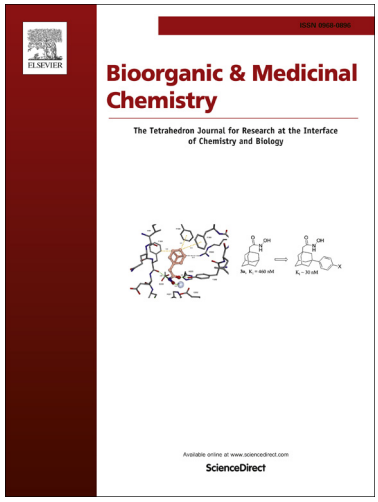

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Synthesis of a novel legumain-cleavable colchicine prodrug with cell-specific toxicity

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Running title: A novel legumain-cleavable colchicine prodrug

Abbreviations: HEK293 cells, human embryonic kidney 293 cells; M38L, monoclonal legumain over-expressing HEK293 cells; M4C, monoclonal cystatin E/M over-expressing HEK293 cells

ABSTRACT

Conventional chemotherapy has undesirable toxic side-effects to healthy tissues due to low cell selectivity of cytotoxic drugs. One approach to increase the specificity of a cytotoxic drug is to make a less toxic prodrug which becomes activated at the tumour site. The cysteine protease legumain have remarkable restricted substrate specificity and is the only known mammalian asparaginyl (Asn) endopeptidase. Over-expression of legumain is reported in cancers and unstable atherosclerotic plaques, and utilizing legumain is a promising approach to activate prodrugs.

In this study we have synthesized the legumain-cleavable peptide sequence *N*-Boc-Ala-Ala-Asn-Val-OH. The peptide was subsequently conjugated to deacetyl colchicine during three steps to produce Suc-Ala-Ala-Asn-Val-colchicine (prodrug) with > 90 % chemical purity. Several cell lines with different expressions and activities of legumain were used to evaluate the general toxicity, specificity and efficacy of the microtubule inhibitor colchicine, valyl colchicine and the legumain-cleavable colchicine prodrug. The prodrug was more toxic to the colorectal cancer HCT116 cells (expressing both the 36 kDa active and 56 kDa proform of legumain) than SW620 cells (only expressing the 56 kDa prolegumain) indicating a relationship between toxicity of the prodrug and activity of legumain in the cells. Also, in monoclonal legumain over-expressing HEK293 cells the prodrug toxicity was higher compared to native HEK293 cells. Furthermore, co-administration of the prodrug either with the potent legumain inhibitor cystatin E/M or the endocytosis inhibitor Dyngo-4a inhibited cell death, indicating that the prodrug toxicity was dependent on both asparaginyl endopeptidase activity and endocytosis. This colchicine prodrug adds to a legumain-activated prodrug strategy approach and could possibly be of use both in targeted anticancer and anti-inflammatory therapy.

1. INTRODUCTION

A major problem with conventional cancer chemotherapy is systemic side-effects due to low specificity towards malignant cells. Thus, it is desirable to develop cytotoxic drugs which could target tumours resulting in fewer side-effects to healthy tissues while retaining toxicity towards the tumour. One approach in developing such cytotoxic drugs is to make a non-toxic prodrug which is activated and becomes toxic at the tumour site. The validity of such an approach has already been shown by exploiting the activity of the cysteine protease legumain which is highly expressed in tumours.[1]

Legumain (asparaginyl endopeptidase) is well conserved throughout evolution and was initially identified in plants.[2] Later, legumain has been identified in parasites and mammals,[3, 4] being the only mammalian member of the C13 family of cysteine proteases and showing a remarkable restricted substrate specificity requiring asparagine (Asn) at the P1 position.[4] Also, legumain is reported to cleave C-terminally of aspartate (Asp) residues at pH below 5.[5] Although ubiquitously expressed in mammalian tissues,[4] legumain is over-expressed in the majority of human solid tumours and in unstable atherosclerotic plaques.[1, 6-8] In tumours, legumain is found both in the malignant cells, endothelial and stromal cells surrounding tumours, and in tumour associated macrophages (TAMs).[1, 9, 10] Also, legumain is suggested to promote tumour progression by participating in processing of cathepsin B, H and L,[11] activating proMMP-2[12] and directly degrading fibronectin.[13] Presence of legumain in colorectal cancer is correlated with a more aggressive tumour type with a poorer prognosis.[14] Legumain is expressed as a 56 kDa proform which is auto-activated at low pH to 47/46 kDa intermediate forms.[15] The intermediate forms are further enzymatically processed by other cysteine proteases to the mature active legumain of 36 kDa.[15] Both the intermediate form (46 kDa) and the mature form (36 kDa) of legumain have enzymatic activity that is inhibited by the endogenous type 2 cystatins such as cystatin C, E/M and F.[16] Prolegumain has been

reported to be secreted to the tumour microenvironments as well as being associated with the $\alpha v\beta 3$ integrin receptor at the cell surface.[1, 15] The secreted prolegumain has also been shown to be internalized and subsequently auto-activated intracellularly.[17] So far, convincing results have been reported for legumain-cleavable prodrugs based on the legumain-specific tripeptide sequence Ala-Ala-Asn linked to doxorubicin, etoposide or auristatin, and cell specific toxicity of these prodrugs has been observed both *in vitro* and *in vivo.* [1, 18-21]

In the present study we have synthesised a legumain-cleavable prodrug as a conjugate of the novel constructs *N*-Boc-Ala-Ala-Asn-Val-OH and deacetyl colchicine. Colchicine is a cytotoxic tricyclic alkaloid extracted from *Colchicum automnale* and binds to the microtubuli resulting in mitotic cell arrest, indicating a potential use in the treatment of cancer. Colchicine is used therapeutically in inflammatory diseases like gout and familial Mediterranean fever due to its anti-inflammatory properties by inhibiting the functions of neutrophils.[22] In atherosclerosis, neutrophils can enter the interstitial space of the atherosclerotic wall and become activated resulting in an aggressive inflammatory response and plaque instability. Recently, a clinical trial showed that 0.5 mg/day colchicine had promising results in prevention of cardiovascular disease.[23] The colchicine prodrug presented herein could have beneficial effects in the prevention of cardiovascular disease by targeting over-expressed legumain in atherosclerotic unstable plaques[6-8] as well as in tumours. The prodrug approach presented in our study could thus reduce the general toxicity and increase specificity of colchicine, thus extend its use against inflammation and be a possible candidate for chemotherapeutic eradication of tumours.

2. RESULTS AND DISCUSSION

2.1. Synthesis of a colchicine prodrug and valyl colchicine

The tripeptide Ala-Ala-Asn has previously been used to construct legumain-activated prodrugs, and was chosen as our core peptide vector.[19, 21] In addition, the lipophilic amino acid valine (Val) was used as a linker between the tripeptide and colchicine, and also served to increase the lipophilicity of the released compound after legumain cleavage (Fig. 1).

