10] Pancreas,[11] Pituitary[12] and of the Prostate.[13] As an example, Prostate cell cancer is usually treated with anti androgen therapy however, in a number of cases, the tumour may become refractory and develop a more aggressive neuroendocrine phenotype.[14] Both androgen sensitive (LnCaP cells) and androgen insensitive (PC3 cells) model prostate cancer cells lines have been shown to express elevated levels of NT receptors.[15] In addition, LnCaP cells have been shown to produce and secrete NT on androgen withdrawal.[15] Although NT production has not been detected by PC3 cells, treatment of this cell line with NT does indeed stimulate DNA synthesis and increase cell number, raising a possible autocrine/paracrine role for NT in prostate cancer.[13] Inhibitors of NT activity would provide molecular biologists with a useful tool to aid in our further understanding of these effects of NT on early (LnCaP) and late (PC3) stage prostate cancer and cancer in general.

The biological effects of NT are thought to be mediated through interaction with at least 3 neurotensin receptors.[16,22] NTR1 and NTR2 are both seven-transmembrane G-protein coupled receptors (GPCR's),[17] whereas NTR3 (showing 100% identity with Sortilin) possess a single transmembrane (TM) domain.[18] Similar to many GPCR's, NTR1 and NTR2 have been shown to activate several signal transduction pathways, up-regulating intracellular cAMP, inositol phosphate, and intracellular calcium levels. Often co-expressed in vivo, identification of various NT receptor subtypes and deconvolution of their intracellular effects remains a rewarding yet challenging area of research.

The identification of these neurotensin receptors and elucidation of their downstream effects has been greatly facilitated by access to agents that can specifically bind to these receptors. Initial investigations utilised NT itself to both identify and purify putative NT receptors by affinity chromatography.[1] Unfortunately, NT has several drawbacks when applied as either a therapeutic or investigative tool. Firstly, as an oligopeptide, NT has poor stability in vivo and is readily degraded by several endopeptidases and metalloproteases.[19] Secondly, its size prevents passage across the blood brain barrier resulting in poor bioavailability requiring injection of NT directly into the central nervous system (CNS).[20] Thirdly, NT and its derivatives are only capable of NT receptor agonism, however, study of these receptors and pharmaceutical development requires access to antagonists of NT receptor (NTR) function.[21] To address

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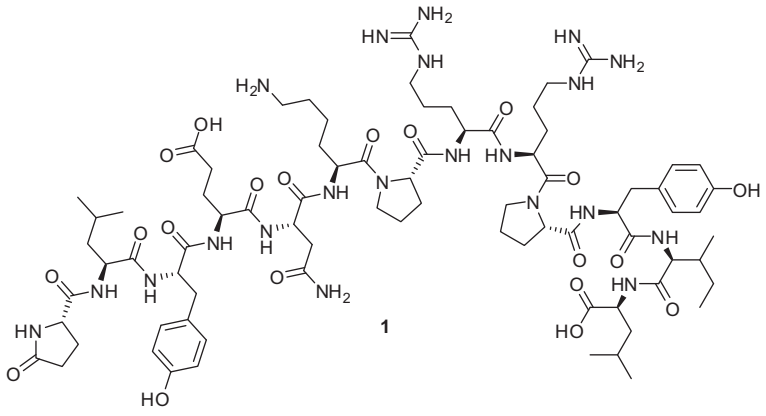


Figure 1. Neurotensin (**1**) and its linear amino acid sequence.

these issues, several small molecule NTR agonists and antagonist have been developed. Amongst these three molecules in particular stand out as valuable entities for the study of NT binding and activity: SR 48692 (**2**), SR 142948A (**3**), and Levocabastine (**4**) (Fig. 2).

SR 48692 (**2**)[22] is a selective and potent NTR1 antagonist demonstrating the ability to displace radiolabeled NT from the receptor in the low nanomolar range (K_i = 2.6 ± 0.2 nM).[23] In addition, **2** has also demonstrated binding to NTR2 (K_i = 418 ± 82.2 nM) and NTR3 (IC_{50} = 238 ± 46 nM) at higher concentrations. In contrast, the biological properties of SR 142948A (**3**) differ significantly from **2**.[24] Compound **3** binds to both NTR1 and NTR2, but is capable of selectively activating NTR2 at much lower concentrations.[25] Finally, the selective histamine H1 receptor antagonist levocabastine (**4**) marketed as Livostin by Novartis is normally used to alleviate symptoms associated with rhinoconjunctivitis but has also shown diagnostic utility in the field of NT research.[26] Indeed, levocabastine is able to discriminate between NTR1 and NTR2, selectively binding NTR2. In conjunction these three molecules represent a valuable small molecular probe tool kit to develop a better understanding of NT receptor action and signaling.[21]

This is especially important with regards to the biological effects of NTR2 agonism and its interaction with SR 142948A which is not fully understood with work continuing in the academic community to disentangle its complexities. Further elucidation of the intricate biological picture concerning NTR2 would greatly benefit from supplies of molecules capable of selective interaction with this receptor.

Although both SR 142948A (**3**) and levocabastine (**4**) are commercially available, when our initial studies began SR 48692 (**2**) was not commercially unavailable, and still remains prohibitory expensive. Sanofi–Aventis have disclosed two routes to SR 48692 (Scheme 1).[22,27] The first approach begins with a Claisen condensation between ketone **5** and diethyl oxalate. Next, an acid mediated condensation of hydrazine **7** with the 1,3-dicarbonyl **6** and saponification of the resultant ester gives acid **9**. Subsequent activation and coupling with amino acid **10** provides the desired product **2**. Alternatively, activation of acid **9** and coupling with amino acid **10** using a mixture of pyridine and CH_2Cl_2 as the solvent yields intermediate **11** which, when treated with TFA/CH_2Cl_2 furnishes **2**. Although this route is relatively short, the final coupling of the unprotected amino acid **9** progresses in a relatively poor yield resulting in a low overall isolation of the desired product. As such, we envisioned that a protected amino acid variant could provide a higher yielding route to SR 48692.

2. Results and discussion

2.1. Chemistry

Synthesis of *tert*-butyl 2-aminoadamantane-2-carboxylate (18; Scheme 4

Our synthetic efforts began with the synthesis of the adamantyl amino acid (**10**) employing the Bucherer–Berg strategy previously described by Nagasawa et al. (Scheme 2).[28] This literature reported that treatment of ketone **12** with a buffered sodium cyanide solution afforded the spirohydantoin **13** which, without purification, could be hydrolyzed to the desired amino acid **10** in good yield. However, in our hands, isolation of clean amino acid **10** could not be achieved, all isolated material (although identical to the reported characterization) was significantly contaminated by unidentifiable inorganic impurities as comprehensively determined by microanalysis and calibrated ^{1}H NMR. Abandoning this approach and in order to avoid the severity of the conditions required for the hydrolysis of the hydantoin, preparation of amino

