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Research paper

Synthesis of functionalized new conjugates of batracylin with tuftsin/retro-tuftsin derivatives and their biological evaluation

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ABSTRACT

New batracylin conjugates with tuftsin/retro-tuftsin derivatives were designed and synthesized using T3P as a coupling agent. The conjugates possess an amide bond formed between the carboxyl group of heterocyclic molecule and the *N*-termini of the tuftsin/retro-tuftsin chain. The *in vitro* cytotoxic activity of the new analogues and their precursors was evaluated using a series of human and murine tumor cells. BAT conjugates containing retro-tuftsin with branched side aminoacid chain, in particular with leucine or isoleucine, were about 10-fold more cytotoxic toward two human tumor cell lines (lung adenocarcinoma (A549) and myeloblastic leukemia (HL-60)). These compounds showed about 10-fold increased cytotoxicity against the two types of tumor cells compared to parent BAT. We have not observed important differences in the mechanism of action between BAT and its cytotoxic tuftsin/retro-tuftsin conjugates. We propose that high biological activity of the most active BAT conjugates is a result of their greatly increased intracellular accumulation.

1. Introduction

Batracylin (8-aminoisoindolo[1,2-b]quinasolin-12(10*H*)-one, BAT) [1] is a heterocyclic amine identified by the drug screening system at the National Institutes of Health (Bethesda, USA) [2]. In preclinical studies, BAT showed high antitumor and cytotoxic activity toward several experimental tumor cell models, including cells which are resistant to standard chemotherapeutics, such as doxorubicin, methotrexate and cisplatin [3–8]. In the recently completed phase I clinical studies, safety profiles of BAT were evaluated in human cancer patients. These studies showed that BAT is well tolerated by human patients up to 400 mg/kg and provided some encouraging data concerning its therapeutic potential [9].

One of the new directions in the synthesis of novel chemical entities with antitumor activity is to combine several different molecules with different functions and/or activities to produce functionalized derivatives [10,11]. The aim of this approach is to obtain compounds with enhanced activity/specificity and improved pharmacologies properties, including increased bioavailability and lowered general toxicity of the conjugate. We recently set out a program aimed at the synthesis of new BAT-tuftsin/retro-tuftsin conjugates which were expected to have improved pharmacological features (such as increased water solubility, lowered general toxicity *in vivo*) and potentially have additional mechanisms of action, including immunostimulatory effect of tuftsin. Tuftsin is a tetrapeptide Thr-Lys-Pro-Arg (TKPR) that been shown to possess immunologic, tumoricidal, and bactericidal activities [12–14]. Accordingly, tuftsin has been successfully used in combination with different antibiotics to treat opportunistic infections caused by bacteria, fungi, and viruses. In addition, it also showed antineoplastics properties [15–32]. Moreover, tuftsin binds to the receptor neuropillin-1 (NRP1) on the surface of cells that participates in several different signalling pathways controlling cell migration and survival [33].

We report here the synthesis of a new series of BAT analogues with tuftsin/retro-tuftsin derivatives containing isopeptide bond between ε-amino group of lysine and carboxyl group of aliphatic amino acids such as Gly, Ala, Val, Leu, Ile. In our method, synthesis of new analogues is based on the modification in the *C*-terminus of the peptide residue by the formation of an amide bond between the

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carboxylic group of the respective peptide and the amine group of BAT. We hypothesized that combination of BAT and tuftsin/retro-tuftsin derivatives will allow us to obtain analogues with increased anticancer activity and improved selectivity toward tumor cells. In this paper, we provide data concerning evaluation of the cytotoxic activity as well as other biological effects induced by these compounds in *in vitro* tests and in tumor cells.

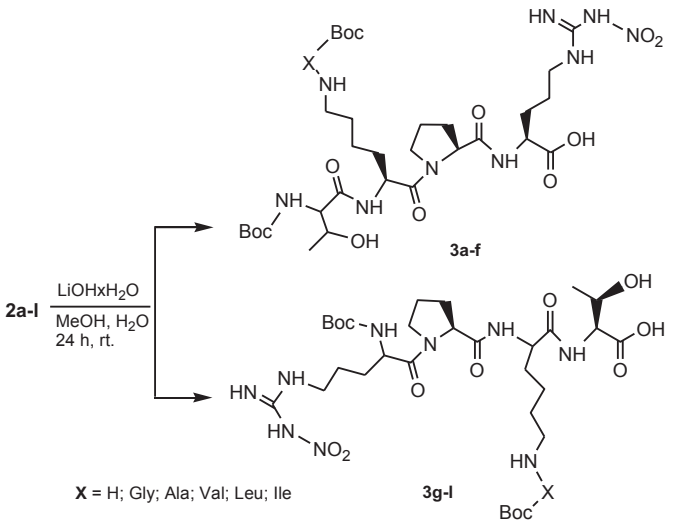
2. Results and discussion

2.1. Chemistry

The synthesis of BAT with tuftsin/retro-tuftsin derivatives was carried out according to reaction presented in Scheme 1, using highly reactive *n*-propanephosphonic acid anhydride (T3P) [34] as a coupling agent in solvent mixture. The condensation between BAT and *C*-termini of tuftsin/retro-tuftsin derivatives **3a**–**l** was achieved during reaction in anhydrous dimethylformamide (DMF) under N_2 for 24 h. T3P (50% solution in DMF) was added to a mixture of **1** and **3a**–**l** in anhydrous pyridine and DMF, and the resulting homogenous solution was held at −15 °C for 4 h. After this time, the reaction was carried out at 45 °C. After 24 h, DMF was evaporated under vacuum. The products **4a**–**l** were purified with preparative TLC, and their identities were confirmed by high resolution ^{1}H NMR (500 MHz) spectroscopy and MALDI-TOF mass spectrometry analysis. The *tert*-butoxycarbonyl (Boc) protecting groups were removed by treatment with HCl in anhydrous Et_2O to give the corresponding hydrochloride as an oil. The presence of final products **5a**–**l** were confirmed by MALDI-TOF mass spectrometry analysis and their purity by HPLC.

