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RESEARCH ARTICLE

Studies of C-terminal naphthoquinone dipeptides as 20S proteasome inhibitors

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Abstract

The ubiquitin proteasome pathway is crucial in regulating many processes in the cell. Modulation of proteasome activities has emerged as a powerful strategy for potential therapies against much important pathologies. In particular, specific inhibitors may represent a useful tool for the treatment of tumors. Here, we report studies of a new series of peptide-based analogues bearing a naphthoquinone pharmacophoric unit at the C-terminal position. Some derivatives showed inhibition in the μM range of the post-acidic-like and chymotrypsin-like active sites of the proteasome.

Keywords

20S Proteasome, naphthoquinone derivatives, pseudodipeptides, synthetic inhibitor

History

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Introduction

The ubiquitin proteasome system is the major pathway for intracellular protein degradation that plays a central role in regulating cellular function and maintaining homeostasis^{1,2}. Polyubiquitinated proteins are degraded by proteasome 26S composed of a core 20S particle capped with two 19S structures. The 20S is assembled in a four stacked rings, two outer rings composed of seven α subunits and two inner rings shaped of seven different β subunits^{3–5}. In eukaryotes, three major proteolytic activities are associated with different subunits: chymotrypsin-like (CT-L) in $\beta 5$, trypsin-like (T-L) in $\beta 2$ and caspase-like (C-L) in $\beta 1$ subunits, respectively^{6,7}. Proteolysis by the β subunits of the 20S core particle occur by γ -hydroxyl function of the N-terminal threonine residue as a nucleophile⁸. The most of cellular proteins are degraded through this pathway involving a broad array of processes such as cell cycle, apoptosis, transcription, DNA repair, protein quality control and antigen presentation. Defects within this pathway are associated with much important pathologies, including cancer⁹. As the essential role of the proteasome in cell function, inhibitors of this multicatalytic complex are potential drugs suitable in various therapeutic applications. Many natural and synthetic molecules have been studied as 20S catalytic subunits inhibitors^{10–19}, among those the boronic pseudodipeptide bortezomib was the first FDA approved proteasome inhibitor for the treatment of multiple myeloma (MM) and recently carfilzomib became the second FDA-approved to treat MM in advanced state^{20,21}.

In previous studies, we have reported the design, synthesis and activity of certain classes of peptide molecules able to inhibit the

proteasome, having different pharmacophoric units, consisting of electrophilic groups potentially able to interact with the catalytic threonine^{22–25}. In this work, we have dealt with the development of a new series of peptide-based derivatives bearing at the C-terminal a unit formed by a 2-chloronaphthoquinone pharmacophoric group. The naphthoquinone is present in molecules with biological activity, e.g. in powerful antidiabetic agents and inhibitors of protein-tyrosine phosphatase^{26–28}. Recently, through library screenings, compounds bearing 2-chloronaphthoquinone unit named PI-083 (NSC-45382), able to express a good inhibition against chymotryptic activity of the proteasome have been identified (compound a in Figure 1). These compounds showed, *in vitro* and *in vivo*, the capacity to selectively inhibit tumor cell proliferation²⁹. Other non-peptide analogues with increased activity compared to the initial prototype, were also developed (general structure b in Figure 1)^{30,31}.

On the basis of these studies, we have designed pseudopeptide compounds having the general structure c shown in Figure 1. The 2-chloronaphthoquinone (CINafQ), which is the pharmacophoric unit able to function as a potential substrate for the threonine catalytic electrophile, is located at the C-terminal of the dipeptidic sequences. Cited studies^{29–31} suggest the proximity of the hydroxyl group of catalytic threonine to the 2-chloronaphthoquinone unit potentially able to attack carbon 2 of the pharmacophoric unit. Our docking studies suggest the same interaction for our compounds making reasonable the nucleophilic substitution of chlorine in position 2. The link between the peptide portion and the pharmacophore is obtained by a spacer consisting of ethylenediamine, chosen for its flexibility and to maintain the minimum distance between the pharmacophoric unit and the P1 position. While the N-terminal end is functionalized with 2-methyl,3-hydroxybenzoyl (Hmb) or *p*-nitrobenzoyl moiety (NBz). Hmb was chosen as N-terminal group since we have previously shown that favors the activity of peptide-based proteasome inhibitors^{23–25}, while *p*-nitrobenzoyl was chosen

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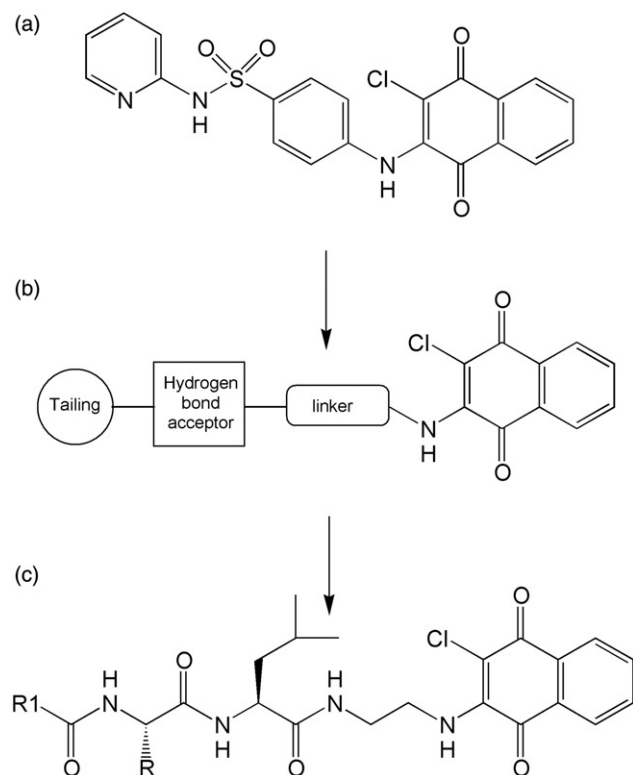