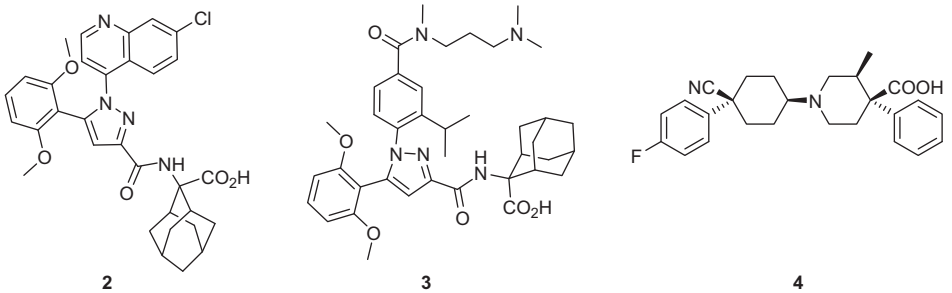
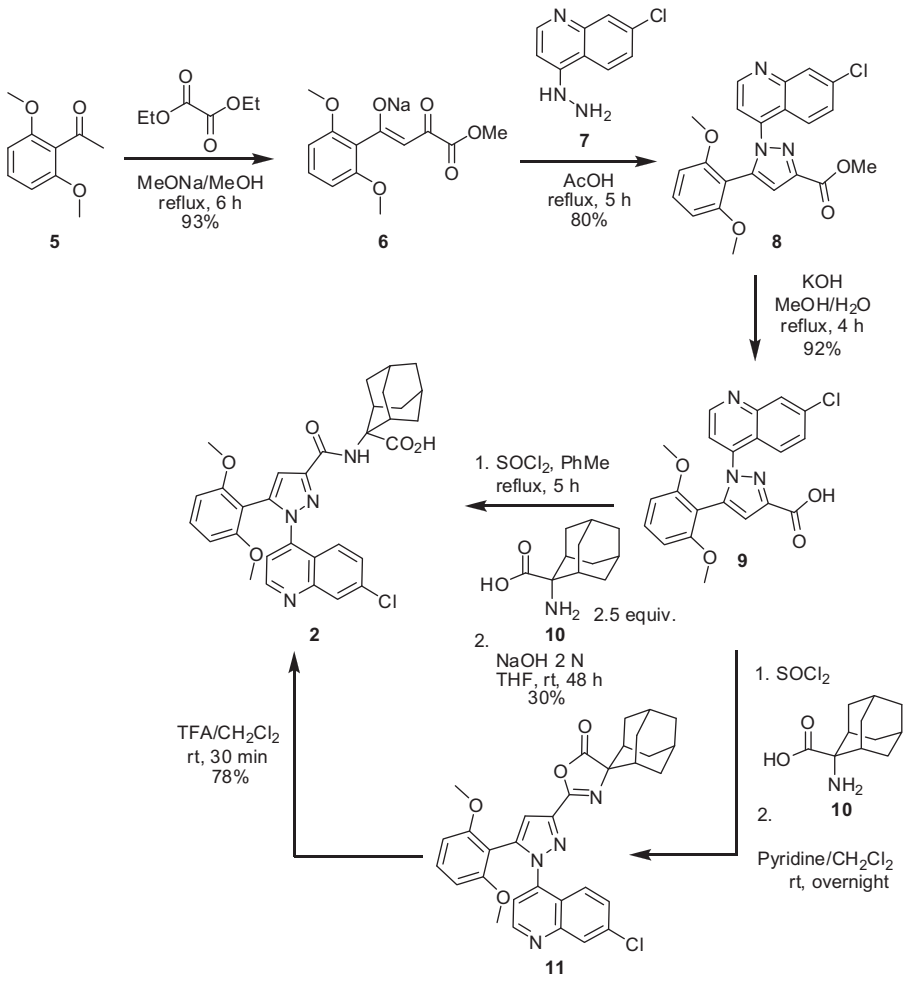
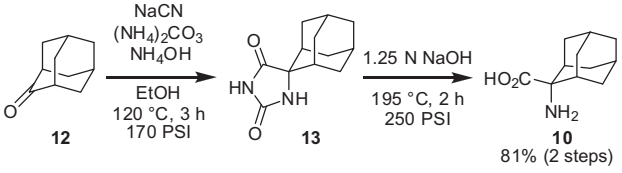


Figure 2. Known NT receptor binders as a diagnostic molecular toolbox.

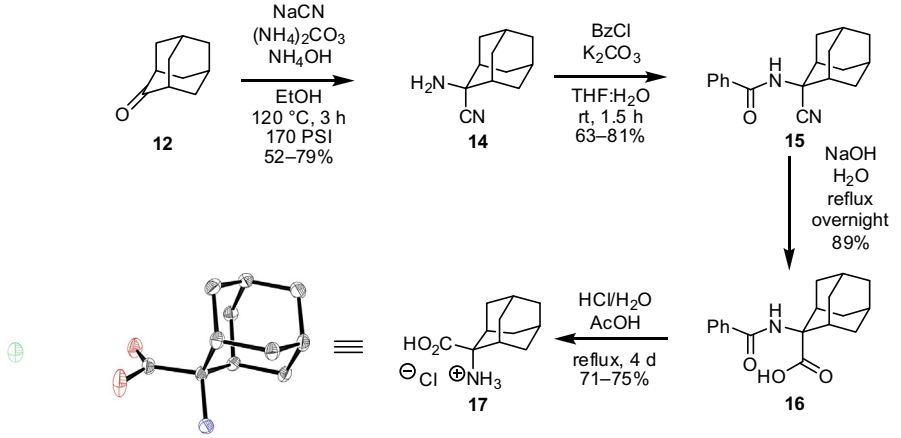


Scheme 1. Routes towards SR 48692 (**2**) reported by Sanofi–Aventis.[22,27]



Scheme 2. Preparation of amino acid 10 via Nagasawa's route.[28]

acid **10** was attempted via a Strecker reaction (Scheme 3). The resultant aminonitrile species **14** is known to be highly resistant to hydrolysis,[29] although employing an intramolecular activation procedure reported by Edward and co-workers,[30] the desired amino acid could be obtained in good yield. This route also had the additional benefit of enabling easier reaction monitoring due to the presence of the aromatic UV chromophore in the intermediate



Scheme 3. Alternative route to the amino acid **17**.

amides **15** and **16**. Consequently, following activation of the aminonitrile **14** by treatment with benzyl chloride, compound **15** was hydrolyzed under basic conditions to the protected acid **16** which under acidic conditions afforded the desired amino acid as its hydrochoride salt (**17**). This material was determined to be completely free of the previously encountered inorganic impurities as evidenced by microanalysis.[31]

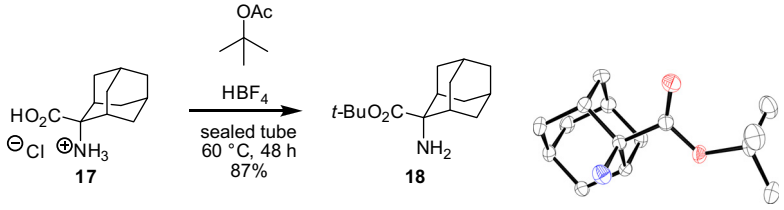
Having gained access to amino acid **17** our attention next turned to its protection. This was undertaken for two key reasons. Firstly, amino acid **10** is highly insoluble in most common solvents that would be suitable for the necessary amide coupling reaction. Secondly, protection of the acid functionality would avoid any possible oligomerisation of the amino acid component during the coupling chemistry. Protection of amino acid **10** as the *tert*-butyl ester had previously been achieved albeit in a very low-yielding multistep procedure.[32] This involved a laborious strategy requiring Cbz protection of the amine, followed by esterification of the acid and then removal of the nitrogen protecting group to reveal the amine once again.

It was noted that this protection could be achieved in one step reaction by treatment of the amino acid salt with *tert*-butyl acetate under acidic conditions. In order to achieve an optimal yield in this transformation it was necessary to ensure that the reaction vessel remained sealed throughout the reaction. Therefore the reaction was run in a sealed microwave tube, presumably preventing the loss of volatile butene from the reaction vessel. Confirmation of the selectivity of this protection could be unambiguously ascertained by X-ray crystallographic methods (see Scheme 4). Although this reaction worked efficiently under the described conditions it proved problematic giving much lower yields when the scale was increased (64% on a 25 g scale and 58% on 50 g).