BAT **1** was synthesized via modified method based on the Czerniak–Einhorn reaction [20]. In this method, a symmetrically protected 1,4-phenylenediamine derivative has undergone the Czerniak–Einhorn reaction, and after hydrolysis of the protecting groups, BAT **1** was obtained.

The protected tuftsin and retro-tuftsin derivatives **2a**–**l**, tetra- and pentapeptides, were synthesized by the mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine (NMM) in anhydrous DMF (Scheme 2) [20–26]. A solution of Boc protected amino acid in anhydrous DMF was cooled to −15 °C and NMM followed by isobutyl chloroformate were added. Five minutes later, amino acid with free carboxyl group or respective peptide in the later stages of a synthesis, neutralized by equivalent amount of NMM or triethylamine (TEA) in anhydrous DMF was added into solution. The reaction mixture was stirred at −15 °C for 4 h then at room temperature for 24 h. After evaporating the solvent *in vacuo*, the crude products were purified by chromatography (SiO_2). Structures of synthesized derivatives **2a**–**l** were established by spectroscopic methods (^{1}H NMR, ^{13}C NMR, MS), optical rotation and melting point.



Scheme 1. Synthesis and chemical structures of BAT analogues **5a-l**.

The Boc protecting groups were removed by treatment with trifluoroacetic acid (TFA). The benzyloxycarbonyl (Z) protecting groups were cleaved by hydrogenolysis (H_2/Pd–C). For recovering free carboxylic group we performed hydrolysis methyl esters of peptides **2a**–**l** (Scheme 3) under mild conditions using LiOH [35] as a reagent.

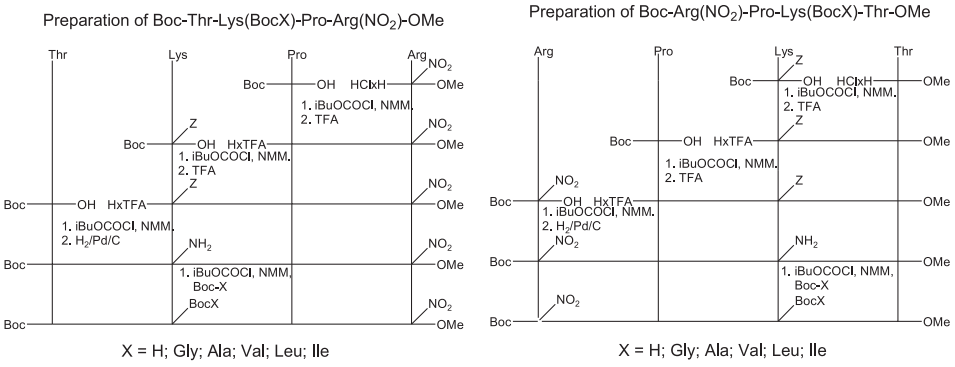
2.2. Cytotoxic properties

We performed cytotoxic activity testing using different human tumor cells: lung adenocarcinoma (A549), two colon carcinomas (HCT116, HT-29), prostate carcinoma (LNCaP), breast carcinoma (MCF-7), human promyelocytic leukemia (HL-60), as well as two murine leukemias (P388, L1210) and two other murine fibroblast-derived cells (WEHI 1640, NIH-3T3). As presented in Table 1, BAT showed moderate cytotoxic properties, represented by its IC_{50} values between 46.3 μM for A549 lung adenocarcinoma and 90.2 μM for LNCaP prostate cancer. Interestingly, no cytotoxicity was observed for human breast cancer cells (MCF-7) and murine leukemia cells (L1210, P388) even at the highest concentration tested.

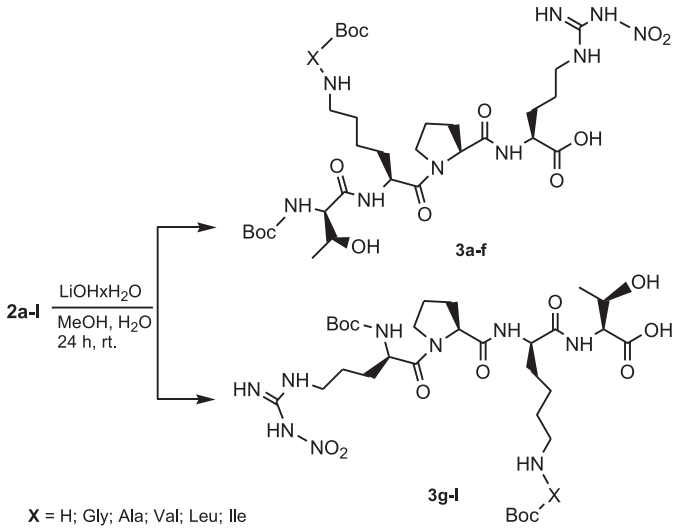
For further investigations, we selected two types of tumor cells, which were the most sensitive to BAT from all tumor cells tested i.e. A549 lung adenocarcinoma cells, as a solid tumor and the most sensitive from leukemia cell lines, human promyelocytic leukemia HL-60 cells. Conjugation of BAT with tuftsin alone did not appreciably change the cytotoxicity of the conjugate, compared to the parent compound. In contrast, retro-tuftsin-BAT conjugate was about 2-fold (for HL-60 cells) and about 5-fold (for A549 cells) more cytotoxic that BAT. The majority of BAT conjugates with branched tuftsin or retro-tuftsin were more cytotoxic toward both investigated cell lines than the parent BAT (see Table 2). This was particularly striking for retro-tuftsin conjugates branched with leucine or isoleucine (compounds **5k** and **5l**) which showed about 10 times or more increased cytotoxicity against both tumor cells than the parent compound BAT and its branched BAT-tuftsin analogues. Importantly, branching tuftsin but also retro-tuftsin with other non-polar aliphatic aminoacids (Gly, Ala, Val) had a very variable effect on the cytotoxic activity of BAT conjugates and this was also tumor cell type dependent.