Thus, the tetrapeptide *N*-Boc-Ala-Ala-Asn-Val-OH was synthesized in 18 % overall yield using solution phase peptide coupling chemistry without further optimisation. Deacetyl colchicine was prepared from colchicine according to literature.[24] The prodrug was synthesized in three steps by attaching the peptide to deacetyl colchicine employing COMU® and Oxyma® in dimethylformamide (DMF), followed by *N*-Boc deprotection with trifluoroacetic acid (TFA) in dichloromethane, and subsequent reaction with succinic anhydride (Scheme 1). The crude prodrug was purified by preparative HPLC, resulting in a 4 % overall yield with > 90 % purity (Supplementary chromatogram 1). The reactions have not been subjected to optimization and the overall yield could possibly be improved by e.g. changing the amount of base in the last condensation with succinic anhydride.

Valyl colchicine was synthesized in 20 % overall yield from deacetyl colchicine and *N*-Boc-Val-OH, utilizing COMU® and Oxyma® in dimethylformamide. The *N*-Boc group was deprotected using trifluoroacetic acid in dichloromethane and precipitated as a yellow salt after treatment with HCl in Et_2O (Scheme 2). All NMR spectra and HPLC chromatograms are given in the supplementary material.

2.2. *In vitro* cleavage of the colchicine prodrug with legumain

Immediate cleavage of the prodrug was observed *in vitro* upon addition of purified bovine legumain, releasing valyl colchicine (Fig. 1). The Ala-Ala-Asn-Val peptide sequence was

cleaved C-terminally to Asn by legumain resulting in colchicine linked to valine (valyl colchicine), a substance not previously described. Valyl colchicine was identified as one of the cleavage products of prodrug by co-injection on HPLC, thus confirming cleavage of the prodrug by legumain to release the active compound and supporting the anticipated structure of the prodrug (see supplementary materials for all chromatograms and data). After 3 hours incubation with legumain, approximately 10 % of the prodrug was cleaved. Surprisingly, only 20 % was cleaved during a prolonged incubation (24 hours). This might suggest inhibition of legumain by the cleavage product as the hydrolysis seemed to slow down. Based on its lipophilicity and the published results of other legumain prodrugs,[1, 19, 21] valyl colchicine was anticipated to be the active cytotoxic agent. Being less lipophilic than the natural compound colchicine, valyl colchicine may be a more convenient compound for therapeutic use because of the disadvantage related to drug formulation of colchicine.[25]

2.3. Legumain over-expressing cells are sensitive to the colchicine prodrug

Native HEK293, M38L (over-expressing legumain) and M4C (over-expressing cystatin E/M) cells showed different levels of legumain expression (Fig. 2A). M38L cells expressed large amounts of both the 56 kDa proform, the 47/46 intermediate forms and the 36 kDa mature form, whereas HEK293 and M4C cells expressed low levels of the pro- and mature forms. Also, predominant detection of prolegumain was observed in the conditioned media from M38L. Prolegumain was also observed in conditioned media from HEK293 and M4C cells but to a much lesser extent than M38L. No immunobands for the legumain inhibitor cystatin E/M was detected either in cell lysates or in conditioned media from HEK293 or M38L cells, whereas M4C cells showed two immunobands of cystatin E/M (14 and 17 kDa) both in lysate and medium (Fig. 2A).

Cell viabilities of native HEK293 and M38L cells were studied after individual treatment with 0.1-100 µM colchicine, valyl colchicine and prodrug. No differences in cell viability were observed between HEK293 and M38L cells after treatment with colchicine or valyl colchicine (Fig. 2B and C, respectively). However, the toxic concentration was 10-fold lower for colchicine than valyl colchicine (EC_{50}-values of 0.03 and 0.3 µM, respectively). The prodrug had the lowest toxicity of the three substances with an EC_{50}-value of 2.7 µM in both cell lines (Fig. 2D). Although these cell lines responded similar to high concentrations of the prodrug, a statistically significantly difference in viability was observed between HEK293 and M38L cells at concentrations lower than 1 µM, reflecting the successful strategy of making a prodrug less toxic than the mother compound. The EC_{25}-values of the prodrug in causing cell death were 1.4 and 0.2 µM in HEK293 and M38L cells, respectively, meaning a 7-fold increased toxicity towards legumain over-expressing cells. The HEK293 and M38L cells responded in the same manner without the presence of serum in the culturing media, however, higher concentrations of the prodrug were needed to reduce viability (data not shown). This could probably be explained by the presence of secretory type 2 cystatins such as cystatin C, E/M and F in serum.[26]

2.4. The colorectal cell line HCT116 was more sensitive to the colchicine prodrug than SW620

Two colorectal cancer cell lines (HCT116 and SW620) with different expression and activity of legumain were investigated for the toxic effect of prodrug treatment. In HCT116 and SW620 cells legumain has recently been shown to be located vesicularly, although surprisingly, HCT116 also showed legumain in the nucleus.[27] HCT116 expressed both the 36 kDa mature legumain and the 56 kDa prolegumain whereas SW620 only expressed prolegumain (Fig. 3A). Both cell lines secreted prolegumain to the conditioned media

whereas only HCT116 cells expressed and secreted cystatin E/M. Also, the HCT116 lysates showed approximately 25 % higher legumain activity than the SW620 lysates (data not shown). Treatment of these cell lines with 10 µM or more prodrug showed reduced cell viability for HCT116 cells at lower concentrations than was observed for SW620 cells (Fig. 3B), thus confirming cell specific toxicity towards cancer cells expressing high levels of legumain in spite of the presence of cystatin E/M.

2.5. Cell toxicity of the colchicine prodrug was partly prevented by cystatin E/M or Dyngo4a

It was of interest to study whether inhibition of legumain could prevent the toxicity of the prodrug. M4C cells (over-expressing cystatin E/M) showed reduced toxicity of the prodrug compared to native HEK293 cells (Fig. 4A). Furthermore, addition of cystatin E/M to the cell culture medium also reduced the toxicity of the prodrug towards native HEK293 and M38L cells by approximately 24 and 33 %, respectively (Fig. 4B). Cystatin E/M is mainly a secretory protein, although we have recently reported that cystatin E/M is able to be internalized and could subsequently inhibit intracellular legumain activity.[17, 28, 29] The reduced toxic effects observed in presence of cystatin E/M indicate extracellular inhibition of legumain, and thus extracellular prodrug cleavage. This is controversial since legumain is stable and active only in low pH, while the extracellular pH is approximately neutral. Legumain have a RGD-motif and interacts with the $\alpha v\beta 3$ integrin receptor located at the plasma membrane.[1, 4] Interestingly, upon legumain binding to the receptor, legumain is stabilized and the pH optimum of activity has been shown to be shifted from pH 5.5 to 6,[30] thus indicating potential legumain activity in slightly less acidic environment. Thus, it was of interest to further study whether the prodrug cleavage appeared intracellularly or extracellularly. Other studies have reported extracellular cleavage of legumain-based prodrugs