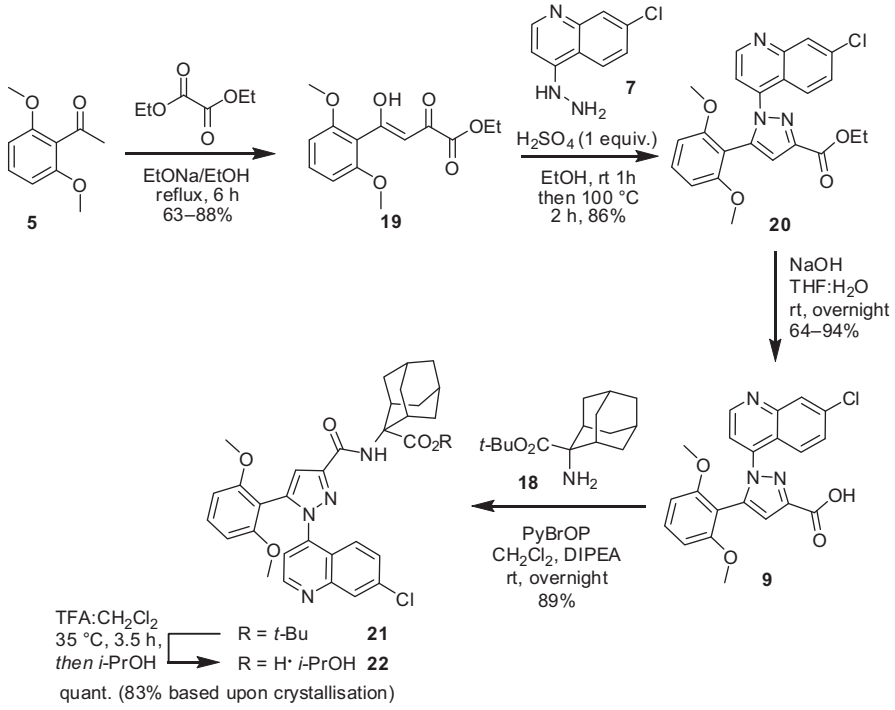
2.2. Synthesis of Meclinertant (SR 48692, 2)

With suitable quantities of the protected amino acid **18** in hand, work began towards the synthesis of the pyrazole core of Meclinertant following the route originally reported by Gully and co-workers.[27] Initial attempts to isolate the 1,3-dicarbonyl **19** by crystallization of the sodium salt[27] as originally described were problematic. This issue could however be easily circumvented by an acidic work-up and extraction of the protonated form of **19**. To reliably achieve high mass recovery it proved necessary to carefully regulate the acidity of the solution maintaining it at pH ~6 throughout the extraction process. Having optimized the procedure (166 mmol scale) the reaction was directly scaled to generate hundreds of grams of the starting material (Scheme 5).

Surprisingly, the condensation reaction between hydrazine **7** and the 1,3-dicarbonyl unit of **19** to furnish the pyrazole **20** also proved initially troublesome. Even at elevated temperatures the reaction was very slow and under a range of conditions we



Scheme 4. *tert*-Butyl protection of amino acid **17**.



Scheme 5. Improved synthesis of Meclinertant (SR 48692, **2**).

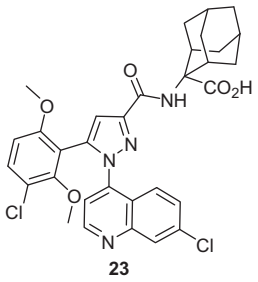


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Figure 3. Proposed structure of the chlorinated by-product.

experienced significant retro Aldol of the 1,3-dicarbonyl unit in **19** as evidence by the appearance of ketone **5** in the reaction mixture. A comprehensive screening of conditions using a microwave synthesizer to evaluate temperature, solvent and a range of catalysts (acids, bases both protic and Lewis) led to the choice of ethanol as the solvent and the addition of 1 equiv of H_2SO_4—which gave superior results over catalytic quantities. Under all conditions complete regioselective addition was observed affording only the desired pyrazole **20**, which was unambiguously confirmed by X-ray crystallography. The subsequent hydrolysis of the ester in **20** to the acid **9** was by comparison straightforward giving a clean transformation and good recovery of material.

Having achieved reliable syntheses to both coupling partners, we investigated the peptide bond forming step which had been indicated as low yielding in the previous literature work.[22,27] Activation of the pyrazole acid **9** via the acid chloride and subsequent treatment with the protected amino acid **18** provided *tert*-butyl protected Meclinertant **21** in 58% yield and in 90% purity after column chromatography. It was found that under these reaction conditions a co-eluting by-product was also being formed. Assignment of this organic impurity was achieved by HRMS and ^{1}H NMR and its structure determined to be the chlorinated analogue of SR 48692 (Fig. 3).[33] This unexpected chlorination product is most likely produced from the thionyl chloride used to initially activation of the carboxylic acid **9**. In order to avoid this issue a variety of alternative coupling strategies were investigated. The activators CDI, and DCC were found to be ineffective at activating the acid **9** to attack by the protected amino acid **18**. However, treatment with PyBrOP affected the transformation in an impressive 89% isolated yield. In addition, unlike in the previous work only a single equivalent of the protected amino ester **18** was required also increasing the ease of work-up and purification.

Next removal of the *tert*-butyl ester protecting group was conducted using a 1:1 mixture of trifluroacetic acid and CH_2Cl_2 (35 °C in a sealed tube) which, after solid powdered K_2CO_3 workup, provided material consistent with the acid, merclinertant **2** (^{1}H/^{13}C NMR, HRMS, LC–MS). Extensive efforts were made to obtain crystals of sufficient quality for study by X-ray diffraction.[34] This analysis revealed that although the organic framework of Meclinertant had indeed been assembled correctly, several unknown metal contaminants were also incorporated within the solid structure. This was further supported by microanalysis of the product. All attempts to remove these impurities by column chromatography were unsuccessful. The problem of inorganic impurities was avoided by modification of the workup procedure.

Following the *tert*-butyl ester deprotection complete removal of the CH_2Cl_2 and trifluroacetic acid was conducted in vacuo. Next, the crude material was directly recrystallised from *iso*-propanol which gave access to the desired compound in a pure form as the mono-solvate (**22**). Although this procedure involving recrystallisation gave high quality material it also gave a low mass recovery (64%). Attempts to use alternative solvents or add an anti-solvent did increase the mass balance but produced material of a much lower grade (poor crystallinity and coloured impurities). An increase in isolated yield could be achieved by harvesting a secondary crop of crystals through partial evaporation (1/2 volume) under vacuum at 40 °C followed by slow cooling. This second collection of material was of identical quality and increased the combined yield to 83%. In addition it was shown that this remained consistent through subsequent batches and scaling (2, 5 and 12 g).

2.3. Biological results

The bioactivity of Meclinertant (**2**) was examined in three different model prostate cancer cells lines. In these studies, the three cell lines were treated with SR 48692 and their proliferation monitored over a 5 day period. From these results it can be concluded that Meclinertant significantly reduced the proliferation of LNCaP cells at concentration of 50 μM and above. In addition, biculamide resistant cell lines (LNCaP-Bic, C4-2b) which display resistance to the common treatment of prostate cell cancer, also displayed reduced proliferation over the same concentration range of Meclinertant dosage. Investigation into the specific IC_{50} value of Meclinertant against the C4-2b cell line revealed that 50% reduction of proliferation of this cell line could be achieved at approximately 30 μM (Fig. 4). The effects of Meclinertant on SK-OV-3 (ovarian cancer) model cell line were also examined. In MTS assays, Meclinertant caused loss of cell viability at 100 μM concentrations, however, at lower concentration no discernible reduction in the number of viable cells was observed.

Taken together, these results show that at relatively high micromolar concentrations Meclinertant is a biologically active compound capable of reducing cell viability in a number of different cancer cell lines, including cell lines unresponsive to other cancer treatments (LNCaP-Bic, C4-2b) confirming its use as a tool in the further study of cancer progression.

In summary, an improved synthesis of SR 48692 has been completed in a five step sequence requiring only one chromatographic step. Unexpected chlorination of **9** by thionyl chloride, a previously unreported contaminant, was avoided by the selection of an alternative coupling agent. This new procedure, combined with the use of the more soluble amino ester **18** leads to increased yields in the key amide bond formation step. Isolation of the desired compound as a crystalline solid can be achieved via the *iso*-propanol solvate of SR 48692 (**22**) and the positive inhibitory response of Meclinertant against cancer cell lines has been demonstrated.