2.3. Effect of studied compounds on the catalytic activity of human type I and II DNA topoisomerases

Previous studies showed that BAT acts as an inhibitor of both type I and II DNA topoisomerases [29,30]. Therefore, we next evaluated whether BAT conjugates with tuftsin and retro-tuftsin, branched with the same chain aminoacids (Leu and Ile) influence the activity of purified DNA topoisomerases. Our results presented on Fig. 1 (upper panel) show that studied compounds did not appreciably inhibit DNA relaxation mediated by type I DNA topoisomerase. Effect of studied compounds on DNA relaxation mediated by topoisomerase IIα enzyme was much more pronounced and drug dose-dependent (Fig. 1, lower panel). In this case, the inhibition of DNA topoiosmerase II correlated with the cytotoxicity toward tumor cells. BAT and its **5f** derivative which showed moderate cytotoxic properties, were able to a certain degree inhibit DNA relaxation *in vitro*, at the concentration 10 μM. In contrast, 100 μM



Scheme 2. Preparation of Boc-Thr-Lys(BocX)-Pro-Arg(NO_2)−OMe **2a-f** and Boc-Arg(NO_2)-Pro-Lys(BocX)-Thr-OMe **2g-l**.



Scheme 3. Deprotection of a methyl ester groups in BAT-tuftsin (**2a-l**) and BAT-retro-tuftsin (**2g-l**) conjugates.

of a much less cytotoxic analog **5e** was sufficient to achieve the same inhibition rate. Two most cytotoxic analogs, **5k** and **5l**, where also the most potent topoisomerase IIα inhibitors, able to inhibit enzyme at a concentration close to 10 μM, that was similar to the effect induced by a positive control, etoposide (VP-16).

2.4. Effect of BAT and its analogues on cell cycle progression and induction of DNA damage

To evaluate the effect induced by studied BAT conjugates in tumor cells, we selected two representative compounds of un-

Table 1
Cytotoxic properties of BAT toward different types of human and murine cells.

Cell lines	BAT − IC_{50} ± SD [μM]
A549	46.3 ± 7.91
HCT 116	88.7 ± 23.46
HT-29	64.3 ± 12.42
LNCaP	90.2 ± 2.58
MCF-7	>100
HL-60	66.6 ± 4.26
P388	>100
L1210	>100
WEHI 1640	58.4 ± 4.17
NIH-3T3	69.9 ± 18.14

Table 2
Cytotoxic properties of new BAT analogs **5a-l** toward tumor cells.

Compound	IC_{50} value [μM]	
	A549 cells	HL-60 cells
BAT	46.3 ± 7.9	66.6 ± 4.4
5a (BAT-tuftsin)	52.8 ± 11.9	77.3 ± 12.6
5b	>100	>100
5c	5.9 ± 0.9	19.6 ± 3.8
5d	>100	>100
5e	>100	83.7 ± 25.8
5f	17.4 ± 2.0	15.4 ± 3.1
5g (BAT-retro-tuftsin)	8.7 ± 2.9	30.8 ± 6.1
5h	>100	28.7 ± 6.1
5i	5.6 ± 0.9	11.1 ± 2.4
5j	13.1 ± 3.9	11.0 ± 1.5
5k	4.0 ± 0.5	5.3 ± 1.8
5l	4.8 ± 0.4	4.7 ± 0.6

branched conjugates (compound **5a**) and Leu-branched conjugate (compound **5k**) and compared it with the effect exerted by the parent compound. Treatment of tumor cells with BAT and its two conjugates, at doses corresponding to their IC_{90} concentrations, resulted in a rapid (within 3 h) phosphorylation of the H2AX histone (Suppl. Fig. 1) which is a typical marker of double-stranded DNA breaks. Analysis of cell cycle progression (Fig. 2) and nuclear morphology (Fig. 3) after treatment of tumor cells with studied compounds revealed that biological action of BAT and its tuftsin or