due to low pH in the extracellular tumour microenvironment or by legumain interacting with up-regulated $\alpha v\beta 3$ integrin receptor on the plasma membrane of tumours.[1, 20] Lack of selectivity and toxicity of the legumain-based prodrug of etoposide due to impaired uptake and activation by legumain in the endosomal/lysosomal compartment has also been reported.[18] In our study, the dynamin inhibitor Dyngo-4a was introduced and used to inhibit endocytosis. A prevention of colchicine prodrug toxicity was observed both for HEK293 and M38L cells treated by Dyngo4a (Fig. 4C) and in a dose-dependent manner. Dyngo4a (50 µM) prevented prodrug toxicity by approximately 30 % and 22 % in HEK293 and M38L cells, respectively, and showed that cell viability at least partly was increased by inhibiting endocytosis. Although this indicates intracellular uptake and cleavage of the prodrug in the endo/lysosmal compartments, extracellular cleavage also seemed to occur since less cytotoxic effects of the prodrug was observed when applied together with cystatin E/M.

3. CONCLUSION

Many chemotherapeutic agents have undesirable cytotoxicity due to a general toxicity towards healthy tissues. One strategy to increase the specificity of chemotherapeutic agents is to target proteins that are highly expressed. Another approach is to exploit a specific enhanced enzyme activity and utilize the enzyme to activate prodrugs.

Legumain is a protease highly expressed in tumours, inflammation and atherosclerotic plaques. In this study a legumain-cleavable prodrug of colchicine has been synthesised and evaluated. The prodrug was more toxic to cells over-expressing active legumain (M38L, HCT116) compared to cells expressing low levels of active or only prolegumain (HEK293, M4C and SW620). Thus, this prodrug adds to a legumain-activated prodrug strategy approach and could be used in a novel targeting therapy.

4. MATERIALS AND METHODS

4.1. Materials

Dulbecco's modified Eagle's medium (DMEM-Glutamax™, 5.5 mM glucose), penicillin–streptomycin (P/S), amphotericin B, XCell SureLock® Mini, NOVEX Tris-Glycine Native Sample Buffer (2X), NOVEX Tris-Glycine Native Running buffer (10X), NuPAGE Bis-Tris 4–12 % gels, NuPAGE MOPS SDS running buffer (20X) and NuPAGE LDS sample buffer (4X) were obtained from Life Technologies (Paisley, UK). Culture plates (96-wells) and 25 cm² flasks were obtained from Corning Life-Sciences (Schiphol-Rijk, The Netherlands). CHAPS, DL-dithiotreitol (DTT) and tryptan blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium pyruvate solution, foetal calf serum (FCS) and trypsin-EDTA were purchased from PAA Laboratories GmbH (Pasching, Austria). Nitrocellulose membranes were from Hybond ECL (Amersham Biosciences, Boston, MA, US). SuperSignal West Dura Extended Duration Substrate and Restore Western Blot Stripping Buffer were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Protein assay reagent, Tween 20, SDS, Precision plus protein standards and goat anti-rabbit IgG HRP-conjugate (cat. no. 170–6515) were purchased from BioRad (Copenhagen, Denmark). Goat anti-human legumain (cat. no. AF2199) and cystatin E/M (cat. no. AF1286) were purchased from R&D Systems (Abingdon, UK). Mouse anti-human GAPDH (cat. no. sc-47724) was from Santa Cruz (Texas, USA). Rabbit anti-goat IgG HRP-conjugate (cat. no. P0160) was purchased from DAKO (Glostrup, Denmark). Z-Ala-Ala-Asn-AMC was purchased from Bachem (Bubendorf, Switzerland). Non-fat dry milk was from Normilk (Levanger, Norway). CellTiter 96® aqueous one solution cell proliferation assay (MTS assay) was obtained from Promega (Madison, Wisconsin, USA). HEK293, SW620 and HCT116 cells were purchased from American Type Culture Collection (ATCC). Stably transfected monoclonal legumain over-expressing (M38L) and cystatin E/M over-expressing (M4C) HEK293 cells, were obtained as

previously described.[17] Colchicine was purchased from TCI (Tokyo, Japan) and all other chemicals and solvents used for synthesis were obtained from Sigma Aldrich (St. Louis, MO, USA). All other reagents and solvents used are of commercial grade and were used without further purifications prior to use.

4.2. Chemistry

4.2.1. General experimental conditions

NMR (^{1}H, ^{13}C) spectra were recorded on a Bruker AVII-400 MHz, a DPX-300 MHz or a DPX-200 MHz spectrometer. Coupling constants (J) are reported in hertz, and chemical shifts are reported in parts per million (δ) relative to $CDCl_3$ (7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C) and [D_6]DMSO (2.50 ppm for ^{1}H and 39.52 ppm for ^{13}C). LC-MS was performed using an Agilent Technologies 1200 Series instrument with diode array detector set at 254 nm and equipped with a C-18 stationary phase and an Agilent Technologies 6310 Ion Trap instrument. Retention time (RT) is reported in minutes and the molecular ion e/z is reported as mass per charge. The ESI source used 60 psi pressure, 11.0 L/min flow at 365 °C. The preparative HPLC used a gradient between the following solvents; water with 0.1 % TFA and acetonitrile with 0.1 % TFA. All NMR spectra and HPLC chromatograms are included in supplementary material.

4.2.2. Synthesis of N-Boc-valyl colchicine

Deacetyl colchicine was prepared from colchicine according to literature.[24] *N*-methyl morpholine was added drop wise to a stirring solution of deacetyl colchicine (250 mg, 0.69 mmol, 1.0 eq), Boc-Val (167 mg, 0.77 mmol, 1.1 eq), COMU® (329 mg, 0.77 mmol, 1.1 eq), Oxyma® (109 mg, 0.77 mmol, 1.1 eq) in dimethylformamide (5 mL) at 0 °C. The mixture was left for 1 h at 0 °C before warming to room temperature and the reaction was left for 4 h.

The mixture was then diluted with H_2O (50 mL) and extracted with EtOAc (5 x 10 mL). The combined organic phases were pooled, washed with 2 x10 mL 0.1 M HCl, 2 x 10 mL 0.5 M $NaHCO_3$ and 10 mL saturated NaCl before it was dried over $MgSO_4$, filtered and concentrated under reduced pressure to give 276 mg (72 %) of an yellow-orange solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 – 7.39 (m, 2H), 7.27 (d, *J* = 10.5 Hz, 1H), 6.80 (d, *J* = 10.9 Hz, 1H), 6.50 (s, 1H), 5.10 (d, *J* = 9.1 Hz, 1H), 4.64 (dd, *J* = 11.8, 6.3 Hz, 1H), 4.08 – 3.80 (m, 8H), 3.64 (s, 2H), 2.50 (dd, *J* = 13.3, 6.2 Hz, 1H), 2.38 (ddd, *J* = 18.9, 12.6, 6.0 Hz, 2H), 2.25 – 1.86 (m, 4H), 1.37 (s, 9H), 0.87 (d, *J* = 6.4 Hz, 3H), 0.79 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, Chloroform-d) δ 179.3, 171.5, 164.1, 156.0, 153.5, 151.2, 141.7, 136.2, 135.1, 134.3, 131.2, 125.7, 112.2, 107.4, 79.7, 61.6, 61.4, 59.2, 56.4, 56.2, 52.2, 36.7, 31.6, 30.0, 28.4, 19.7, 17.3.