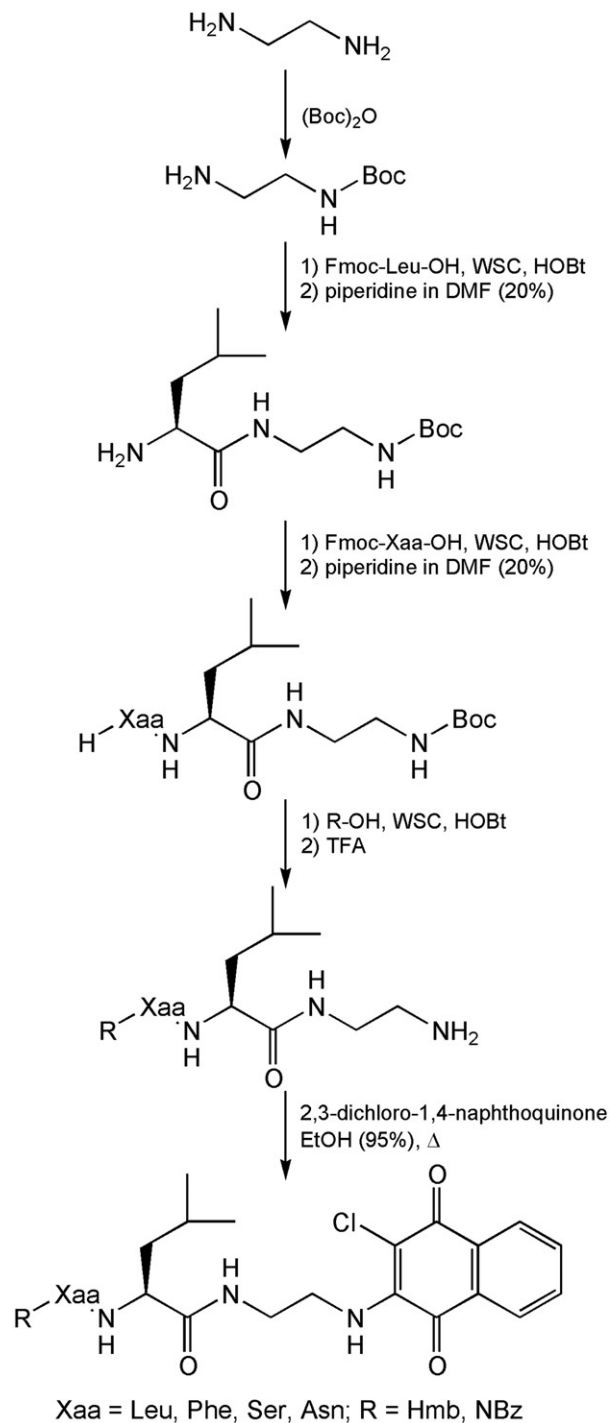
Figure 1. PI-083 (a), schematic structure of the naphthoquinone non-peptidic compounds (b) and general structure of the naphthoquinone dipeptide derivatives (c).

because it is a suitable substituent in the non-peptidic naphthoquinone derivatives of the phenotypic structure PI-083³⁰. The central dipeptidic sequences have the constant presence of a leucine residue at C-terminal, while the N-terminal position provides residues with different characteristics such as serine, phenylalanine, leucine or asparagine.

Materials and methods

Chemistry – general

Amino acids, amino acid derivatives and chemicals were purchased from Bachem, Novabiochem and Fluka (Switzerland). Crude products were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C₁₈ (30 cm × 4 cm, 300 Å, 15 μm spherical particle size column). The column was perfused at a flow rate of 30 ml/min, with a mobile phase-containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0% to 100% of solvent B (60%, v/v, acetonitrile in 0.1% TFA); 30 min was the time adopted for elution of the compounds. HPLC analysis was performed using a Beckman System Gold with a Hypersil BDS C18 column (5 μm; 4.6 mm × 250 mm). Analytical determination and retention time (*T_r*) of the peptides were assayed via HPLC conditions in the above solvent system (solvents A and B), programmed at flow rates of 1 ml/min, using the following linear gradients: (a) from 0% to 90% B for 25 min and (b) from 30% to 100% B for 25 min. No pseudo-dipeptide showed >1% impurity when monitored at 220 and 254 nm. The molecular weights of the compounds were determined by electrospray ionization (ESI) (MICROMASS ZMD 2000), and the values are expressed as [M + H]⁺. HR-MS spectra were recorded by Agilent ESI-Q-TOF 6520 instrument. TLC was performed on pre-coated plates of silica gel F254 (Merck, Darmstadt, Germany), exploiting the following solvent systems: (c) AcOEt/*n*-hexane (1:1, v/v), (d) CH₂Cl₂/methanol



Scheme 1. Synthesis of naphthoquinone dipeptide molecules 1–8.

(9.5:0.5, v/v), (e) CH₂Cl₂/methanol (9:1, v/v) and (f) CH₂Cl₂/methanol/toluene (17:2:1, v/v/v). Ninhydrin (1%) or chlorine iodine spray reagents were employed to detect the peptides. Melting points were determined by a Kofler apparatus and are uncorrected. Optical rotations were determined by a Perkin-Elmer 141 polarimeter with a 10-cm water-jacketed cell. ¹H NMR spectroscopy was obtained using a Varian 400 MHz spectrometer.

Synthesis

To obtain all the compounds, a synthetic strategy, allowing the lowest number synthetic steps, was used (Scheme 1). Specifically, the ethylenediamine was mono-protected on an amine functionality by reaction with di-tert-butyl dicarbonate. Fmoc-leucine was

condensed using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (WSC) and *N*-hydroxybenzotriazole (HOBT) as acylating agents, to give the intermediate functionalized with the appropriate linker. After fluorenylmethoxycarbonyl removal with piperidine, the sequence is completed by condensation of the selected Fmoc-protected residue (Leu, Phe, Ser and Asn), always for activation of the carboxyl group by WSC/HOBT. After removal of the Fmoc, the *N*-functionalization is carried out by acylation with 2-methyl-3-hydroxybenzoic acid or *p*-nitrobenzoic acid using WSC/HOBT as a coupling agent. After the Boc deprotection with TFA, the synthesis proceed to the final condensation with 2,3-dichloro-1,4-naphthoquinone in EtOH (95%) at reflux, for an average duration of 3 days.

General synthetic procedures

Condensation with Fmoc-amino acids, methyl-2-3-hydroxybenzoic acid or *p*-nitrobenzoic acid

To 1 mmol of carboxylic component dissolved in 10 ml of DMF were added 1.1 mmol of HOBT, 1.1 mmol of WSC and 1.1 mmol of the amine component. The reaction mixture is put under stirring for 1 h at 0 °C and overnight at room temperature. The DMF is evaporated, the residue is taken with AcOEt and washed successively with 10% citric acid, 5% NaHCO₃ and again with brine. The organic phase is dried with Na₂SO₄, filtered and evaporated.