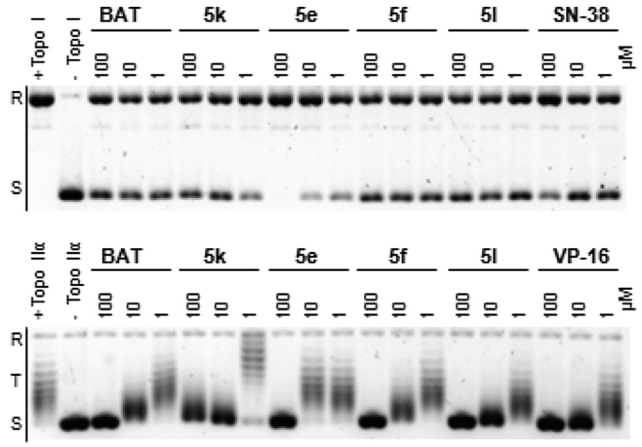


Fig. 1. *In vitro* inhibition of the catalytic activity of type I (upper panel) and type IIα (lower panel) DNA topoisomerases human pure enzymes by batracylin and its tuftsin conjugates. For comparison, effect of two standard inhibitors of the two enzymes (active metabolite of irinotecan, compound SN-38, and etoposide) is shown, as a positive. R − relaxed DNA; T − DNA topoisomers; S − supercoiled DNA.

retro-tuftsin analogs was cell-type specific. In A549 cells, these compounds arrested cell cycle progression in G2/M phase already after 24h, and this effect was further increased after the next four days of treatment. Additionally, for **5a** and **5k** analogs an increasing fraction of cell debris was observed in drug-treated cell population, which can be attributed to the induction of cell death by these compounds and fragmentation of dying cells (Fig. 2). The **5a** BAT-tuftsin conjugate increased debris fraction by about 3.5-fold, compared to **BAT**. Lack of mitotic cell in drug-treated tumor cells, as revealed by fluorescence microscopy after DNA staining with Hoechst 33342 (Fig. 3), together with our results obtained by flow cytometry analysis, indicated that A549 cells arrest their proliferation in G2 phase. Interestingly, A549 cells which did not undergo apoptosis after further prolonged drug exposure finally acquired features of premature senescent cells. As can be seen on Fig. 3, cell nuclei become progressively much larger after drug exposure than these observed in non-treated cells, also majority of cells become flat and greatly enlarged (Suppl. Fig. 2). Furthermore, increased activity of senescence-associated β-galactosidase was observed in drug-treated cells (Suppl. Fig. 3). All these features confirm that premature senescence was induced by BAT and its tuftsin/retro-tuftsin conjugates in A549.

In contrast to A549 tumor cell model, HL-60 leukemia cells after 24 h treatment with BAT and **5a** and **5k** analogs do not stop their cell cycle in G_2 phase similar to A549 cells, but enter mitosis. Lack of prolonged G_2M arrest in HL-60 after cell may be related to the absence of p53 in this cells, which leads to aberrant mitoses and polyploidy [31]. As shown on Fig. 1 and Suppl. Fig. 2, a high fraction of abnormal mitotic figures was observed in HL-60 cells treated with studied compounds. These included lagging telophase cells, cells with abnormal segregation of chromosomes during mitosis as well as multipolar mitoses. Most of these abnormal cell divisions resulted in mitotic catastrophe and led eventually to cell death by apoptosis. However, some of drug-treated HL-60 cells underwent polyploidization or micronucleation and only those cells had intact cell membrane and were still alive after 120 h of treatment (stained with fluorescein diacetate) (Suppl. Fig. 2). Similarly to A549, both investigated conjugates induced cell death and increased cell debris fraction by about two times compared to BAT. At the same time, both conjugates induced much lower fraction of polyploid or micronucleated cells as compared to BAT.

3. Summary

We describe here the synthesis of a series of new BAT analogs combined with tuftsin/retro-tuftsin derivatives. The aim of our design and synthesis these compounds as they should have increased water solubility and lower general toxicity that results from acetylated amino group of BAT. In our conjugates, this amine position is used to covalently link tuftsin/retro-tuftsin derivatives the BAT so its acetylation and consequently acetyl-BAT toxicity should be decreased. We confirmed experimentally our initial hypothesis that by conjugation of BAT with tuftsin and in particular retro-tuftsin derivatives we were able to increase the cytotoxicity of these compounds more that 10-fold.

We showed that BAT but also cytotoxic tuftsin/retro-tuftsin conjugates inhibit the catalytic activity of type II DNA topoisomerase and at cytotoxic concentrations produce rapidly DNA damage (double stranded DNA breaks) in drug-treated tumor cells. This was associated with the activation DNA damage response and induction of cells death by apoptosis or premature senescence, depending of the type of tumor cells used and p53 functionality. If this is true, induction of p53 by DNA damage produced by BAT and its conjugates in A549 cells (functional p53 pathway) should lead to premature senescence [32] but in cells with inactivated p53 (HL-60) where G2 arrest induced in the presence of DNA damage is only transient, drug-treated tumor cells enter abnormal mitosis and undergo cells death. We concluded that the increased cytotoxicity of tuftsin/retro-tuftsin conjugates of BAT is most probably not associated with important differences in the mechanism of their action on the cellular level. We propose this greatly enhanced cytotoxic effect should be rather related to increased bioavailability of new BAT derivatives that leads to their much higher accumulation in tumor cells thus much effective tumor cell killing.

The molecular mechanism of increased accumulation of BAT-tuftsin/retro-tuftsin conjugates can only be speculated and requires further studies. Tuftsin was shown to be translocated into living cells, most probably by endocytosis [33]. Tuftsin receptor has been isolated and partially characterized many years ago [35] but its function and particularly amino acid sequence remains still obscure although it has been found to be expressed in human tumor cells [36]. More recent studies have shown that tuftsin binds specifically to neuropilin-1 receptor (NRP-1), a tyrosine kinase VEGF co-receptor, and this binding inhibits the interaction between NRP-1 and VEGFR [37]. It was even proposed that biological effect of tuftsin is directly associated with its binding to NRP-1 [38]. There is an intriguing possibility that interaction of tuftsin with NRP-1 may stimulate tuftsin internalization and lead to the increased bioavailability of tuftsin conjugates.