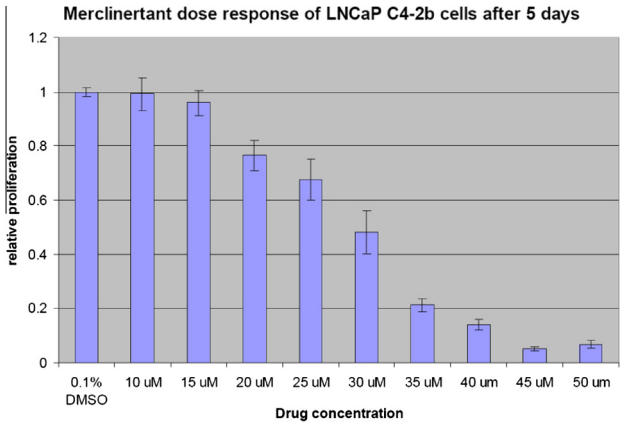

Figure 4. Meclinertant (**2**) dose–response curve.

3. Experimental

3.1. Chemistry

^{1}H NMR spectra were recorded on a Bruker Avance DPX-400, DRX-600, Avance 400 QNP Cryo, or Avance 500 Cryo spectrometer with the residual solvent peak as the internal reference ($CDCl_3$ = 7.26 ppm, DMSO-d_6 = 2.50 ppm). ^{1}H resonances are reported to the nearest 0.01 ppm. ^{13}C NMR Spectra were recorded on the same spectrometers with the central resonance of the solvent peak as the internal reference ($CDCl_3$ = 77.16 ppm, and DMSO-d_6 = 39.52 ppm). All ^{13}C resonances are reported to the nearest 0.1 ppm. DEPT 135, COSY, HMQC, and HMBC experiments were used to aid structural determination and spectral assignment. Where specified, NOESY and gradient NOE spectra were used to aid the assignment of ^{1}H spectra. The multiplicity of ^{1}H signals are indicated as: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, or combinations thereof. Coupling constants (J) are quoted in Hz and reported to the nearest 0.1 Hz. Where appropriate, averages of the signals from peaks displaying multiplicity were used to calculate the value of the coupling constant. Infrared spectra were recorded neat on a PerkinElmer Spectrum One FT-IR spectrometer using Universal ATR sampling accessories. Letters in parentheses refer to the relative absorbency of the peak: w = weak, less than 40% of the most intense peak; m = medium, ca. 41–69% of the most intense peak; s = strong, greater than 70% of the most intense peak. Unless stated otherwise, reagents were obtained from commercial sources and used without prior purification. Laboratory reagent grade EtOAc, petroleum ether 40–60, and CH_2Cl_2 were obtained from Fischer Scientific and distilled before use. The removal of solvent under reduced pressure was carried out on a rotary evaporator or a Vapoutec V-10 evaporator. All column chromatography was carried out using Silica Gel (0.040–0.063 mm), purchased from Breckland Scientific Supplies. TLC analysis was performed on Merck 60 F_{254} silica gel plates and visualised using both short and long waved ultraviolet light in combination with standard laboratory stains; acidic potassium permanganate and acidic ammonium molybdate. Melting points were performed on a Stanford Research Systems MPA100 (OptiMelt) automated melting point system and are uncorrected. High resolution mass spectrometry (HRMS) was performed using a Waters Micromass LCT Premier™ spectrometer using time of flight with positive ESI, or conducted by Mr. Paul Skelton (Department of Chemistry, University of Cambridge) on a Bruker BioApex 47e FTICR spectrometer using positive ESI or EI at 70 ev to within a tolerance of 5 ppm of the theoretically calculated value. LC–MS analysis was performed on an Agilent HP 1100 series chromatography (Mercury Luna 3u C18 (2) column) attached to a Waters ZQ2000 mass spectrometer with ESCi ionization source in ESI mode. Elution was carried out at a flow rate of 0.6 mL/min using a reverse phase gradient of MeCN and H_2O containing 0.1% formic acid. The gradient run is as described in Table 1. Retention time (R_t) is given in min to the nearest 0.1 min and the m/z value is reported to the nearest mass unit (m.u.).

X-ray crystal structures were determined by Dr. John Davies (Department of Chemistry, University of Cambridge). CIF numbers are reported as part of compound characterization. Elemental analyses within a tolerance of ±0.3% of the theoretical values were determined by Mr. Alan Dickerson and Mrs. Patricia Irele in the microanalytical laboratories (Department of Chemistry, University of Cambridge).

Table 1
LC–MS conditions

Time (min)	CH_3CN (%)	Flow rate (mL/min)
0.00	5	1
3.00	95	1
5.00	95	1
5.50	5	1
8.00	5	1

3.2. Spiro[adamantane-2,4′-imidazolidine]-2′,5′-dione hydrate (13)[28]

To a mixture of 2-adamantone (**12**) (25.0 g, 0.17 mol) and $(NH_4)_2CO_3$ (24.0 g, 0.25 mol) in EtOH/H_2O (400 mL; 3:1) was added NaCN (17.9 g, 0.37 mol) and the resultant slurry was stirred at ambient temperature (the dispensing vessel was a three necked flask maintained under a positive pressure using a nitrogen balloon and maintained as a suspension with continuous stirring). The reaction mixture was processed in a CEM Voyager Stop-Flow Microwave unit in 50 mL batches heating at 150 °C for 2 h per batch. A wash solution of EtOH/H_2O (50 mL; 3:1) was used to clean the reaction vessel in between cycles. On completion, the resulting suspension was diluted with H_2O (400 mL) and the solids collected, washed with H_2O (200 mL), acetone (100 mL), and Et_2O (100 mL) to give a white solid (230.8 g) which was used without purification. Recrystallisation of a portion (20 g) of this material from THF (3 recrystallisations) afforded the title compound as a white crystalline solid (2.16 g, 9.07 mmol, 11%).

Mp: 296–299 °C; ^{1}H NMR (400 MHz, DMSO-d_6): 8.35 (1H, s, NH-2′), 4.13 (2H, br s, hydrate·H_2O), 2.54 (2H, d, J = 11.9 Hz, H-4), 2.02 (2H, d, J = 12.5 Hz, H-6), 1.77 (4H, m, H-5/8/3), 1.68 (2H, s, H-7), 1.56 (4H, m, H-4/6); ^{13}C NMR (100 MHz, DMSO-d_6): 177.2 (C-9), 155.9 (C-1), 65.6 (C-2), 37.3 (C-7), 34.6 (C-3), 32.9 (C-6), 31.5 (C-4), 26.3 (C-5 or 8), 25.6 (C-5 or 8); IR (neat) cm^{-1}: 3484.4 (w), 3423.9 (w), 3340.9 (m), 2916.5 (m), 2763.7 (m), 1736.2 (w), 1704.9 (s), 1421.0 (m); HRMS: calcd for $[C_{12}H_{17}O_2N_2]^+$ = 221.1296, found 221.1296, Δ = 0.0 ppm; Microanalysis: calcd (found) for $C_{12}H_{16}O_2N_2.H_2O$ (as the monohydrate); C = 60.49% (60.24%), H = 7.61% (7.61%), N = 11.52% (11.76%).

3.3. 2-Aminoadamantane-2-carbonitrile (14)[29]

2-Adamantone (**12**) (100 g, 0.67 mol), NaCN (32.68 g, 0.67 mol), NH_3 (132 mL), and NH_4Cl (71.30 g, 1.33 mol), were dissolved in H_2O (665 mL) and EtOH (1.2 L). The resultant solution (pH >10) was heated at 55 °C for 8 h. The reaction was allowed to cool to ambient temperature and was extracted with Et_2O (2 × 1 L), concentrated (approx. 350 mL), to generate a precipitate, the solid was isolated by filtration, washed with H_2O (2 × 150 mL), and dried to give a white solid (93.0 g, 0.53 mol, 79% yield).