Removal of the Fmoc

The Fmoc is removed with piperidine solution in DMF 20%. After 1 h of treatment, the solvent is evaporated and proceeds with precipitation by use of ethyl ether. The precipitate is then separated by centrifugation and collected.

Removal of the Boc

The Boc are removed with 90% aqueous TFA for 50 min (1 ml for mmol of Boc-protected compound). After evaporation, the residue is triturated with ethyl ether several times, and separated by centrifugation.

Condensation with 2,3-dichloro-1,4-naphthoquinone

About 0.3 mmol of amine are dissolved in ~15 ml 95% EtOH and treated with NMM (*N*-methyl-morpholine), then were added 0.6 mmol of 2,3-dichloro-1,4-naphthoquinone and allowed to react at reflux for 3 days at 115 °C. The solvent is evaporated and precipitated with ethyl ether and then isolate the solid by centrifugation.

Preparation of Boc-ethylenediamine

At 10 mmol of ethylenediamine dissolved in 20 ml of *t*-ButOH/H₂O (2:1) were added 7 mmol of (Boc)₂O and allowed to react for 2 h under stirring at RT. Water (20 ml) was added and extracted for 2 times with 10 ml of *n*-pentane in a separatory funnel. The aqueous phase was focused and extracted with 50 ml of AcOEt. The organic phase is dried with anhydrous Na₂SO₄ and evaporated to yield the title compound as a colorless oil (0.96 g, 85%); ¹H NMR (CDCl₃) δ: 5.98 (bs, 2H), 3.08 (m, 2H), 2.69 (m, 2H), 1.75 (bs, 2H), 1.39 (s, 9H); MS (M + H⁺) 161.2; HPLC (T_r) 6.54 min. Spectroscopic data are consisted with to previously reported³².

H-Leu-NH-(CH₂)₂-NH-Boc

The common intermediate Fmoc-Leu-NH-(CH₂)₂-NH-Boc was prepared for acylation of the Boc-ethylenediamine by

Fmoc-Leu-OH following general procedure: (a) to give the title compound. Fmoc-protected intermediate was then treated on the basis of method and (b) to give H-Leu-NH-(CH₂)₂-NH-Boc as a white solid (yield 77%). MP = 78–81 °C; [α]_D²⁰ –34.8 (*c* = 1, MeOH); ¹H NMR, (CDCl₃) δ: 3.49–3.41 (m, 2H), 3.15 (m, 1H), 3.03 (m, 1H), 2.90 (m, 1H), 1.77 (m, 1H), 1.39 (s, 9H), 1.34 (m, 2H), 0.94 (d, 3H, *J* = 7.4 Hz), 0.89 (d, 3H, *J* = 7.4 Hz); MS (M + H⁺) 274.3; HPLC (T_r) 13.87 min.

H-Xaa-Leu-NH-(CH₂)₂-NH-Boc

The dipeptides Fmoc-Xaa-Leu-NH-(CH₂)₂-NH-Boc was prepared for acylation of the H-Leu-NH-(CH₂)₂-NH-Boc by Fmoc-Xaa-OH following general procedure: (a) to give the title compound. Fmoc-protected intermediate was then treated on the basis of method and (b) to give H-Xaa-Leu-NH-(CH₂)₂-NH-Boc.

H-Leu-Leu-NH-(CH₂)₂-NH-Boc (yield 65%). MP = 118–122 °C; [α]_D²⁰ –19.4 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 4.06, (m, 1H), 3.66, (m, 2H), 3.31–3.24 (m, 1H), 3.20 (m, 1H), 3.12–3.00 (m, 2H), 2.68 (m, 2H), 1.81–1.73 (m, 3H), 1.39 (s, 3H), 1.28 (m, 1H), 0.95–0.85 (m, 12H); MS (M + H⁺) 387.6; HPLC (T_r) 18.37 min.

H-Phe-Leu-NH-(CH₂)₂-NH-Boc (yield 71%). MP = 111–114 °C; [α]_D²⁰ –17.7 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.22–7.18 (m, 5H), 4.25 (m, 1H), 3.86–3.75 (m, 2H), 3.56 (m, 1H), 3.37 (m, 1H), 2.74–2.61 (m, 2H), 2.49 (m, 1H), 1.74 (m, 2H), 1.50 (m, 1H), 1.37, (s, 9H), 0.93 (d, 3H, *J* = 7.6 Hz), 0.87 (d, 3H, *J* = 7.6 Hz); MS (M + H⁺) 421.5; HPLC (T_r) 19.16 min.

H-Ser-Leu-NH-(CH₂)₂-NH-Boc (yield 75%). MP = 145–150 °C; [α]_D²⁰ –21.5 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 4.75 (bs, 1H), 4.30 (m, 1H), 4.10–4.05 (m, 2H), 3.71–3.64 (m, 2H), 3.49 (m, 1H), 3.13–3.07 (m, 2H), 2.97 (m, 1H), 1.74 (m, 2H), 1.47 (m, 1H), 1.39 (s, 9H), 0.92 (d, 3H, *J* = 7.2 Hz), 0.86 (d, 3H, *J* = 7.2 Hz); MS (M + H⁺) 361.4; HPLC (T_r) 13.08 min.

H-Asn-Leu-NH-(CH₂)₂-NH-Boc (yield 63%). MP = 121–126 °C; [α]_D²⁰ –23.8 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 4.16 (m, 1H), 4.02 (m, 1H), 3.67–3.60 (m, 1H), 3.02–2.95 (m, 2H), 2.62–2.56 (m, 1H), 2.46–2.34 (m, 2H), 1.74 (m, 2H), 1.50 (m, 1H), 1.39 (s, 9H), 0.93 (d, 3H, *J* = 7.6 Hz), 0.87 (d, 3H, *J* = 7.6 Hz); MS (M + H⁺) 388.4; HPLC (T_r) 10.14 min.

R-Xaa-Leu-NH-(CH₂)₂-NH₂

N-terminal functionalized dipeptides R-Xaa-Leu-NH-(CH₂)₂-NH-Boc was prepared for acylation of the H-Xaa-Leu-NH-(CH₂)₂-NH-Boc by Hmb-OH or NBz-OH following general procedure: (a) to give the title compound. Boc protection was removed by described procedure (b) to give R-Xaa-Leu-NH-(CH₂)₂-NH₂.