4. Experimental section

Melting points (uncorrected) were determined on the Kofler-block apparatus. All chemicals and solvents were of reagent grade

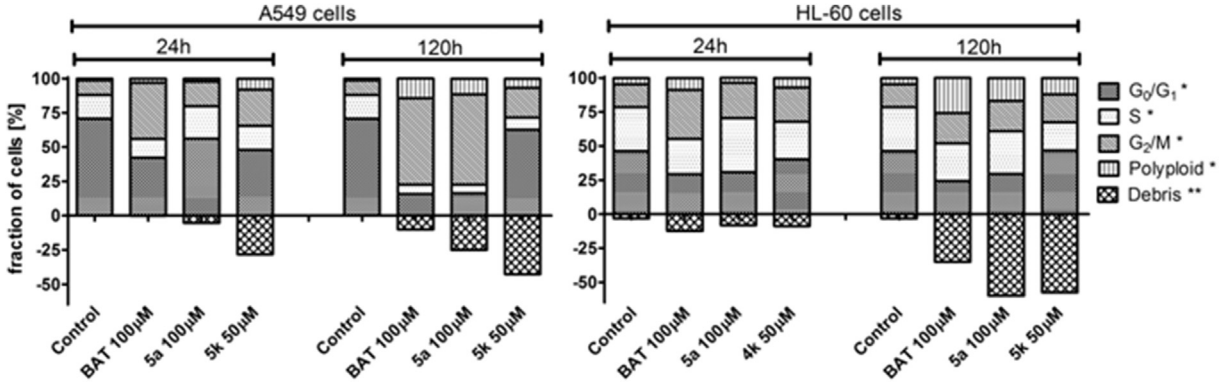


Fig. 2. Effect of studied compounds on the cell cycle distribution of tumor cells. Cells were exposed to specified drug doses for the time indicated and analyzed by flow cytometry. * Measured as a fraction of living cells; ** Measured as a fraction of sub-G1 of the total counted events.

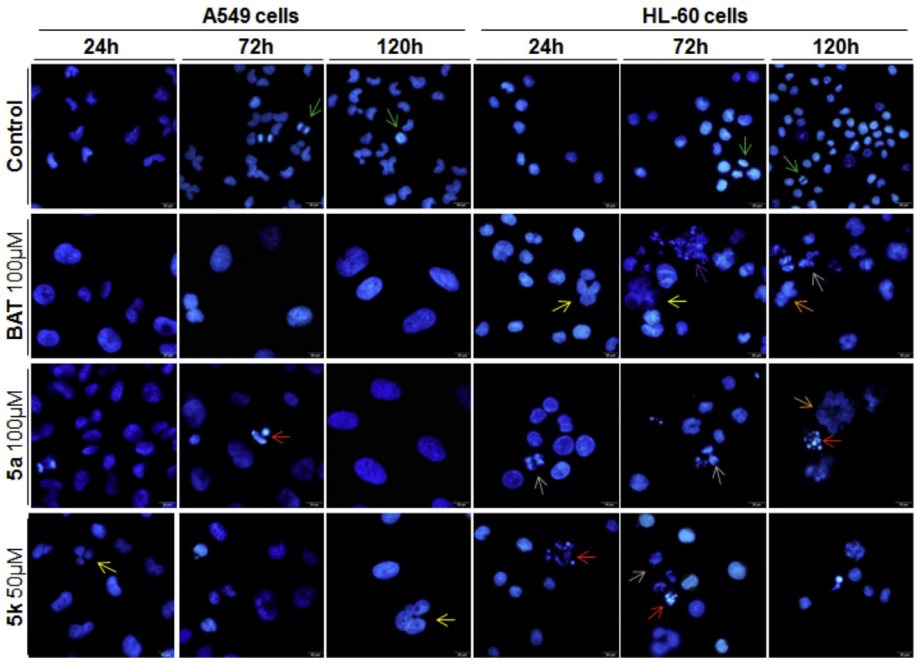


Fig. 3. Nuclear morphology of tumor cells treated with **BAT** and **5a**, **5k** analogs for the time indicated. After drug treatment cells were stained with Hoechst 33342 and analyzed by fluorescence microscopy. Green arrow ⟶ mitoses; yellow arrow ⟶ polyploid cells; orange arrow ⟶ multiple micronuclei; gray arrow ⟶ lagging telophases; purple arrow ⟶ mitotic catastrophes; red arrow ⟶ apoptotic bodies; scale bar 20 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and were used without further purification. The reactions were monitored by TLC on Merck F254 silica gel pre-coated plates. The following solvent systems (by vol.) were used for TLC development: $CHCl_3$–MeOH (7:1, v/v) (A), $CHCl_3$–MeOH (9:1, v/v) (B), $CHCl_3$–MeOH (10:1, v/v) (C), $CHCl_3$–MeOH (20:1, v/v) (D). The detection was carried out using UV and ninhydrin. MS spectra were recorded on matrix-assisted laser desorption/ionization-time on flight mass spectrometry (MALDI-TOF MS, Biflex III Bruker). Proton and carbon NMR spectra were recorded in DMSO-d_6 (isotopic enrichment 99.95%) solutions at 293 K using a Varian Unity 500 Plus spectrometer (500.13 MHz for ^{1}H, 125.76 MHz for ^{13}C) with a sample concentrations collected in Table 1 (Suppl.), using 5 mm inverse detection broadband probes and deuterium lock. The central peak of DMSO-d_6 signals (2.49 ppm for ^{1}H and 39.50 ppm for ^{13}C) was used as the internal reference standard.