4.2.3. Synthesis of valyl colchicine

TFA (2 mL) was added to a stirring solution of *N*-Boc-Val colchicine (100 mg, 0.18 mmol) in dichloromethane (18 mL) at 0 °C. The mixture was left for 1 h at 0 °C before warming to room temperature where the reaction was left for 4 h. The mixture was then concentrated under reduced pressure to give a yellow semisolid, which was dissolved in dichloromethane and precipitated by drop-wise addition of 2 M HCl in Et_2O. The 25 mg product (28 %) was isolated by filtration after washing the solid with cold Et_2O and drying. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.30 (d, *J* = 6.9 Hz, 1H), 8.13 (s, 3H), 7.19 – 7.00 (m, 2H), 6.79 (s, 1H), 4.38 (dt, *J* = 12.4, 6.5 Hz, 1H), 4.01 – 3.68 (m, 8H), 3.72 – 3.58 (m, 1H), 3.54 (s, 2H), 2.64 (dd, *J* = 13.4, 6.2 Hz, 1H), 2.39 – 1.84 (m, 3H), 0.93 (dd, *J* = 21.8, 6.8 Hz, 5H). ^{13}C NMR (101 MHz, DMSO) δ 177.6, 167.6, 163.6, 153.0, 150.4, 149.9, 140.8, 134.8, 134.6, 134.1, 130.5, 125.3, 120.1, 112.1, 107.9, 60.8, 60.7, 57.4, 56.1, 55.9, 51.9, 35.5, 29.7, 29.1, 18.6, 17.4. HRMS: (m/z) calculated for $C_{25}H_{33}N_2O_6$: (M-Cl) 527.2339, found 457.2338.

4.2.4. Synthesis of colchicine prodrug

The tetrapeptide *N*-Boc-Ala-Ala-Asn-Val-OH was prepared using standard solution phase peptide chemistry employing Boc-protected amino acids and the ethyl esters of the corresponding amino acids. The peptide *N*-Boc-L-Ala-L-Ala-L-Asn-L-Val-OH (400 mg, 0.84 mmol, 1 eq) and deacetyl colchicine (300 mg, 0.84 mmol, 1 eq) were dissolved in dimethylformamide (2 mL) under nitrogen at 0 °C. N-methyl morpholine (92 µL, 0.84 mmol, 1 eq), COMU® (360 mg, 0.84 mmol, 1.2 eq) and Oxyma® (119 mg, 0.84 mmol, 1 eq) were subsequently added with a small amount of dimethylformamide. The mixture was left at 0 °C for 45 min and at room temperature for an additional 4 h. The mixture was then diluted with EtOAc (100 mL) and washed with 1 M HCl (2 x 20 mL), 1 M $NaHCO_3$ (2 x 20 mL), and saturated NaCl (2 x 20 ml). The organic phase was then dried over $MgSO_4$, filtered and concentrated under reduced pressure to give 198 mg of crude product. LC-MS shows two peaks (RT=8.5 and 8.9 min) with m/z 813.5 (M+1) and m/z 835.5 (M+23) which indicated racemization.

Crude *N*-Boc-L-Ala-L-Ala-L-Asn-L-Val-colchicine (198 mg, 0.12 mmol, 1 eq) was dissolved in dichloromethane (18 mL) and cooled to 0 °C before trifluoroacetic acid (2 mL) was added. After 15 min at 0 °C, the mixture was allowed to warm to room temperature where it was left for 2 h. The mixture was then concentrated under reduced pressure to give a quantitative recovery. LC-MS shows two peaks (RT=6.6 and 7.2 min) with m/z 713.4 (M+1) and m/z 735.5 (M+23) which indicated racemization.

Crude NH_2-L-Ala-L-Ala-L-Asn-L-Val-colchicine trifluoroacetate salt (>100 mg, 0.12 mmol, 1 eq) was dissolved in dichloromethane (1 mL). Et_3N (17 µL, 0.12 mmol, 1 eq), dimethyl aminopyridine (2 mg, 0.01 mmol, 0.1 eq) and succinic anhydride (121 mg, 1.2 mmol, 10 eq) were added to the mixture at room temperature. An additional amount of succinic anhydride (30 mg, 0.30 mmol, 2.5 eq) was added four times during 1 h (a total 10 eq). The reaction mixture was stirred for an additional 3 h before it was concentrated under

reduced pressure. The crude mixture gave a number of peaks in the LC-MS spectrum, with at least 3 peaks (RT=8.4, 10.2 and 11.1 min) showing m/z 813.2 (M+1). The crude mixture was purified by preparative HPLC to give 27 mg prodrug (4 % yield) with > 90 % chemical purity. CID-MS: (m/z) 358.1, 457.1, 571.2, 642.2, 713.2 and 813.2 (Supplementary materials).

4.3. Pharmacology

4.3.1. In vitro cleavage of prodrug by legumain

The cleavage of the prodrug was studied using HPLC. Prodrug (1 mg/mL) in acetate buffer (pH 5.0) was incubated with and without (control) purified bovine legumain (0.48 µg/µL). Samples were made alkaline by addition of 1 M NaOH to pH > 8 and analysed by HPLC. Cleaved prodrug was confirmed by co-injection of valyl colchicine. All HPLC chromatograms are included in the supplementary materials, as well as a plot showing how the concentrations of the prodrug and the cleaved prodrug change during the experiment.

4.3.2. Cell culturing and harvesting of cell lysates and conditioned medium

HEK293, M38L, M4C, HCT116 or SW620 cells were seeded onto 96-wells plates ($2x10^4$ cells/well) or 75 cm^2 flasks ($2x10^6$ cells/flask) in DMEM containing 1 mM sodium pyruvate, 10 % FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. M38L and M4C was added 800 µg/mL G418 or 150 µg/mL zeocin, respectively. After 24 h, 0-100 µM colchicine, valyl colchicine or prodrug with or without 50 % cystatin E/M-enriched medium or 1-50 µM Dyngo-4a were added, and incubated for 48 h. The cells were analysed by cell viability (MTS) assay or the cells were harvested in lysis buffer containing 100 mM sodium citrate, 1 mM disodium-EDTA, 1 % n-octyl-β-D-glucopyranoside, pH 5.8. Cell lysates were freeze-

thawed 3 times before analysis by immunoblotting, legumain activity and total protein measurements.