Hmb-Leu-Leu-NH-(CH₂)₂-NH₂ (yield 61%). MP = 149–152 °C; [α]_D²⁰ –25.7 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.13–7.08 (m, 2H), 6.92 (m, 1H), 4.24 (m, 1H), 4.12 (m, 1H), 3.26 (m, 1H), 2.83–2.74 (m, 2H), 2.39 (m, 1H), 2.25 (s, 3H), 1.80–1.63 (m, 5H), 1.50 (m, 1H), 0.94–0.86 (m, 12H); MS (M + H⁺) 421.3; HPLC (T_r) 13.22 min.

Hmb-Phe-Leu-NH-(CH₂)₂-NH₂ (yield 64%). MP = 119–121 °C; [α]_D²⁰ –19.1 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.19 (m, 5H), 7.06–6.98 (m, 2H), 6.88–6.84 (m, 1H), 4.88 (m, 1H), 4.21 (m, 1H), 3.24–3.14 (m, 2H), 3.02–2.96 (m, 1H), 2.79–2.57 (m, 3H), 2.21 (s, 3H), 1.74 (m, 2H), 1.45 (m, 1H), 0.93 (d, 3H), 0.84 (d, 3H). MS (M + H⁺) 439.4; HPLC (T_r) 17.41 min.

Hmb-Ser-Leu-NH-(CH₂)₂-NH₂ (yield 71%). MP = 167–170 °C; [α]_D²⁰ –28.4 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.25–7.22 (m, 1H), 7.12 (m, 1H), 6.89 (m, 1H), 4.62 (m, 1H), 4.26 (m, 1H), 3.66–3.60 (dd, 1H, *J* = 6.8 Hz, *J* = 3.2 Hz), 3.55–3.49 (dd, 1H, *J* = 6.8 Hz, *J* = 3.2 Hz), 3.35 (m, 1H), 2.64 (m, 3H), 2.27 (s, 3H), 1.87–1.72 (m, 3H), 0.94–0.85 (m, 6H); MS (M + H⁺) 395.3; HPLC (T_r) 8.83 min.

Hmb-Asn-Leu-NH-(CH₂)₂-NH₂ (yield 54%). MP = 131–135 °C; $[\alpha]_D^{20}$ –28.1 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.23–7.12 (m, 3H, Ar), 6.90 (m, 1H, Ar), 4.18–4.08 (m, 2H), 3.04 (m, 1H), 2.86 (m, 1H), 2.79–2.62 (m, 2H), 2.39–2.29 (m, 2H), 2.27 (s, 3H), 1.74 (m, 2H), 1.50 (sept, 1H, *J* = 7.4 Hz), 0.93 (d, 3H, *J* = 7.4 Hz), 0.84 (d, 3H, *J* = 7.4 Hz); MS (*M* + *H*⁺) 422.5; HPLC (*T_r*) 6.67 min.

NBz-Leu-Leu-NH-(CH₂)₂-NH₂ (yield 71%). MP = 143–145 °C; $[\alpha]_D^{20}$ –29.1 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.87 (m, 2H), 7.59 (m, 1H), 7.48 (m, 1H), 4.29 (m, 1H), 4.20 (m, 1H), 3.00 (m, 1H), 2.44–2.26 (m, 3H), 1.80–1.72 (m, 4H), 1.58–1.45 (m, 2H), 0.94–0.80 (m, 12H); MS (*M* + *H*⁺) 391.4; HPLC (*T_r*) 15.38 min.

NBz-Phe-Leu-NH-(CH₂)₂-NH₂ (yield 73%). MP = 131–135 °C; $[\alpha]_D^{20}$ –20.5 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.86 (m, 2H), 7.61 (m, 1H), 7.50 (m, 2H), 7.19 (s, 5H), 4.86 (m, 1H), 4.17 (m, 1H), 3.22 (m, 1H), 3.06 (m, 1H), 2.98–2.82 (m, 2H), 2.68 (m, 1H), 2.41 (m, 1H), 1.74 (m, 2H), 1.54 (sept, 1H, *J* = 7.4 Hz), 0.95 (d, 3H, *J* = 7.4 Hz), 0.88 (d, 3H, *J* = 7.4 Hz); MS (*M* + *H*⁺) 440.3; HPLC (*T_r*) 16.24 min.

NBz-Ser-Leu-NH-(CH₂)₂-NH₂ (yield 75%). MP = 154–158 °C; $[\alpha]_D^{20}$ –27.1 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.89 (m, 2H), 7.65 (m, 1H), 7.54 (m, 2H), 4.59 (m, 1H), 4.25 (m, 1H), 3.53–3.43 (m, 2H), 2.98 (m, 1H), 2.66 (m, 2H), 2.35 (m, 1H), 1.75 (m, 2H), 1.62 (sept, 1H, *J* = 7.4 Hz), 0.94 (d, 3H, *J* = 7.4 Hz), 0.87 (d, 3H, *J* = 7.4 Hz); MS (*M* + *H*⁺) 365.3; HPLC (*T_r*) 12.94 min.

NBz-Asn-Leu-NH-(CH₂)₂-NH₂ (yield 62%). MP = 119–124 °C; $[\alpha]_D^{20}$ –23.6 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.83 (m, 2H), 7.59 (m, 1H), 7.48 (m, 2H), 4.23–4.14 (m, 2H), 3.08–2.89 (m, 2H), 2.85–2.64 (m, 2H), 2.38–2.29 (m, 2H), 1.72 (m, 2H), 1.53 (m, 1H), 0.89 (m, 6H); MS (*M* + *H*⁺) 392.2; HPLC (*T_r*) 9.38 min.

R-Xaa-Leu-NH-(CH₂)₂-NH-CINafQ

The compounds **1–8** were obtained for reaction by R-Xaa-Leu-NH-(CH₂)₂-NH₂ with 2,3-dichloro-1,4-naphthoquinone following general procedure (d). All the products, after purification by RP-HPLC, were analyzed by mass spectrometry (HRMS) and NMR.

Hmb-Leu-Leu-NH-(CH₂)₂-NH-CINafQ (**1**) (yield 32%). MP = 97–102 °C; $[\alpha]_D^{20}$ –23.6 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.24–7.18 (m, 2H), 7.12–7.07 (m, 2H), 6.91 (m, 1H), 6.67 (s, 2H), 4.28 (m, 1H), 4.13 (m, 1H), 3.55 (m, 1H), 3.20–3.07 (m, 2H), 2.51 (m, 1H), 2.10 (s, 3H), 1.80–1.72 (m, 2H), 1.38 (m, 2H), 0.93 (m, 6H), 0.87 (m, 6H); HRMS (*M* + *H*⁺) Calc: 561.2474, found: 561.2485; HPLC (*T_r*) 18.63 min.