Mp: 191–192 °C; ^{1}H NMR (400 MHz, $CDCl_3$): 2.23 (2H, d, J = 12 Hz, H-4 or H-6), 1.98 (2H, d, J = 12.4 Hz, H-4 or H-6), 1.90 (2H, s, H-7), 1.67–1.70 (3H, m, H-3, H-4 or H-6), 1.45 (2H, d, J = 12.4 Hz, H-4 or H-6); ^{13}C NMR (100 MHz, $CDCl_3$): 125.2 (C-1), 55.9 (C-2), 37.1 (C-7), 35.4 (C-3), 34.4 (C-4 or 6), 29.7 (C-4 or 6), 26.3 (C- C-5 or C-8), 26.0 (C- C-5 or C-8); IR (neat) cm^{-1}: 3379.0 (w), 2904.9 (s), 2858.7 (m), 2217.9 (w), 1719.3 (w), 1610.3 (br w), 1450.8 (m); HRMS: calcd for $[C_{11}H_{17}N_2]^+$ = 177.1392, found 177.1396, Δ = 2.3 ppm.

3.3. *N*-(2-Cyanoadamantan-2-yl)benzamide (15)[30]

To a solution of compound **14** (50.0 g, 0.284 mol) dissolved in THF (500 mL) was added a solution of K_2CO_3 (59.5 g, 0.43 mol) in H_2O (800 mL). To the resulting solution was added benzoyl chloride (48.1 mL, 0.415 mol) and the mixture stirred at ambient temperature. After 15 min a white precipitate began to form. After 1.5 h, the white solid was isolated by filtration, triturated with

EtOAc (250 mL), THF (300 mL), and the solid dried in vacuo to afford a white solid (64.3 g, 0.229 mol, 81%).

Mp: 191−192 °C; LC–MS: R_t = 4.57, $[M]^+$ = 253.88 (frag); ^{1}H NMR (400 MHz, $CDCl_3$): 8.57 (1H, s, NH), 7.85 (2H, d, J = 7.3 Hz, H-3′), 7.58 (1H, t, J = 7.3 Hz, H-5′), 7.50 (2H, t, J = 7.3 Hz, H-4′), 2.69 (2H, s, H-3), 2.01-2.10 (4H, m, H-4 and H-6), 1.88–1.91 (3H, m, H-4 or 6 and H-5 or 8), 1.80 (1H, s, H-5 or 8), 1.74 (2H, s, H-7), 1.64 (2H, d, J = 12.9 Hz, H-4 or 6); ^{13}C NMR (100 MHz, $CDCl_3$): 167.0 (C-1′), 134.1 (C-2′), 131.7 (C-5′), 128.3 (C-4′), 127.7 (C-3′), 120.8 (C-1), 56.8 (C-2), 36.5 (C-7), 33.6 (C-4 or 6), 32.3 (C-3), 30.6 (C-4 or 6), 25.8 (C-5 or 8), 25.6 (C-5 or 8); IR (neat) cm^{-1}: 3349.0 (m), 2909.1 (m), 2227.0 (w), 1663.0 (w), 1646.0 (s), 1512.1 (s), HRMS: calcd for $[C_{18}H_{21}N_2O]^+$ = 281.1654, found 281.1659, Δ = 1.8 ppm; Microanalysis: calcd (found) for $C_{18}H_{20}N_2O$; C = 77.11% (77.19%), H = 7.14% (7.14%), N = 9.99% (10.11%); The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 860426.

3.4. 2-Benzamidoadamantane-2-carboxylic acid (16)[30]

Compound **15** (20.0 g, 71.1 mmol) was suspended in a solution of concd hydrochloric acid (350 mL) and THF (45 mL) and stirred at ambient temperature for 18 h at which stage the reaction had reached completion as judged by LC−MS. The solution was slowly poured in to ice H_2O (500 mL) and left to stand for 1 h. The resultant solid was isolated by filtration and dried in vacuo overnight to give the product as a white solid (18.95 g, 63.3 mmol, 89% yield) in good enough purity for further synthesis. An analytical sample could be obtained by recrystallization from acetone.

Mp: 234−236 °C; LC–MS: R_t = 4.28, $[MNa]^+$ = 322.14; ^{1}H NMR (400 MHz, DMSO-d_6): 12.17 (1H, br s, COOH), 8.10 (1H, NH, s), 7.79 (2H, d, J = 7.2 Hz, H-3′), 7.52 (1H, t, J = 7.2 Hz, H-1′), 7.44 (2H, t, J = 7.2 Hz, H-2′), 2.63 (2H, s, H-7), 2.12 (4H, m, H-4 and H-6), 1.80 (2H, s, H-5 and H-8), 1.65–1.69 (4H, m, H-3 and H-4 or H-6), 1.56 (2H, d, J = 12.7 Hz, H-4 or H-6); ^{13}C NMR (100 MHz, DMSO-d_6): 173.8 (C-1), 166.4 (C-5′), 135.1(C-4′), 131.1 (C-1′), 128.1 (C-3′), 127.7 (C-2′), 63.1 (C-2), 37.5 (C-3), 33.5 (C-4 or 6), 32.8 (C-4 or C-6), 31.5 (C-7), 26.5 (C-5 or C-8), 26.4 (C-5 or C-8); IR (neat) cm^{-1}: 3389.7 (w), 2905.7 (m), 1740.0 (s), 1626.8 (m), 1526.3 (s); HRMS: calcd for $[C_{18}H_{21}NO_3Na]^+$ = 322.1419, found 322.1421, Δ = 1.8 ppm; The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 860427.

3.5. 2-Aminoadamantane-2-carboxylic acid hydrochloride (17)[30]

Compound **16** (30.0 g, 0.10 mol) was dissolved in conc. hydrochloric acid (50 mL), H_2O (150 mL) and acetic acid (350 mL) and heated at reflux for 3 days when the reaction was judged to be complete by ^{1}H NMR. The reaction mixture was concentrated in vacuo and the solid obtained was triturated with hot Et_2O (2 × 200 mL), hot MeCN (2 × 150 mL). The solid was dissolved in the minimum volume of MeOH (∼60 mL), added to Et_2O (500 mL) and left to crystallise at 0 °C overnight. The resulting solid was isolated by filtration to afford the title compound as a white crystalline solid (17.33 g, 74.7 mmol, 75% yield).

Mp: >258 °C dec; ^{1}H NMR (400 MHz, DMSO-d_6): 9.07 (1H, br s, COOH), 2.21 (2H, s, H-3), 2.12 (2H, d, J = 13.2 Hz, H-4 or H-6), 1.92 (2H, d, J = 12.4 Hz, H-4 or H-6), 1.73–1.80 (4H, m, H7 and H-4 or H-6), 1.63–1.67 (4H, m, H- H-4 or H-6); ^{13}C NMR (100 MHz, DMSO-d_6): 171.0 (C-1), 63.4 (C-2), 37.01 (C-3), 33.9 (C-4 or C-6), 31.7 (C-7), 30.7 (C-4 or C-6), 25.7 (C-5 or C-8), 25.7 (C-5 or C-8); IR (neat) cm^{-1}: 3220.3 (w), 3168.38 (w), 2775.9 (s), 1725.8 (s), 1587.8 (s), 1506.6 (s), 1468.5 (s); Microanalysis: calcd (found) for $C_{11}H_{18}NO_2Cl$; C = 57.02 (56.77%), H = 7.83 (7.70%), N = 6.04 (5.94%). HRMS: calcd for $[C_{11}H_{18}NO_2]^+$ = 196.1332, found 196.1327, Δ = 1.1 ppm; The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 860428.

3.6. *tert*-Butyl-2-aminoadamantane-2-carboxylate (18)[32]

Compound **17** (1.16 g, 5 mmol) was suspended in a solution of *t*-butyl acetate (15 mL) and tetrafluoroboric acid (2.5 mL, 48 wt% in H_2O) was added. The resultant suspension was stirred at 60 °C in a seal 20 mL Biotage microwave vial for 48 h during which time it slowly became homogeneous. The reaction mixture was then carefully poured into an ice cold solution of KOH (2.5 N, 100 mL) and extracted with CH_2Cl_2 (3 × 50 mL), dried ($MgSO_4$), and the solvent removed in vacuo to afford the title compound as a pale yellow solid (1.09 g, 4.3 mmol, 87%). An analytical sample could be obtained by recrystallization from CH_2Cl_2.