Cystatin E/M-enriched medium was obtained by culturing M4C cells ($2x10^6$) in 75 cm^2 flasks and serum-free medium for 48 h. The conditioned medium was centrifuged at 1000 G for 5 min and the supernatant used for experiments. The cystatin E/M-enriched medium was analysed by ELISA described elsewhere,[17] and contained 45 ng/mL cystatin E/M.

4.3.3. Legumain activity measurements

Legumain activity was measured by recording the cleavage of the peptide substrate Z-Ala-Ala-Asn-AMC described elsewhere.[4, 31] Briefly, 20 µL of cell lysate was added to black 96-well microtiter plates. A kinetic measurement based on increase in fluorescence over 10–60 min was performed after addition of 100 µL legumain assay buffer and 50 µL peptide substrate solution (10 µM). The measured legumain activity was divided by total protein content in each sample. Total protein concentrations were determined by a procedure described elsewhere,[32] and standard curves were established using albumin.

4.3.4. Immunoblotting

Samples of cell lysates were prepared for NuPAGE electrophoresis according to the manufacturer's recommendations. Briefly, samples were mixed with 0.5 M DTT and NuPAGE LDS sample buffer and run along with 5 µL Precision plus protein standard on NuPAGE 4–12 % gels in a container with NuPAGE MOPS SDS running buffer. Blotting was performed using 20 % methanol, 25 mM Tris, and 0.2 M glycine, pH 8.3. Nitrocellulose membranes were blocked with 5 % non-fat milk in Tris-buffered saline containing 0.05 % Tween 20 (TBS-T) for 1 h at room temperature and incubated overnight at 4 °C with goat

anti-human legumain (1:1000), cystatin E/M (1:1000), or GAPDH (1:10000). Further incubation for 1 h was performed with appropriate HRP-conjugate of secondary antibodies. After 4 times of washing for 10 min in T-TBS, immunoreactive bands on the membranes were detected by SuperSignal West Dura Extended Duration Substrate. Membranes were reprobed after using Restore Western Blot Stripping Buffer as described by the manufacturer.

4.3.5. Cell viability (MTS) assay

Cell viability assays were carried out by adding 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS reagent) to each well, and incubated for 4 h before absorbance was measured at 490 nm in a microplate reader Wallac Victor3 (PerkinElmer) according to the manufacturer's recommendations.

4.4. Statistics

The data are represented as mean ± SEM. Student t-test or student paired t-test were performed when appropriate, and statistical significance was considered at $p<0.05$

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Figure legends

Figure 1. Proposed structure of the colchicine prodrug. The colchicine prodrug was generated by conjugating colchicine to a legumain-cleavable peptide (Suc-Ala-Ala-Asn-Val). The legumain cleavage site at asparagine (Asn) is indicated by a red arrow in the prodrug. Legumain cleavage of the prodrug results in release of valyl colchicine.

Figure 2. Cell toxicity is increased in legumain over-expressing cells treated with a legumain-cleavable colchicine prodrug. A. Native, legumain over-expressing (M38L) and cystatin E/M over-expressing (M4C) HEK293 cells were cultured for 48 h before cell lysates and conditioned media were collected. Equal amounts of total proteins (10 µg) were used in immunoblot analysis of legumain, cystatin E/M and GAPDH. **B**, **C** and **D**. Native HEK293 or M38L cells (20 000 cells/well) were incubated for 48 h with or without 0.1-100 µM colchicine (**B**), valyl colchicine (**C**) or prodrug (**D**) before MTS-reagent was added and absorbance measured at 490 nM after 4 h. The data are normalized to untreated controls (0.1 % DMSO) and presented as mean ± SEM (n=3-6).

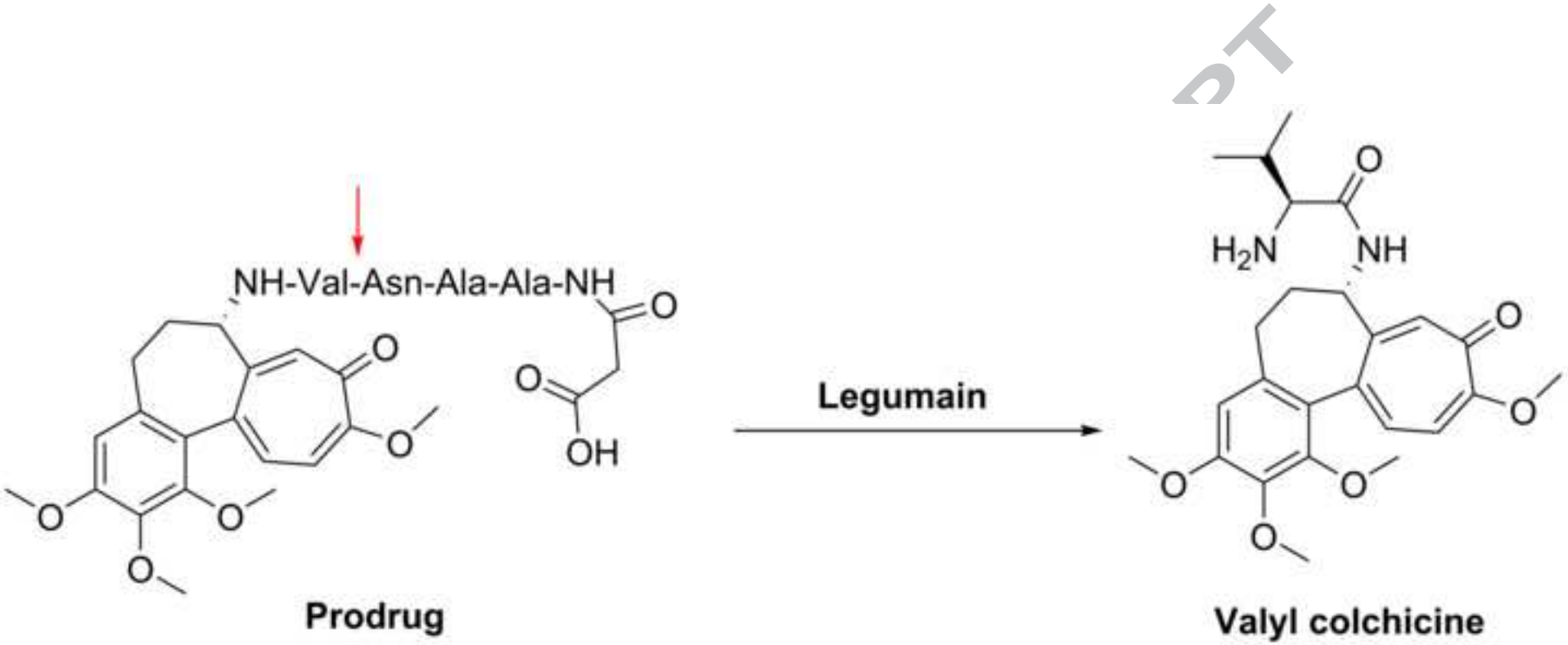
Figure 3. The colchicine prodrug is more toxic to HCT116 than SW620 A. HCT116 and SW620 cells (20 000 cells/well) were cultured for 72 h before cell lysates and conditioned media (last 48 h) were collected. Equal amounts of total proteins (10 µg) were used for immunoblot analysis of legumain, cystatin E/M and GAPDH. **B.** HCT116 and SW620 cells (20 000 cells/well) were cultured and incubated for 48 h with or without 1-50 µM prodrug before MTS-reagent were added. Absorbance was measured at 490 nM after 4 h. The data are normalized to untreated controls (0.1 % DMSO) and presented as mean ± SEM (n=3, student t-test, *$p<0.05$ vs. SW620).