Hmb-Phe-Leu-NH-(CH₂)₂-NH-CINafQ (**2**) (yield 44%). MP = 116–121 °C; $[\alpha]_D^{20}$ –16.2 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 7.26–7.15 (m, 8H), 6.94 (m, 1H), 6.85 (m, 2H), 6.65 (d, 2H, *J* = 1.2 Hz), 5.00 (m, 1H), 4.16 (m, 1H), 3.34–3.15 (m, 2H), 2.98–2.76 (m, 4H), 2.26 (s, 3H), 1.74 (m, 1H), 1.54 (m, 2H), 0.94 (d, 3H, *J* = 7.4 Hz), 0.89 (d, 3H, *J* = 7.4 Hz); HRMS (*M* + *H*⁺) Calc: 595.2317, found: 595.2302; HPLC (*T_r*) 19.08 min.

Hmb-Ser-Leu-NH-(CH₂)₂-NH-CINafQ (**3**) (yield 41%). MP = 129–132 °C; $[\alpha]_D^{20}$ –18.5 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 0.82 (d 6H); 1.49 (m 1H); 1.62 (1H d); 1.64–1.79 (m 1H); 2.28 (s 3H); 3.31 (m 1H); 3.21–3.88 (m 1H); 4.22 (m 1H); 4.55 (m 1H); 4.68 (s 1H); 4.81 (m 1H); 5.61 (bs 1H); 5.78 (bs 1H); 6.21 (bs 1H); 7.01 (dd 1H); 7.30 (dd 1H); 7.41 (m 1H); 7.62 (m 2H); 7.71 (m 2H); 7.97 (bs 1H); HRMS (*M* + *H*⁺) Calc: 535.1954, found: 535.1941; HPLC (*T_r*) 9.09 min.

Hmb-Asn-Leu-NH-(CH₂)₂-NH-CINafQ (**4**) (yield 34%). MP = 122–126 °C; $[\alpha]_D^{20}$ –20.5 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 0.82 (d 6H); 1.49 (m 1H); 1.25–1.98 (m 1H); 2.28 (s 3H); 2.80–3.26 (m 1H); 3.47 (m 1H); 3.31 (m 1H); 4.72 (m 1H); 4.80 (m 1H); 5.28 (bs 1H); 5.61 (bs 1H); 6.27 (bs 1H);

6.99 (dd 1H); 7.30 (dd 1H); 7.40 (m 1H); 7.59 (m 2H); 7.68 (m 2H); 7.94 (bs 1H); HRMS (*M* + *H*⁺) Calc: 562.2063, found: 562.2074; HPLC (*T_r*) 6.89 min.

NBz-Leu-Leu-NH-(CH₂)₂-NH-CINafQ (**5**) (yield 33%). MP = 92–95 °C; $[\alpha]_D^{20}$ –24.2 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 0.85 (d 6H); 1.47 (m 1H); 1.62 (m 2H); 2.09 (m 2H); 3.48 (m 2H); 3.86 (m 2H); 4.32 (m 1H); 4.50 (m 1H); 5.21 (bs 1H); 5.66 (bs 1H); 6.32 (bs 1H); 7.17 (bs 1H); 7.62 (m 2H); 7.71 (m 2H); 8.62 (d 2H); 8.87 (d 2H); HRMS (*M* + *H*⁺) Calc: 576.2219, found: 576.2234; HPLC (*T_r*) 19.77 min.

NBz-Phe-Leu-NH-(CH₂)₂-NH-CINafQ (**6**) (yield 40%). MP = 93–97 °C; $[\alpha]_D^{20}$ –22.3 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 0.83 (d 6H); 1.49 (m 1H); 1.97 (m 2H); 3.11–3.71 (m 6H); 4.55 (m 1H); 5.56 (m 1H); 6.41 (bs 1H); 6.88 (bs 1H); 7.22 (m 5H); 7.28 (bs 1H); 7.66 (m 2H); 7.75 (m 2H), 8.13 (d 2H), 8.28 (d 2H); HRMS (*M* + *H*⁺) Calc: 610.2063, found: 610.2069; HPLC (*T_r*) 23.44 min.

NBz-Ser-Leu-NH-(CH₂)₂-NH-CINafQ (**7**) (yield 37%). MP = 124–128 °C; $[\alpha]_D^{20}$ –18.5 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 8.30–8.10 (m, 5H), 6.70 (d, 2H, *J* = 1.2 Hz), 4.61 (m, 1H), 4.37 (m, 1H), 3.94 (m, 1H), 3.40 (m, 1H), 3.26–2.92 (m, 3H), 2.46 (m, 1H), 1.75–1.62 (m, 2H), 0.90 (m, 6H); HRMS (*M* + *H*⁺) Calc: 550.1699, found: 550.1705; HPLC (*T_r*) 19.67 min.

NBz-Asn-Leu-NH-(CH₂)₂-NH-CINafQ (**8**) (yield 29%). MP = 106–111 °C; $[\alpha]_D^{20}$ –24.2 (*c* = 1, MeOH); ¹H NMR (CDCl₃) δ: 8.30 (d, 2H, *J* = 7.2 Hz), 8.11 (d, 2H, *J* = 7.2 Hz), 6.88 (bs, 2H), 6.61 (s, 2H), 4.30 (m, 1H), 4.12 (m, 1H), 3.15 (m, 1H), 3.02–2.82 (m, 2H), 2.68 (m, 1H), 2.53–2.42 (m, 2H), 1.74 (m, 1H), 1.50 (sept, 1H, *J* = 7.2 Hz), 0.93 (d, 3H, *J* = 7.6 Hz), 0.87 (d, 3H, *J* = 7.6 Hz); HRMS (*M* + *H*⁺) Calc: 577.1808, found: 577.1815; HPLC (*T_r*) 17.02 min.

Biological assays

Proteasome purification proteasome subunit inhibition

Proteasomes were isolated from lymphoblastoid cell lines (LCL) as previously described³³.

Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC (Sigma) were used to measure CT-L, T-L and post-acidic proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min with proteasomes, untreated or pre-treated with 0.1–100 μM of test compounds, in activity buffer. Fluorescence was determined by a fluorimeter (Spectrafluor plus, Tecan, Salzburg, Austria), using an excitation of 360 nm and emission of 465 nm. Activity was evaluated in fluorescence units and the inhibitory activity of the compounds is expressed as IC₅₀.