The analysis HPLC compounds **5** were performed using Agilent liquid chromatograph series 1290 (Agilent Technology, Waldbronn, Germany) consisting of binary pump G4220A, autosampler G4226A, thermostated column compartment G1316C, diode-array detector G1315C. Chromatographic column: Supelcosil C-18, (4.6 × 150 mm), 3 μm, Supelco. Analytes were dissolved in a mixture of DMSO/MeOH (1:1; *v/v*) and 2 μL of the solution were injected onto the chromatographic column. A mixture of **A:** 0.1% HCOOH in water **B:** 0.1% HCOOH in ACN/MeOH (1:1; v/v) was used as a mobile phase in gradient mode at flow rate of 2.0 mL min^{-1}. Gradient: at 5 min 20% B; at 15 min 100% B for analyzed compounds. The UV–Vis detector was operated at 254, 210 and 580 nm (DAD in single wavelength mode). All analyses were thermostated at 40 °C.

4.1. 8-Aminoisoindolo[1,2-b]quinazolin-12(10H)-one (BAT) *1*

This compound was prepared according to the method described by Dzierzbicka et al. [20] from *N*-[2-(phthalimidomethyl)-1,4-acetyl]phenylenediamine and recrystallized from DMF.

4.2. Tuftsin/retro-tuftsin derivatives *2* and *3*

The procedure for the synthesis of compounds **2a**, **2c**–**d**, **2g** and **2i** by the mixed anhydride method has been published previously [19–26]. The compounds **2b**, **2e,f**, **2h**, **2j**–**l** were prepared to the same procedure. The Boc-protecting groups in Boc-Pro-Arg(NO_2)–OMe or Boc-Lys(Z)-Thr-OMe were removed by treatment with TFA and then coupling with Boc-Lys(Z)-OH or Boc-Pro-OH gave compounds: Boc-Lys(Z)-Pro-Arg(NO_2)–OMe or Boc-Pro-Lys(Z)-Thr-OMe. Next the Boc-protecting groups were removed and then coupling with Boc-Thr-OH or Boc-Arg(NO_2)–OH gave tetrapeptides. The Z-protecting groups were removed from Boc-Thr-Lys(Z)-Pro-Arg(NO_2)–OMe or Boc-Arg(NO_2)-Pro-Lys(Z)-Thr-OMe by catalytic hydrogenation over 10% palladium on charcoal and then coupled with Boc-X-OH (X: H, Gly, Ala, Val, Leu, Ile) to give pentapetides Boc-Thr-Lys(Boc-X)-Pro-Arg(NO_2)–OMe (**2a**–**f**) or Boc-Arg(NO_2)-Pro-Lys(BocX)-Thr-OMe (**2g**–**l**). Finally, the OMe-protecting group was removed from peptides **2a**–**l** by treatment with LiOH/MeOH to give the free *C*-terminal groups derivatives **3a**–**l**. The chemical characteristics of new compounds are presented below.

4.2.1. Boc-Thr-Lys(BocGly)-Pro-Arg(NO_2)–OMe *2b*

Yield 46%, mp. 106–111 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.04 (d, J = 8.4 Hz, 3H, γ-T4), 1.29 (m, 2H, γ-K4), 1.31 (m, 2H, δ-K5), 1.37 (s, 9H, Boc), 1.43 (m, 1H, β-K3a), 1.56 (m, 1H, γ-R4a), 1.56 (m, 1H, β-K3b), 1.74 (m, 1H, γ-R4b), 1.74 (m, 2H, β-R3), 1.91 (m, 2H, γ-P4), 2.03 (m, 2H, β-P3), 2.29 (m, 1H, ε-K6a), 3.07 (m, 1H, ε-K6b), 3.20 (m, 2H, δ-R5), 3.54 (s, 1H, R2-OH), 3.60 (m, 2H, α-G2), 3.63 (m, 2H, δ-P5), 3.78 (m, 1H, β-T3), 3.86 (d, J = 7.6 Hz, 1H, α-T2)), 4.24 (m, 1H, α-R2), 4.29 (d, J = 6.3 Hz, 1H, α-P2), 4.39 (m, 1H, α-K2), 6.96 (d, J = 7.0 Hz, 1H, α-TN), 7.40 (d, J = 7.0 Hz, 1H, α-GN), 7.47 (m, 1H, δ-KeN), 7.80 (d, J = 7.0 Hz, 1H, α-KaN), 8.37 (bs, 1H, δ-RdN), 8.36 (d, J = 7.5 Hz, 1H, α-RaN). MS $[M+H]^+$ *m/z* calcd for $C_{34}H_{60}N_{10}O_{13}$ 816.43, found 816.80. R_f = 0.52 (B). $[\alpha]_D^{20}$ −28 (c 0.5, $CHCl_3$).