Figure 4. Cystatin E/M prevents the cytotoxic effect of the colchicine prodrug. Native, legumain over-expressing (M38L) or cystatin E/M over-expressing (M4C) HEK293 cells (20 000 cells/well) were cultured and incubated for 48 h with or without 0.1-10 µM prodrug. **A.** A dose-dependent effect of the prodrug on cell viability of HEK293 and M4C cells is shown (n=3). **B.** HEK293 and M38L cells were incubated for 48 h with 5 µM prodrug with (black bars) or without (open bars) cystatin E/M (50 % M4C-conditioned medium) before measuring cell viability. The data are presented as mean ± SEM (n=3, student t-test, *$p<0.05$ vs. prodrug). **C.** HEK293 and M38L cells were incubated for 48 h with 10 µM prodrug and 0-50 µM Dyngo4a before measuring cell viability (n=3-9). The data are normalized to untreated controls (0.1 % DMSO) and presented as mean ± SEM (n=3-6). **A-C.** Absorbance was measured at 490 nM after 4 h incubation with MTS-reagent.

Scheme 1. Synthesis of prodrug from deacetyl colchicine

Scheme 2. Synthesis of valyl colchicine from deacetyl colchicine

Figure 1



Prodrug

Valyl colchicine

Figure 2

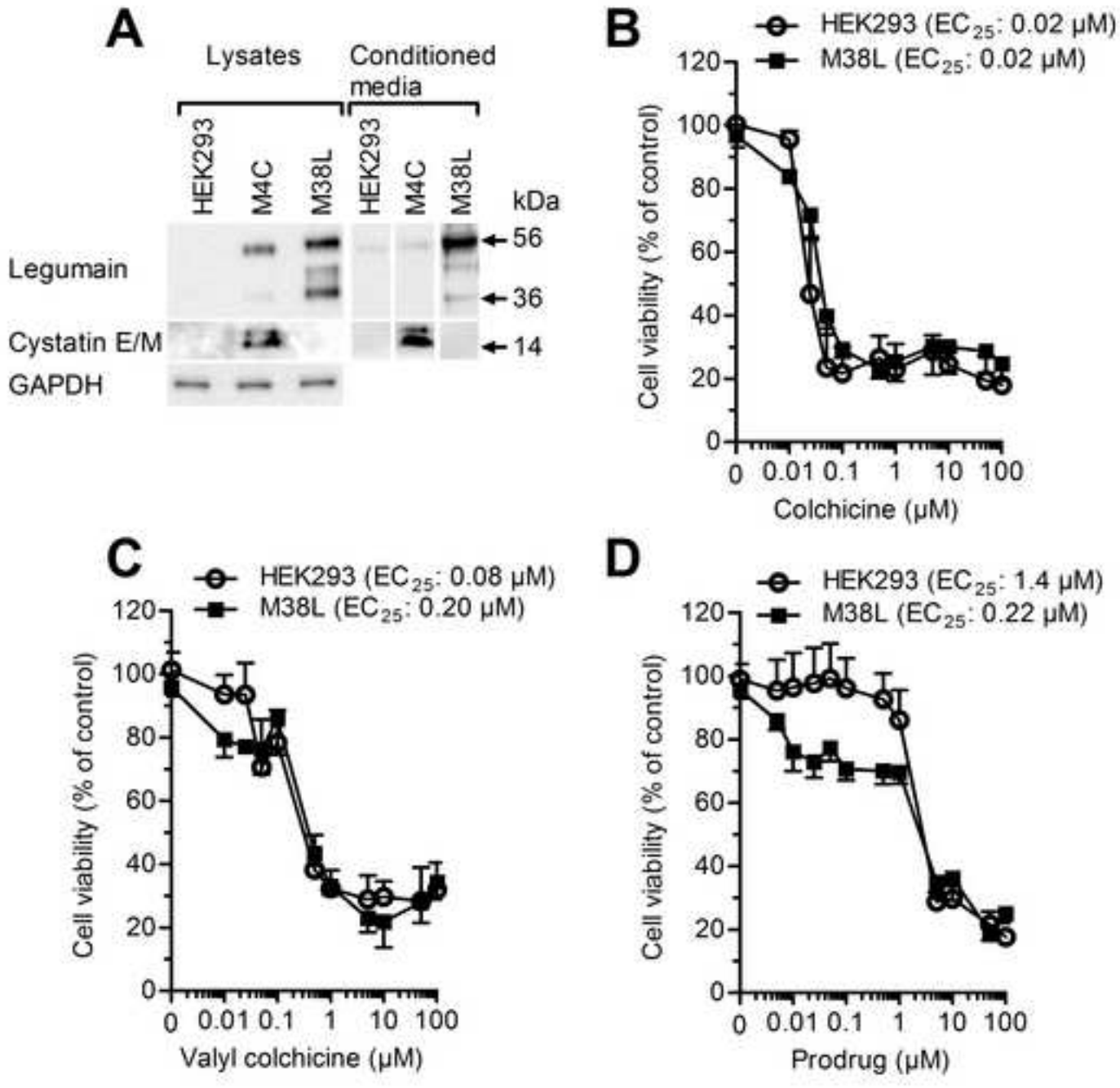


Figure 3

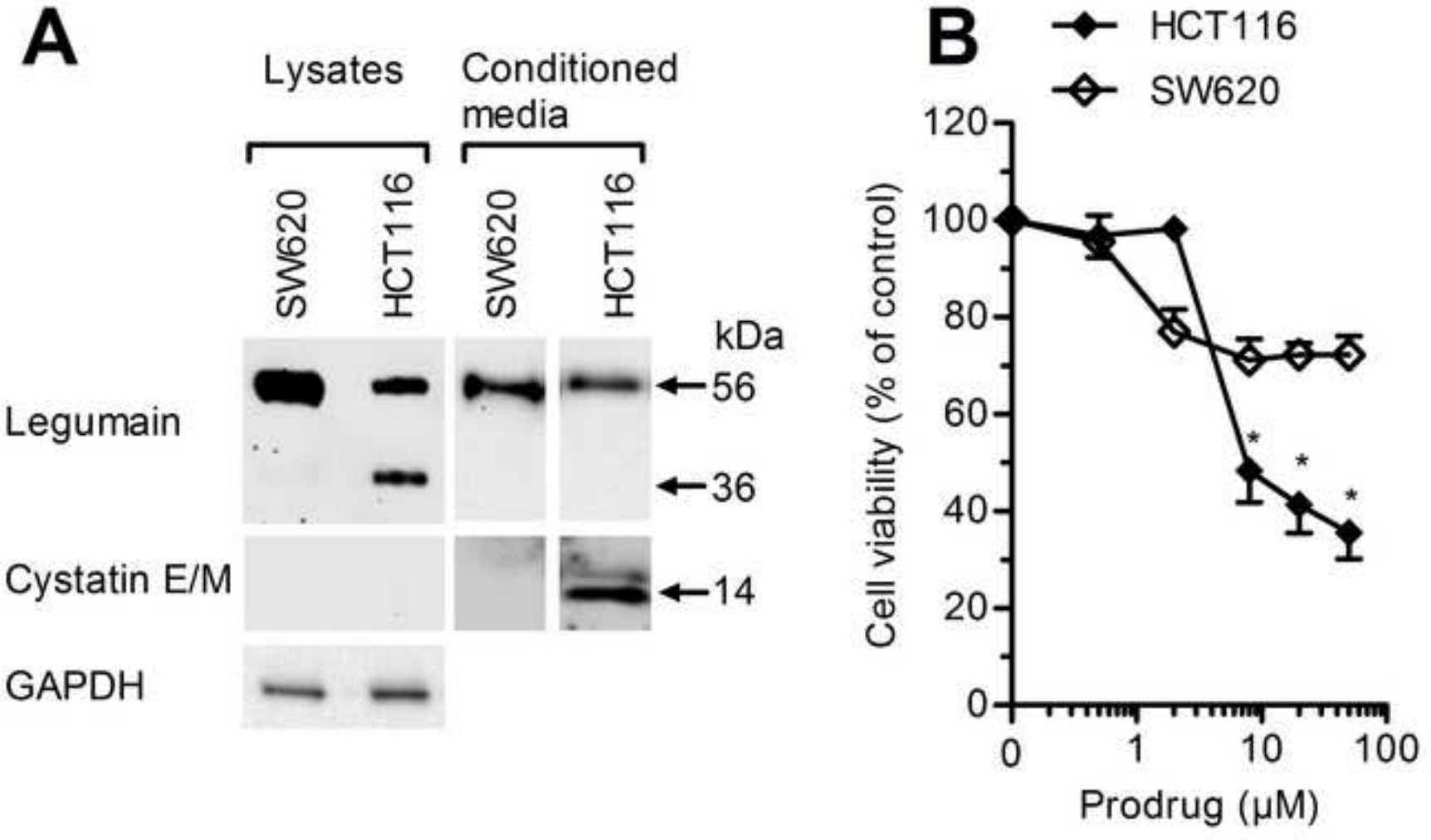
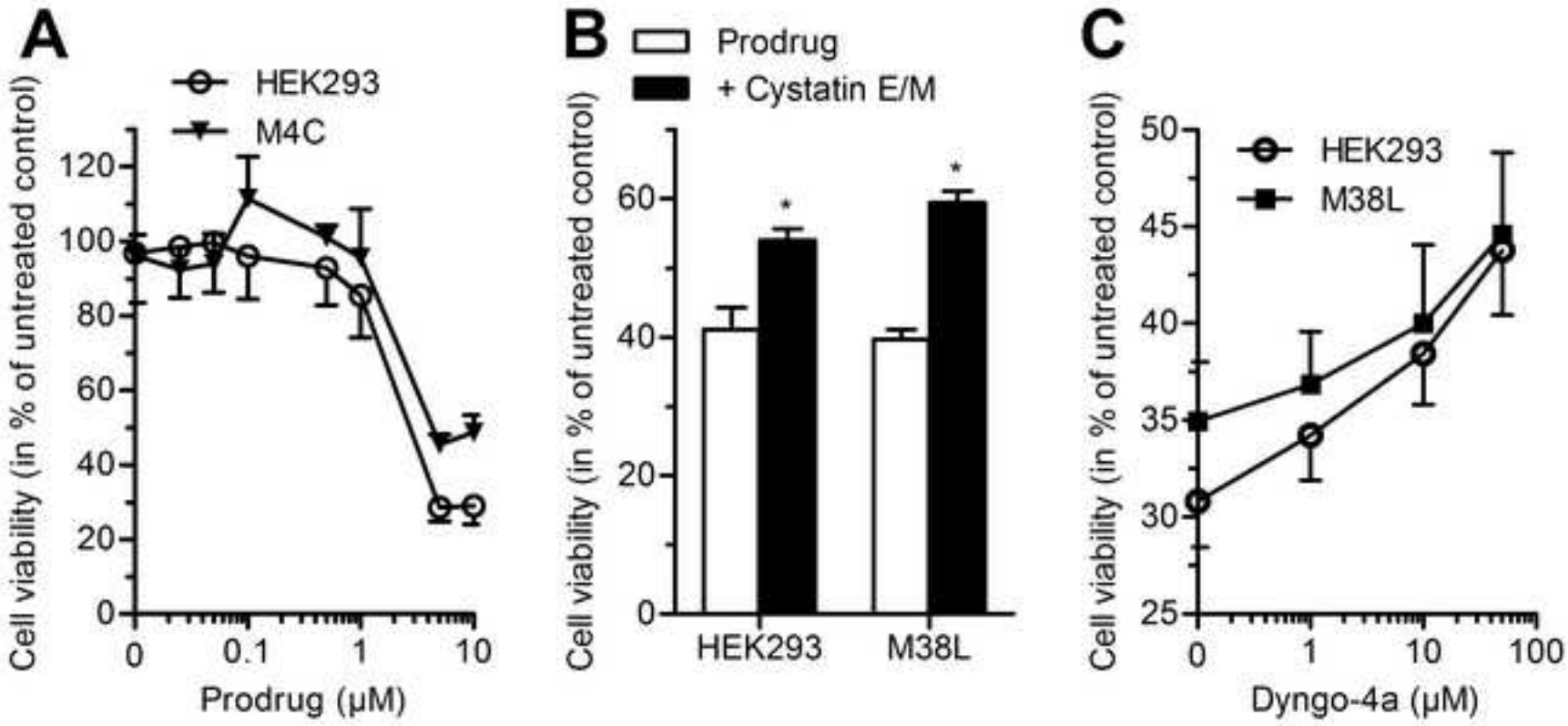
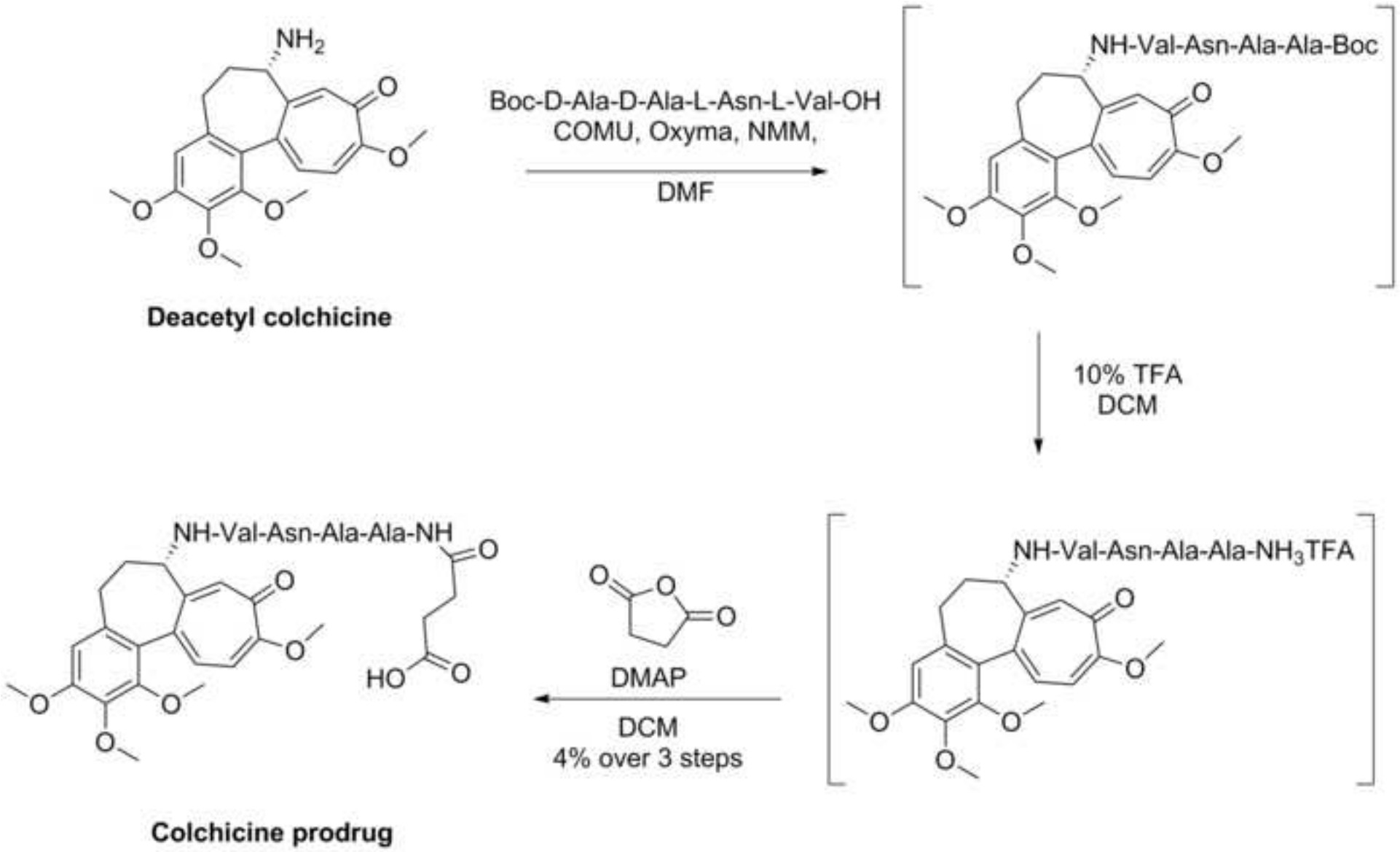