Growth inhibition assays

Cell growth inhibition assays were carried out using the breast cancer cell line MDA and ovarian cancer cell line A2780. Cell lines were obtained from ATCC and maintained in DMEM and RPMI, respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U ml⁻¹), streptomycin (100 U ml⁻¹) and glutamine (2 mM); incubation was performed at 37 °C in a 5% CO₂ atmosphere. Cells were routinely passaged every 3 days at 70% confluence; 0.05% trypsin-EDTA was used. The anti-proliferative activity of compounds was tested with the MTT assay. The cells were seeded in triplicate in 96-well trays at a density of 25 × 10³ in 50 μl of complete medium. Stock solutions (10 mM) of each compound were made in DMSO and diluted in complete medium to give final concentrations of 10 and 100 μM. MG-132 was employed as a control. Untreated cells were placed in every plate as a negative control. The cells were exposed to the compounds, in 100 μl total volume, for 72 h, and then 25 μl of a 12-mM solution of

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) was added. After 2 h of incubation, 100 μ l of lysing buffer (50% DMF + 20% SDS, pH 4.7) was added to convert the MTT solution into a violet-colored formazan. After an additional 18 h, the solution absorbance, proportional to the number of live cells, was measured by spectrophotometer at 570 nm and converted into % of growth inhibition³⁴.

Docking

The equilibrium geometry of molecule **5** was obtained using semi-empirical PM3 calculations and subsequently used in a docking simulation to both β 1 and β 5 binding sites of 20S proteasome. The simulation was performed utilizing the crystal structures of PDB codes **1G65**³⁵ and **4LQI**³⁶ for β 1 and β 5 binding sites, respectively. Before the simulation, hydrogen atoms were added to the inner part of the enzyme and the energy of the structure was minimized keeping fixed the atoms of the mainframe and using the MMFF94 molecular mechanics forcefield³⁷. Molecule **5** was placed in β 1 and β 5 binding sites using a pharmacophore query derived from the bound inhibitors epoxomicin (structure **1G65**) and omuralide (structure **4LQI**), respectively, as a filter for docking placement. A pharmacophore is a set of structural features in a ligand that are directly related to the ligand's recognition at a receptor site and its biological activity. Out of 50 unique poses obtained, the 10 having the highest score on the base of the value assumed by the enthalpic contribution to the free energy of binding were retained. These poses were in turn rescored on the base of the estimation

of the free energy of binding of the ligand, i.e. the sum of the electrostatic and dispersive interaction energy between the ligand and the target as well as the intramolecular energy of the ligand due to changes of its conformation. All the calculations were performed using MOE-Dock integrated in the MOE system of programs [MOE, Chemical Computing Group, release 2013.0802].

Results and discussion

Napthoquinone pseudodipeptides were synthesized following the strategy reported in Scheme 1 and tested as proteasome inhibitors.

Inhibition of β 1, β 2 and β 5 active sites of the 20S proteasome, purified from lymphoblastoid cell lines²⁵, was determined using fluorogenic substrates specific for the three main proteolytic activities of the enzymatic complex. Suc-LLVY-AMC, Boc-LRR-AMC and Z-LLE-AMC were used to measure CT-L, T-L and C-L proteasome activities, respectively. Substrates were incubated, at 37 °C for 30–180 min, with the proteasome, pre-treated with incremented concentrations (from 0.1 to 100 μ M) of the new naphthoquinone derivatives and compared with the well-known proteasome inhibitor MG132 in activity buffer. Substrate degradation was evaluated in fluorescence units, and the inhibitory activity of all the compounds is expressed here as IC₅₀. From the data reported in Table 1, after 30 min of incubation it can be observed that some of the naphthoquinone derivatives present an interesting biological profile. The inhibition of the post-acidic (PGPH) and chymotryptic (ChT-L) activities is significant for some of the derivatives. In particular, the activity against the β 1

Table 1. Inhibition of the proteasome subunits by naphthoquinone dipeptides **1–8** and reference compound MG132.

Compound	ChT-L	IC ₅₀ (μ M)* T-L	PGHP
1 . Hmb-Leu-Leu-NH-(CH ₂) ₂ -NH-CINafQ	>100	>100	4.51 \pm 0.32
2 . Hmb-Phe-Leu-NH-(CH ₂) ₂ -NH-CINafQ	46.15 \pm 3.15	91.40 \pm 9.04	20.29 \pm 1.43
3 . Hmb-Ser-Leu-NH-(CH ₂) ₂ -NH-CINafQ	87.24 \pm 6.54	77.49 \pm 8.53	25.10 \pm 2.26
4 . Hmb-Asn-Leu-NH-(CH ₂) ₂ -NH-CINafQ	34.2 \pm 3.02	>100	15.82 \pm 0.97
5 . NBz-Leu-Leu-NH-(CH ₂) ₂ -NH-CINafQ	39.40 \pm 2.97	>100	0.73 \pm 0.08
6 . NBz-Phe-Leu-NH-(CH ₂) ₂ -NH-CINafQ	>100	>100	13.74 \pm 0.77
7 . NBz-Ser-Leu-NH-(CH ₂) ₂ -NH-CINafQ	74.35 \pm 5.45	93.6 \pm 10.67	60.28 \pm 4.89
8 . NBz-Asn-Leu-NH-(CH ₂) ₂ -NH-CINafQ	24.79 \pm 1.98	>100	1.94 \pm 0.22
MG132	0.002	1.08	>10

*The values reported are the mean \pm SE of three independent determinations.