*4.2.2. Boc-Thr-Lys(BocLeu)-Pro-Arg(NO_2)–OMe **2e**. Yield 39%, mp. 109–114 °C*

^{1}H NMR (500 MHz, DMSO) δ ppm: 0.81 (d, *J* = 3.7 Hz, 3H, δ1-L5), 0.84 (d, *J* = 4.2 Hz, 3H, δ2-L6), 1.00 (d, *J* = 8.0 Hz, 3H, γ-T4), 1.28 (m, 2H, γ-K4), 1.32 (d, *J* = 3.9 Hz, 2H, β-L3), 1.35 (m, 2H, δ-K5), 1.36 (s, 9H, Boc), 1.46 (m, 1H, β-K3a), 1.54 (m, 1H, γ-R4a), 1.57 (d, *J* = 5.2 Hz, 1H, γ-L4), 1.60 (m, 1H, β-K3b), 1.72 (m, 1H, γ-R4b), 1.72 (m, 2H, β-R3), 1.83 (m, 2H, γ-P4), 2.03 (m, 2H, β-P3), 2.69 (m, 1H, ε-K6a), 2.99 (m, 1H, ε-K6b), 3.13 (m, 2H, δ-R5), 3.59 (s, 1H, R2-OH), 3.63 (m, 2H, δ-P5), 3.85 (m, 1H, β-T3), 3.85 (d, *J* = 7.9 Hz, 1H, α-T2)), 4.09 (m, 1H, α-L2), 4.21 (m, 1H, α-R2), 4.31 (d, *J* = 6.3 Hz, 1H, α-P2), 4.47 (m, 1H, α-K2), 6.38 (d, *J* = 7.2 Hz, 1H, α-TN), 7.58 (d, *J* = 7.6 Hz, 1H, α-LN), 7.67 (m, 1H, δ-KeN), 7.84 (d, *J* = 7.0 Hz, 1H, α-KaN), 8.26 (d, *J* = 7.9 Hz, 1H, α-RaN), 8.58 (bs, 1H, δ-RdN). MS $[M+H]^+$ *m/z* calcd for $C_{38}H_{68}N_{10}O_{13}$ 872.50, found 873.80. R_f = 0.51 (B). $[\alpha]_D^{20}$ −26 (c 0.5, $CHCl_3$).

*4.2.3. Boc-Thr-Lys(BocIle)-Pro-Arg(NO_2)–OMe **2f***

Yield 43%, mp. 108–112 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.88 (brs, 6H, γ1-IMe3, δ-IMe5), 1.14 (d, *J* = 4.0 Hz, 2H, γ2-I4), 1.07 (d, *J* = 8.2 Hz, 3H, γ-T4), 1.27 (m, 2H, γ-K4), 1.33 (m, 2H, δ-K5), 1.36 (s, 9H, Boc), 1.48 (m, 1H, β-K3a), 1.54 (m, 1H, γ-R4a), 1.54 (m, 1H, β-K3b), 1.70 (m, 1H, γ-R4b), 1.70 (m, 2H, β-R3), 1.95 (m, 2H, γ-P4), 2.10 (m, 2H, β-P3), 2.31 (m, 1H, ε-K6a), 3.04 (m, 1H, ε-K6b), 3.18 (m, 2H, δ-R5), 3.57 (s, 1H, R2-OH), 3.60 (m, 2H, δ-P5), 3.80 (m, 1H, β-T3), 3.83 (m, 1H, β-I3), 3.84 (d, *J* = 7.0 Hz, 1H, α-T2), 3.90 (m, 1H, α-I2), 4.26 (m, 1H, α-R2), 4.29 (d, *J* = 6.0 Hz, 1H, α-P2), 4.40 (m, 1H, α-K2), 6.20 (d, *J* = 9.0 Hz, 1H, α-IN), 6.97 (d, *J* = 7.1 Hz, 1H, α-TN), 7.52 (m, 1H, δ-KeN), 7.84 (d, *J* = 7.7 Hz, 1H, α-KaN), 8.41 (bs, 1H, δ-RdN), 8.44 (d, *J* = 7.4 Hz, 1H, α-RaN). MS $[M+H]^+$ *m/z* calcd for $C_{38}H_{68}N_{10}O_{13}$ 872.50, found 873.10. R_f = 0.52 (B). $[\alpha]_D^{20}$ −38 (c 0.5, $CHCl_3$).

*4.2.4. Boc-Arg(NO_2)-Pro-Lys(BocGly)-Thr-OMe **2h***

Yield 42%, mp. 107–110 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.05 (d, *J* = 8.4 Hz, 3H, γ-T4), 1.36 (m, 2H, γ-K4), 1.37 (s, 9H, Boc), 1.40 (m, 2H, δ-K5), 1.49 (m, 2H, β-R3), 1.53 (m, 2H, β-K3), 1.59 (m, 2H, γ-R4), 1.84 (m, 2H, β-P3), 1.91 (m, 1H, γ-P4), 2.97 (m, 1H, ε-K6), 3.13 (m, 2H, δ-R5), 3.43 (d, *J* = 6.4 Hz, 1H, α-P2), 3.55 (m, 2H, δ-P5), 3.60 (m, 2H, α-G2), 3.63 (s, 3H, T-$COOCH_3$), 4.12 (m, 1H, β-T3), 4.16 (m, 1H, α-R2), 4.28 (m, 1H, α-K2), 4.29 (d, *J* = 6.8 Hz, 1H, α-T2), 5.00 (s, 1H, T3-OH), 6.92 (d, *J* = 7.0 Hz, 1H, α-RaN), 7.38 (d, *J* = 8.0 Hz, 1H, α-GN), 7.80 (d, *J* = 6.8 Hz, 1H, α-TN), 7.83 (m, 1H, δ-KeN), 8.01 (d, *J* = 7.0 Hz, 1H, α-KaN), 8.49 (bs, 1H, δ-RdN). MS $[M+H]^+$ *m/z* calcd for $C_{34}H_{60}N_{10}O_{13}$ 816.43, found 817.10. R_f = 0.50 (B). $[\alpha]_D^{20}$ −18 (c 0.5, $CHCl_3$).