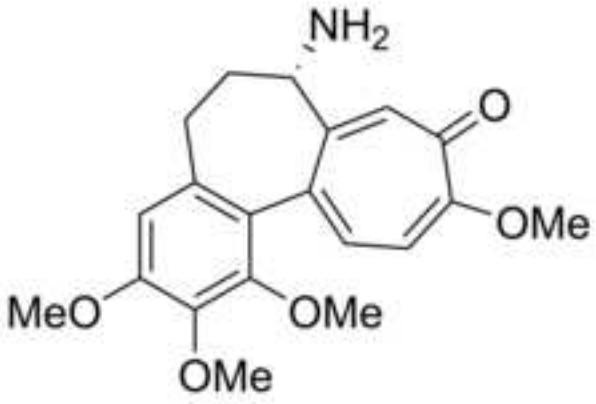
Figure 4



Scheme 1



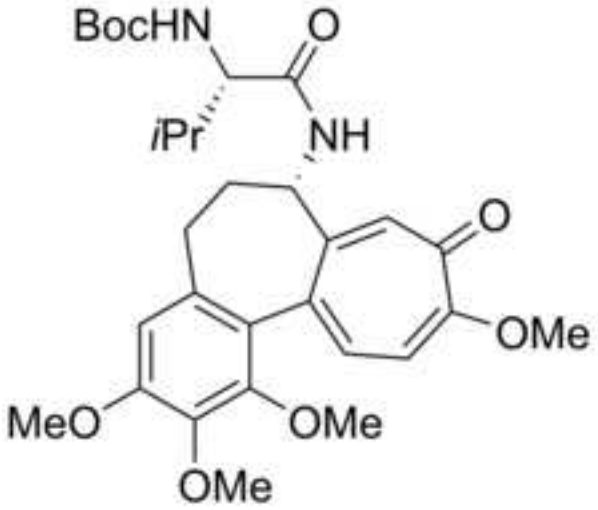
Scheme 2



Deacetyl colchicine

N-Boc-Val-OH
COMU, Oxyma, NMM

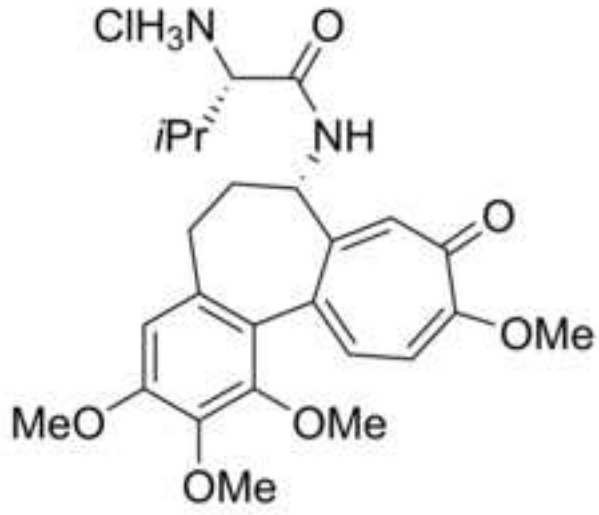
DMF, 0°C-r.t.
72%



Boc-valyl colchicine

1) TFA, DCM 0°C-r.t.
2) HCl, Et_2O

28 %



Valyl colchicine HCl

*Graphical Abstract (for review)