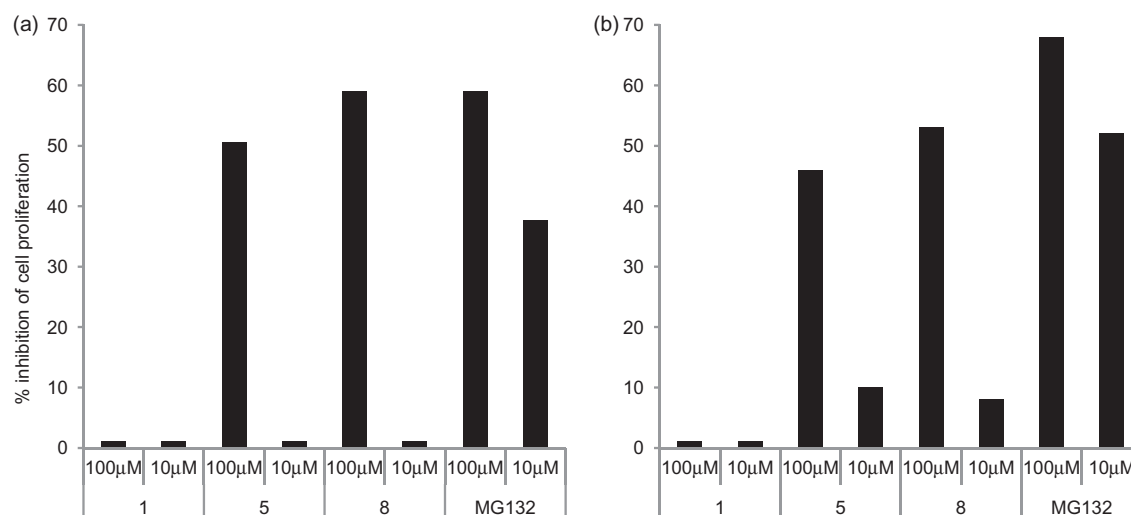


Figure 2. Effect of compounds **1**, **5** and **8** on cell proliferation. MDA (panel a) and A2780 (panel b) tumor cells were cultured for 3 days in the presence or absence of the indicated concentrations of compounds **1**, **5** and **8**. Results are expressed as % inhibition proliferation calculated in respect to untreated cells. The means of three independent experiments performed in duplicate are shown.

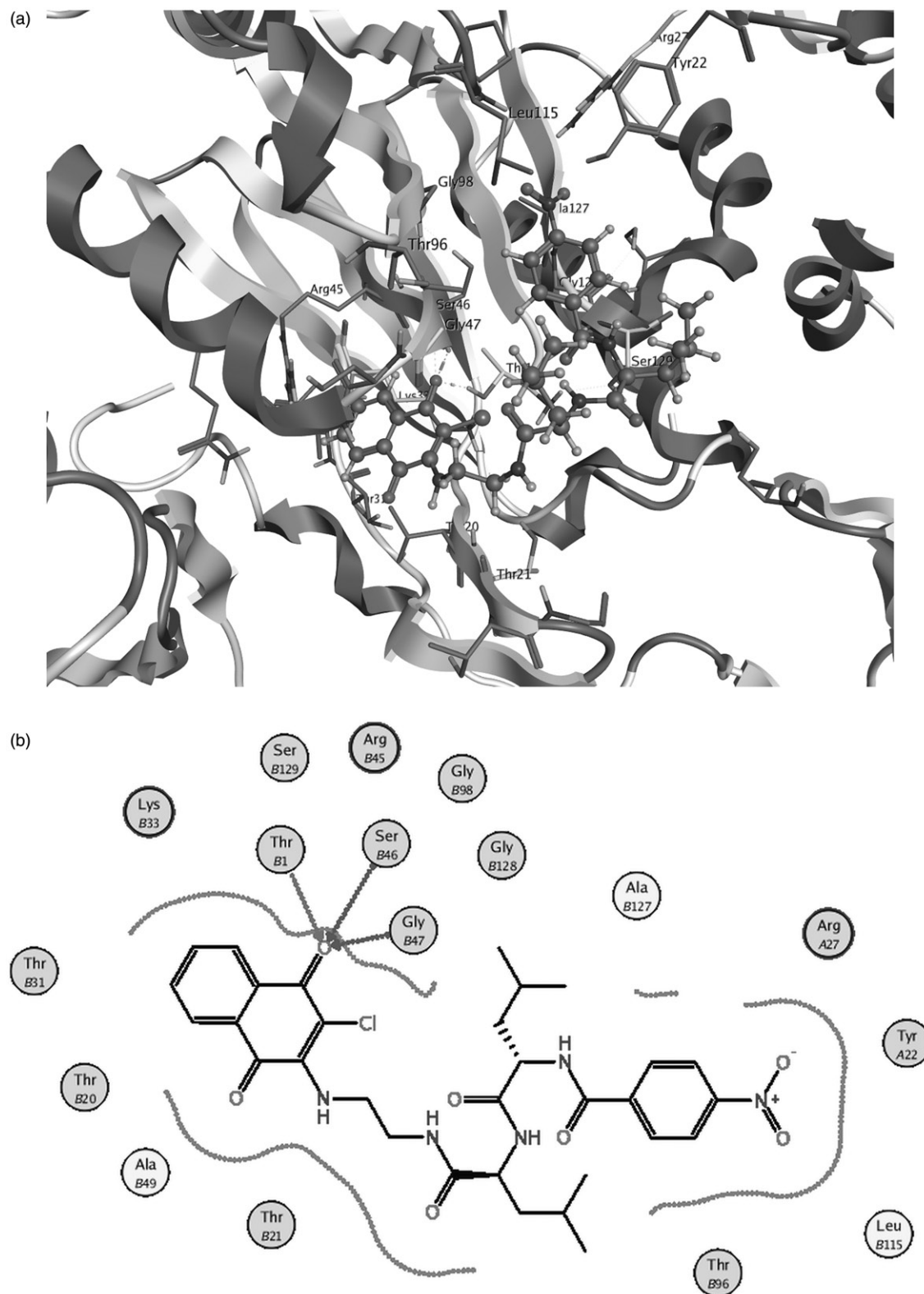


Figure 3. Molecule 5 in the $\beta 1$ binding pocket (a) and a schematic diagram of the inhibitor-protein interactions (b).

subunit is remarkable for the analogs **1**, **5** and **8** with IC_{50} values around μM unit. In addition, compounds **5** and **8**, but not compound **1**, were able to some extent to inhibit the chymotryptic activity. None of the compounds were able to inhibit the tryptic activity. The most efficient compounds of the series are **5** and **8**, bearing the central Leu-Leu and Asn-Leu dipeptide sequence, respectively. The different N-terminal functionalizations appear not determinant for biological response.