Mp: 102.5−104 °C; LC–MS: R_t = 3.44, $[M]^+$ = 252.36; ^{1}H NMR (500 MHz, DMSO-d_6): 3.32 (2H, s, NH_2), 2.24 (2H, d, J = 12.1 Hz, H-4), 1.91 (2H, s, H-3), 1.72 (2H, m, H-5/8), 1.67 (4H, m, H-6), 1.60 (2H, m, H-7), 1.40-1.42 (11H, m, H-4/H10); ^{13}C NMR (125 MHz, DMSO-d_6): 175.2 (C-1), 78.8 (C-9), 61.0 (C-2), 37.3 (C-7), 34.6 (C-6), 33.5 (C-3), 31.4 (C-4), 27.5 (C-10), 26.7 (C-5 or 8), 26.3 (C-5 or 8); ^{1}H NMR (400 MHz, $CDCl_3$): 2.22 (2H, d, J = 12.4 Hz), 2.03 (2H, s), 1.71–1.78 (6H, m), 1.66 (2H, m) 1.54 (2H, d, J = 12.4 Hz), 1.46 (9H, s), 1.43(2H, s); ^{13}C NMR (100 MHz, $CDCl_3$): 176.1, 80.1, 62.0, 37.9, 35.3, 34.3, 32.1, 28.1, 27.3, 27.0; IR (neat) cm^{-1}: 2901.1 (s), 2851.7 (m), 1710.3 (s), 1450.5 (m), 1365.3 (m); HRMS: calcd for $[C_{15}H_{26}O_2N]^+$ = 252.1964, found 252.1952, Δ = 4.8 ppm; Microanalysis: calcd (found) for $C_{15}H_{25}O_2N$; C = 71.67% (71.37%), H = 10.02% (10.05%), N = 5.57% (5.69%); The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 860425.

3.7. Ethyl 4-(2,6-dimethoxyphenyl)-4-hydroxy-2-oxobut-3-enoate (19)

Sodium (11.4 g, 496 mmol) was dissolved in EtOH (450 mL) using an ice bath to control the exotherm. To this solution, diethyl oxalate (45 mL, 332 mmol) was added in one portion, followed by a slurry of 2′,6′-dimethoxyacetophenone (30.0 g, 166 mmol) in EtOH (250 mL). The resulting suspension was allowed to stir at ambient temperature for 6 h then the solvent removed in vacuo. The mixture was extracted with EtOAc (8 × 350 mL) ensuring the pH remained below 6 between each extraction (addition of concd hydrochloric acid). The organic phases were combined, dried ($MgSO_4$), and the solvent removed in vacuo. Following recrystallisation from EtOH, the title compound was as a glassy crystalline solid (41.2 g, 147 mmol, 88% yield).

Mp: 102−104 °C; ^{1}H NMR (400 MHz, $CDCl_3$): 14.33 (1H, br s, OH), 7.34 (1H, t, J = 8.4 Hz, H-1), 6.60 (1H, s, H-6), 6.59 (1H, d, J = 8.4 Hz, H-2), 4.35 (2H, q, J = 7.1 Hz, H-1′), 3.82 (6H, s, H-3′), 1.37 (3H, t, J = 7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 195.8 (C-5), 164.0 (C-7), 162.6 (C-8), 158.0 (C-3), 132.4 (C-1), 117.2 (C-4), 106.4 (C-6), 104.3 (C-2), 62.5 (C-1′), 56.2 (C-3′), 14.2 (C-2′); LC–MS: R_t = 4.19, $[MNa]^+$ = 302.83; IR (neat) cm^{-1}: 1735.7 (m), 1625.1 (m), 1586.8 (s), 1475.3 (m), 1257.5 (s); HRMS: calcd for $[C_{14}H_{16}O_6Na]^+$ = 303.0845, found 303.0847, Δ = 0.7 ppm; Microanalysis: calcd (found) for $C_{14}H_{16}O_6$; C = 59.99% (59.99%), H = 5.75% (5.72%), N = 0.00% (0.00%); The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 913971.

3.8. Ethyl 1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carboxylate (20)

Procedure 1: To a solution of compound **19** (14.4 g, 51.4 mmol) dissolved in EtOH (900 mL) was added hydrazine **7** (10.0 g, 51.6 mmol) in one portion. To this solution, was added 98% sulfuric acid (2.8 mL, 52 mmol) and the resultant solution heated under reflux for 4 h. Following this, the solvent was removed in vacuo and the residue dissolved in CH_2Cl_2 (500 mL), washed with 10% NaOH (2 × 100 mL), dried (Na_2SO_4) and concentrated in vacuo. The crude product was redissolved in the minimum volume of warm MeOH and precipitated with Et_2O. This was repeated 3 times, affording the product as a pale yellow solid (12.76 g, 29.1 mmol, 56% yield).

Procedure 2: Compound **19** (280 mg, 0.10 mmol), **7** (193 mg, 0.1 mmol), and 98% sulfuric acid (0.05 mL) were added to a 20 mL Biotage microwave vial containing EtOH (12 mL) and stirred at ambient temperature for 1 h. The resulting suspension was heated under microwave irradiation at 100 °C for 2 h. The solution was concentrated to dryness in vacuo. The crude product was purified by column chromatography on silica affording the product as a pale yellow solid (376 mg, 86% yield). The reaction was run in series using the automated robot on the microwave conducting 48 sequential reactions (6 entire cycles using the 8 place reactor base EZ8) giving an aggregated combined yield of 17.4 g (83%). An analytical sample could be obtained by recrystallization utilizing slow evaporation from a CH_2Cl_2/MeOH solution.

Mp: 147–149 °C; ^{1}H NMR (600 MHz, DMSO-d_6): 8.91 (1H, d, J = 4.5 Hz, H-9), 8.17 (1H, d, J = 1.8 Hz, H-16), 7.72 (1H, dd, J = 9.0, 1.8 Hz, H-14), 7.68 (1H, d, J = 9.0 Hz, H-13), 7.26 (1H, t, J = 8.4 Hz, H-1), 7.23 (1H, d, J = 4.5 Hz, H-10), 7.04 (1H, s, H-6), 6.54 (2H, d, J = 8.4 Hz, H-2), 4.35 (2H, q, J = 7.1 Hz, H-1′), 3.40 (6H, s, H-3′), 1.33 (3H, t, J = 7.1 Hz, H-2′); ^{13}C NMR (100 MHz, DMSO-d_6): 161.4 (C-8), 157.5 (C-3), 151.7 (C-9), 149.1 (C-17), 144.5 (C-7), 143.3 (C-15), 139.2 (C-5), 134.8 (C-11), 132.0 (C-1), 128.1 (C-14), 127.7 (C-16), 125.7 (C-13), 122.1 (C-12), 118.3 (C-10), 111.6 (C-6), 105.4 (C-4), 104.0 (C-2), 60.6 (C-1′), 55.4 (C-3′), 14.2 (C-2′); LC–MS: R_t = 4.58, $[MH]^+$ = 437.93; IR (neat) cm^{-1}: 1719.4 (m), 1590.0 (m), 1476.6 (s), 1434.8 (m), 1232.7 (s); HRMS: calcd for $[C_{23}H_{21}N_3O_4Cl]^+$ = 438.1221, found 438.1219, Δ = 0.5 ppm. Microanalysis: calcd (found) for $C_{23}H_{20}N_3O_4Cl$; C = 63.09% (62.80%), H = 4.60% (4.63%), N = 9.60% (9.58%). The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 913972.