Since proteasomes play a key role in cell viability and proliferation, we evaluated the anti-proliferative activities of three selected compounds (**1**, **5** and **8**) on MDA and A2780 tumor cells. To this end, cells were treated with 10 or 100 μM concentrations of compounds **1**, **5** or **8**. After 3 days, cell proliferation was evaluated and as shown in Figure 2a and b, only compounds **5** and **8** at 100 μM demonstrated the capacity to inhibit cell proliferation at levels similar to the proteasome inhibitor MG132. No anti-

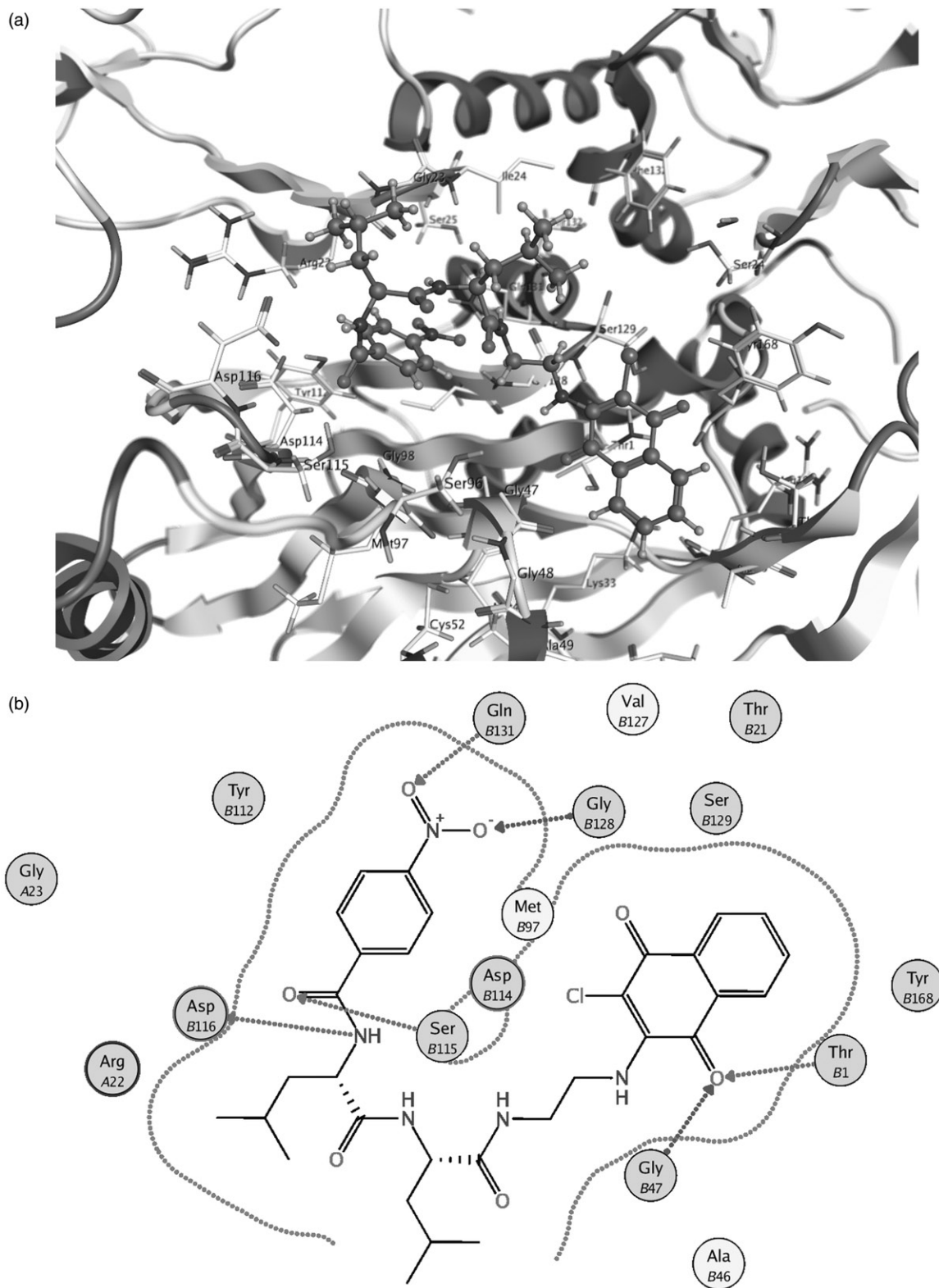


Figure 4. Molecule **5** in the $\beta 5$ binding pocket (a) and a schematic diagram of the inhibitor–protein interactions (b).

proliferative activity was observed at 10 μM . Of note, compound **1** that inhibits only the post-acidic activity of the proteasome did not show any anti-proliferative activity.

Structure–activity relationship provided suggests that the C-terminal pharmacophoric is a favorable electrophilic substrate able to undergo nucleophilic attack by the enzymatic threonine. The secondary interactions that stabilize the enzyme–inhibitor binding are most effective when the dipeptide sequence has features more hydrophobic. As shown by docking studies, the

enzyme–inhibitors interaction should be not covalent, this data suggest a reversible inhibition, confirmed by the decreasing of IC_{50} values during the time of incubation of the naphtoquinone derivatives with the isolated proteasome.

The results of the docking study of compound **5** to both $\beta 1$ and $\beta 5$ binding sites of 20S proteasome seem to support the previous considerations; actually, in both sites, the naphtoquinonic unit is positioned near the active Thr1 residue. In the $\beta 1$ pocket, the O_2 quinonic oxygen is located near Thr1 and Gly47 residues at a

quite short distance of some 2.6–2.7 Å, compatible with the formation of hydrogen bonding interactions (Figure 3). Conversely, in the β5 site the O1 oxygen of the naphthoquinonic unit is found at a higher distance with respect to the active Thr1 residue (some 3.1 Å) but a number of additional interactions can be observed between the functional groups of the N-terminal fragment and Ser115, Gln131, Gly128 and Asp116 residues (Figure 4) at ligand–protein distances in the range 3.0–3.5 Å which contribute to the binding stability.

Conclusions

This work reports the design, synthesis, biological activities and molecular modeling analysis of pseudo-dipeptide molecules assayed as inhibitors of three major catalytic activities of the proteasome. These compounds have at the C-terminal position a 2-chloronaphthoquinone group pharmacophoric unit primary responsible for the interaction with the active subunits of the enzymatic complex. The same scaffold is present in non-peptidic molecules previously reported as inhibitors of the 20S proteasome. Data on the biological response of some analogs of this new series showed an interesting inhibition of the proteasome. Derivatives 5 and 8 were the most active against the β1 and β5 subunits, with a biological profile that makes them potentially capable of operating such as antineoplastic agents. The molecular structure of the new inhibitors allows further studies on the structure–activity relationship, maintaining the C-terminus naphthoquinonic unit as electrophilic substrate.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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