3.9. 1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carboxylic acid (9)

To a suspension of the pyrazole ethyl ester 20 (10.0 g, 22.8 mmol) in THF (50 mL) was added NaOH (1.10 g, 27.5 mmol) and H_2O (5 mL) and the mixture was stirred overnight. The reaction mixture was concentrated in vacuo and the residue portioned between CH_2Cl_2 (220 mL) and H_2O (100 mL). The aqueous phase was neutralised by addition of conc. hydrochloric acid and extracted with CH_2Cl_2 (3 × 150 mL), dried ($MgSO_4$) and concentrated in vacuo to give a yellow solid (9.34 g, 22.7 mmol, 94% yield). An analytical sample could be obtained by recrystallisation utilising slow evaporation from a CH_2Cl_2/MeOH solution.

^{1}H NMR (400 MHz, DMSO-d_6): 13.08 (1H, s, COOH), 8.89 (1H, d, J = 4.6 Hz, H-9), 8.16 (1H, d, J = 1.9 Hz, H-16), 7.73 (1H, d, J = 9.0 Hz, H-13), 7.70 (1H, dd, J = 9.0, 1.9 Hz, H-14), 7.26 (1H, t, J = 8.4 Hz, H-1), 7.20 (1H, d, J = 4.6 Hz, H-10), 6.99 (1H, s, H-6), 6.54 (2H, d, J = 8.5 Hz, H-2), 3.39 (6H, s, H-3′); ^{13}C NMR (100 MHz, DMSO-d_6): 162.9 (C-8), 157.5 (C-3), 151.6 (C-9), 149.1 (C-17), 145.4 (C-7), 143.3 (C-15), 138.9 (C-5), 134.7 (C-11), 131.8 (C-1), 128.0 (C-14), 127.7 (C-16), 125.9 (C-13), 122.1 (C-12), 118.1 (C-10), 111.7 (C-6), 105.6 (C-4), 104.0 (C-2), 55.3 (C-3′); LC–MS: R_t = 4.35, $[MH]^+$ = 410.25; IR (neat) cm^{-1}: 1687.7 (m), 1575.2 (s), 1479.2 (s), 1456.9 (m), 1434.7 (s); HRMS: calcd for $[C_{21}H_{17}N_3O_4Cl]^+$ = 410.0908, found 410.0921, Δ = 3.2 ppm. Microanalysis: calcd (found) for $C_{21}H_{16}N_3O_4Cl$; C = 61.54% (61.30%), H = 3.94% (4.00%), N = 10.25% (10.17%), Cl = 8.65% (8.58%). The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 913973.

3.10. 1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carbonyl chloride

Pyrazole acid **9** (445 mg, 1.1 mmol) was dissolved in thionyl chloride (5 mL) and the resultant solution stirred at ambient temperature for 3 h. The excess thionyl chloride was removed in vacuo and the residue further azetroped with toluene (15 mL) and used directly in the subsequent amide formation.

^{1}H NMR (600 MHz, $CDCl_3$)*: 9.23 (1H, d, J = 5.7 Hz, H-9), 8.92 (1H, d, J = 1.7 Hz, H-16), 8.34 (1H, d, J = 9.2 Hz, H-13), 7.88 (1H, dd, J = 9.2, 1.7 Hz, H-14), 7.59 (1H, d, J = 5.7 Hz, H-10), 7.37 (1H, t, J = 8.5 Hz, H-1), 7.30 (1H, s, H-6), 6.53 (2H, d, J = 8.5 Hz, H-2), 3.52 (1H, s, H-3′); ^{13}C NMR (150 MHz, $CDCl_3$): 161.8 (C-8), 157.3 (C-3), 150.6 (C-9), 149.0 (C-17), 144.6 (C-7), 142.0 (C-15), 141.2 (C-5), 140.3 (C-11), 133.1 (C-1), 131.9 (C-14), 127.2 (C-16), 122.6 (C-13), 121.0 (C-10), 117.3 (C-6), 114.3 (C-4), 104.1 (C-2), 55.6 (C-3′); LC–MS: R_t = 5.00, $[MH]^+$ = 428.06 (dec. observed); HRMS: calcd for $[C_{21}H_{16}N_3O_3Cl_2]^+$ 428.0569, found 428.0571, Δ = 0.5 ppm; IR (neat) cm^{-1}: 1758.7 (s), 1633.9 (m), 1593.8 (s), 1475.8 (m), 1444.1 (m).

*Standardised against CH_3 peak (δ = 2.36 ppm) of toluene impurity due to overlap of reference peaks in aromatic region.

3.11. *tert*-Butyl 2-(1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carboxamido)adamantane-2-carboxylate (21)

Procedure 1: To a solution of the acid chloride prepared using the above procedure in CH_2Cl_2 (15 mL) was added triethylamine (0.3 mL, 2.2 mmol) followed by a solution of the protected amine **18** (300 mg, 1.1 mmol) in CH_2Cl_2 (10 mL) and the resultant solution was stirred at ambient temperature overnight. The mixture was concentrated in vacuo and purified directly by column chromatography (Eluent CH_2Cl_2 with 2% MeOH) to afford a mixture of the desired compound and starting amine **18**. The combined evaporated fractions were redissolved in CH_2Cl_2 (10 mL) and passed through a SCX-II cartridge, washing with CH_2Cl_2 (2 × 10 mL). Concentration of the resultant solution gave the title compound as a light orange foam (413 mg, 0.64 mmol, 58% yield).

Procedure 2: To a solution of **9** (2.00 g, 4.89 mmol), DIPEA (1.8 mL, 10.7 mmol) and PyBrOP (2.50 g, 5.38 mmol) in CH_2Cl_2 (150 mL) and DMF (2.5 mL) was added amine **18** (1.34 g. 5.38 mmol). The resulting mixture was stirred at ambient temperature overnight. The reaction mixture was transferred to a separating funnel and washed with satd aq LiCl (2 × 100 mL), 10% aq NaOH (100 mL), passed through a SCX-II cartridge, dried ($MgSO_4$) and concentrated in vacuo. The crude product could be purified by column chromatography (Eluent 10% MeOH/CH_2Cl_2) to afford the title compound as a white solid (2.79 g, 4.34 mmol, 89% yield). An analytical sample could be obtained by recrystallization by slow evaporation from CH_2Cl_2.

Mp: 219–222 °C; LC–MS: R_t = 5.86, $[MH]^+$ = 643.42; ^{1}H NMR (400 MHz, $CDCl_3$): 8.76 (1H, d, J = 4.6 Hz, H-9), 8.13 (1H, d, J = 2.0 Hz, H-16), 7.95 (1H, d, J = 9.0 Hz, H-13), 7.48 (1H, dd, J = 2.0, 9.0 Hz, H-14), 7.21 (1H, t, J = 8.0 Hz, H-1), 7.10 (1H, br s, C-2″NH), 7.07 (1H, s, H-6), 7.04 (1H, d, J = 4.6 Hz, H-10), 6.39 (2H, d, J = 8.0 Hz, H-2) 3.39 (6H, s, H-3′) 2.66 (2H, s, H-3″), 2.14 (2H, d,

J = 12.9 Hz, H-4″ or H-6″) 2.05 (2H, d, J = 12.9 Hz, H-4″ or H-6″), 1.65-1.87 (8H, m, H-5″/8″/7″/4″/6″), 1.51 (9H, s, H-10″); ^{13}C NMR (100 MHz, $CDCl_3$): 171.6 (C-1″), 160.6 (C-8), 158.0 (C-3), 151.0 (C-9), 150.2 (C-17), 148.6 (C-7), 144.5 (C-15), 139.4 (C-5), 136.0 (C-11), 131.6 (C-1), 128.5 (C-16), 128.1 (C-14), 126.4 (C-13), 122.9 (C-12), 117.5 (C-10), 110.7 (C-6), 106.9 (C-4), 103.8 (C-2), 80.9 (C-9″), 64.1 (C-2″), 55.4 (C-3′), 38.1 (C-7″), 34.2 (C-4″ or C-6″), 33.4 (C-4″ or C-6″), 33.0 (C-3″), 28.2 (C-10″), 27.2 (C-5″ or C-8″), 26.9 (C-5″ or C-8″); IR (neat) cm^{-1}: 3413.7 (w), 2908.8 (w), 1743.8 (w), 1731.9 (m), 1706.2 (w), 1685.8 (m), 1532.4 (m), 1100.9 (s); HRMS: calcd for $[C_{36}H_{40}N_4O_5Cl]^+$ = 643.2687, found 643.2711, Δ = 3.7 ppm. The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 913974.

3.12. 2-(1-(7-Chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1*H*-pyrazole-3-carboxamido)adamantane-2-carboxylic acid (2)

Compound **21** (2.1 g, 3.3 mmol) was dissolved in a mixture of CH_2Cl_2 (25 mL) and TFA (10 mL) and monitored by TLC. After 3.5 h the reaction was complete, the reaction mixture was concentrated in vacuo and azeotroped with toluene (50 mL) to afford the title compound as a tan coloured oil. After one round of recrystallization from *i*-PrOH, the title compound could be isolated as the *iso*-propyl solvate as a white crystalline solid (1.43 g, 64% yield), a second crop could be attained after evaporation of ~1/2 the *iso*-propanol solvent (406 mg, 19% yield, combined yield 83%).

3.12.1. Merclineritant data

^{1}H NMR (500 MHz, DMSO-d_6): 12.39 (1H, br s, C-1″ COOH), 8.91 (1H, d, J = 4.3 Hz, H-9), 8.15 (1H, d, J = 1.1 Hz, H-16), 7.78 (1H, d, J = 8.9 Hz, H-13), 7.68 (1H, dd, J = 1.1, 8.9 Hz, H-14), 7.54 (1H, br s, C-2″NH), 7.28 (1H, d, J = 4.3 Hz, H-10), 7.24 (1H, t, J = 8.4 Hz, H-1), 6.92 (1H, s, H-6), 6.53 (2H, d, J = 8.4 Hz, H-2), 3.42 (6H, s, H-3′), 2.59 (2H, s, H-3″), 2.10 (2H, m, H-4″/6″), 1.99 (2H, m, H-4″/6″), 1.77 (2H, m, H-5/8), 1.60-1.70 (4H, m, H-7″/4″/6″), 1.58 (2H, m, H-4″/6″); LC–MS: R_t = 5.09, $[MH]^+$ = 587.38; IR (neat) cm^{-1}: 3405.7 (w), 2910.8 (m), 2860.2 (w), 1728.5 (m), 1666.9 (s), 1592.5 (m); HRMS: calcd for $[C_{32}H_{32}N_4O_5Cl]^+$ = 587.2061, found 587.2065, Δ = 0.7 ppm.

3.12.2. Data as the *iso*-propyl solvate

^{1}H NMR (500 MHz, DMSO-d_6): 12.37 (1H, br s, C-1″ COOH), 8.92 (1H, d, J = 4.6 Hz, H-9), 8.15 (1H, d, J = 2.1 Hz, H-16), 7.78 (1H, d, J = 9.1 Hz, H-13), 7.68 (1H, dd, J = 2.1, 9.1 Hz, H-14), 7.59 (1 h, br s, C-2″NH), 7.29 (1H, d, J = 4.6 Hz, H-10), 7.24 (1H, t, J = 8.5 Hz, H-1), 6.93 (1H, s, H-6), 6.53 (2H, d, J = 8.5 Hz, H-2), 4.34 (1H, d, J = 4.1 Hz, i-PrOH), 3.77 (1H, m, *i*-PrOH CH), 3.42 (6H, s, H-3′), 2.59 (2H, s, H-3″), 2.10 (2H, d, J = 12.0 Hz, H-4″/6″), 1.99 (2H, d, J = 12.5 Hz, H-4″/6″), 1.77 (2H, m, H5″/8″), 1.60-1.70 (4H, m, H-7″/4″/6″), 1.59 (2H, d, J = 12.5 Hz, H-4″/6″), 1.04 (6H, d, J = 6.1 Hz, *i*-PrOH CH_3). ^{13}C NMR (125 MHz, DMSO-d_6): 173.3 (C-1″), 160.1 (C-8), 157.5 (C-3), 151.8 (C-9), 149.1 (C-17), 148.0 (C-7), 143.4 (C-11), 139.2 (C-5), 134.7 (C-15), 131.9 (C-1), 128.0 (C-14), 127.7 (C-16), 125.9 (C-13), 122.2 (C-12), 118.5 (C-10), 109.7 (C-6), 105.7 (C-4), 104.0 (C-2), 62.7 (C-2″), 62.0 ($HOCH(CH_3)_2$), 55.4 (C-3′), 37.3 (C-7″), 33.4 (C-4″/6″), 32.6 (C-4″/6″), 31.7 (C-3″), 26.4 (C-5″/8″), 26.1 (C-5″/8″), 25.5 ($HOCH(CH_3)_2$); IR (neat) cm^{-1}: 3403.3 (w), 2924.7 (m), 2860.6 (w), 1724.2 (m), 1673.9 (s), 1591.1 (m); HRMS: calcd for $[C_{32}H_{32}N_4O_5Cl]^+$ = 587.2061, found 587.2084, Δ = 3.9 ppm. The structure was unambiguously confirmed by X-ray crystallography and the structure deposited at the CCDC with the unique reference 913975.

3.13. Biology

3.13.1. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich. Casodex® (Bicalutamide) was purchased from Toronto Research Chemicals Inc., SR142948 was purchased from Tocris biosciences, LNCaP cells were available from CRUK cell line bank and the American Type Culture Collection (ATTC)®. LNCaP C4-2 and C4-2b were a kind gift from Dr. D. Prowse, Centre for Cutaneous Research, Barts and The London, Queen Mary's School of Medicine and Dentistry. LNCaP-bicalutamide resistant (LNCaP-Bic) cells were a kind donation from Professor Z. Culig, Department of Urology, University of Innsbruck.

3.13.2. Cell culture

LNCaP cells were maintained in RPMI-1640 with L-Glutamine media (Invitrogen™) supplemented with 10% FBS (Hyclone). Frozen cells were thawed in a 37 °C water bath, resuspended in pre-warmed (37 °C) media and centrifuged (1300*g*, 3 min, 21 °C). The supernatant was discarded and the pellet was resuspended thoroughly before seeding into culture flasks. Cells were routinely sub-cultured 1:2 or 1:3 2–3 times a week using .05% Trypsin–EDTA (Invitrogen™). Cell counts were conducted using a haemocytometer.

3.13.3. CellTiter 96® Aqueous One Solution cell proliferation assay

Cells were seeded at a density of 3000 cells/100 μL/well. 24 h after seeding, the media was aspirated and replaced with the relevant treatments. All experiments were produced in quadruplicate. Drug concentrations were made from serial dilutions in media, from a stock solution of the drug dissolved in an organic solvent or water vehicle. Controls were set as the highest vehicle concentration used, typically 0.1% solvent. At the relevant time points, CellTiter 96® Aqueous One Solution reagent (Promega) (20 μL) was added directly to cultured wells with minimal exposure to light. Plates were incubated for 1–2 h (37 °C, 5% CO_2) and the formazan absorption was measured at 492 nm using an anthoslucy2 spectrometer. The mean absorbance of drug-treated wells was displayed as optical density or expressed as a percentage of control to estimate the proliferation status:

$$\begin{aligned}\text{Percentage of control}(\%) = {} & (\text{Absorbance treatment group} \\ & - \text{Absorbance blank}) \\ & \times (\text{Absorbance control} \\ & - \text{Absorbance blank}) \times 100\%.\end{aligned}$$

$$\text{Absorbance treatment group}: \ \text{HUVECs} + \text{medium} + \text{Drug} + \text{MTS reagent}.$$

$$\text{Absorbance blank}: \ \text{medium} + \text{MTS reagent}.$$

$$\text{Absorbance control}: \ \text{HUVECs} + \text{medium} + \text{MTS reagent}.$$

Values were expressed as the mean ± the standard deviation (SD). Where necessary, one obvious outlying result was removed.

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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.bmc.2013.04.075.

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