*4.2.5. Boc-Arg(NO_2)-Pro-Lys(BocVal)-Thr-OMe **2j***

Yield 32%, mp. 136–138 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.97 (d, *J* = 4.9 Hz, 6H, δ1-V4, δ2-V5), 0.97 (d, *J* = 4.6 Hz, 1H, β-V3), 1.05 (d, *J* = 7.6 Hz, 3H, γ-T4), 1.31 (m, 2H, γ-K4), 1.38 (s, 9H, Boc), 1.40 (m, 2H, δ-K5), 1.51 (m, 2H, β-K3), 1.55 (m, 2H, γ-R4), 1.62 (m, 2H, β-R3), 1.84 (m, 2H, β-P3), 1.91 (m, 1H, γ-P4), 2.97 (m, 1H, ε-K6), 3.14 (m, 2H, δ-R5), 3.57 (m, 2H, δ-P5), 3.63 (s, 3H, T-$COOCH_3$), 4.13 (m, 1H, β-T3), 4.16 (m, 1H, α-R2), 4.29 (m, 1H, α-K2), 4.26 (d, *J* = 6.3 Hz, 1H, α-T2), 4.32 (d, *J* = 5.9 Hz, 1H, α-P2), 4.99 (s, 1H, T3-OH), 5.04 (m, 2H, α-V2), 7.01 (d, *J* = 7.9 Hz, 1H, α-RaN), 7.02 (d, *J* = 7.0 Hz, 1H, α-VN), 7.81 (d, *J* = 7.2 Hz, 1H, α-TN), 7.84 (m, 1H, δ-KeN), 8.22 (d, *J* = 7.0 Hz, 1H, α-KaN), 8.49 (bs, 1H, δ-RdN). MS $[M+H]^+$ *m/z* calcd $C_{37}H_{56}N_{10}O_{13}$ 858.48, found 856.60. R_f = 0.54 (B); $[\alpha]_D^{20}$ −21 (c 0.5, $CHCl_3$).

*4.2.6. Boc-Arg(NO_2)-Pro-Lys(BocLeu)-Thr-OMe **2k***

Yield 39%, mp. 106–110 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.82 (d, *J* = 6.4 Hz, 3H, δ1-L5), 0.83 (d, *J* = 6.4 Hz, 3H, δ2-L6), 1.03 (d, *J* = 6.1 Hz, 3H, γ-T4), 1.34 (m, 2H, γ-K4), 1.37 (m, 2H, δ-K5), 1.41 (s, 9H, Boc), 1.46 (m, 2H, β-L3), 1.48 (m, 2H, δ-K5), 1.51 (m, 1H, β-R3), 1.52 (m, 1H, β-K3), 1.55 (m, 2H, γ-R4), 1.6 (m, 2H, γ-L4), 1.70 (m, 1H, β-R3), 1.72 (m, 1H, β-K3), 1.82 (m, 1H, β-P3), 1.93 (m, 2H, γ-P4), 2.06 (m, 1H, β-P3), 3.05 (m, 2H, ε-K6), 3.13 (m, 2H, δ-R5), 3.45 (m, 1H, δ-P5), 3.55 (m, 1H, δ-P5), 3.55 (s, 3H, T-$COOCH_3$), 4.0 (dt, *J* = 5.2 Hz, *J* = 9.0 Hz, 1H, α-L2), 4.13 (m, 1H, β-T3), 4.18 (m, 1H, α-R2), 4.33 (dd, *J* = 3.4 Hz, *J* = 8.5 Hz, α-T2), 4.29 (m, 1H, α-K2), 4.4 (dd, *J* = 4.0 Hz, *J* = 8.3 Hz, 1H, α-P2), 5.00 (s, 1H, T3-OH), 7.37 (m, 1H, δ-KeN), 7.55 (bs, 1H, α-LN), 7.74 (d, *J* = 7.6 Hz, 1H, α-TN), 7.78 (d, *J* = 8.5 Hz, 1H, α-TN), 7.92 (d, *J* = 7.3 Hz, 1H, α-KaN), 7.98 (d, *J* = 7.3 Hz, 1H, α-RaN), 8.18 (bs, 1H, δ-RdN), 8.03 (t, *J* = 5.5 Hz, ε-K6), 8.1 (d, *J* = 7.8 Hz, 1H, α-KN), 8.44 (bs, 1H, δ-R5). MS $[M+H]^+$ *m/z* calcd for $C_{38}H_{68}N_{10}O_{13}$ 872.50, found 873.60. R_f = 0.52 (B). $[\alpha]_D^{20}$ −24 (c 0.5, $CHCl_3$).

*4.2.7. Boc-Arg(NO_2)-Pro-Lys(BocIle)-Thr-OMe **2l***

Yield 37%, mp. 101–104 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.82 (brs, 6H, γ1-IMe3, δ-IMe5), 1.04 (d, *J* = 7.9 Hz, 3H, γ-T4), 1.21 (m, 2H, γ-K4), 1.38 (m, 2H, δ-K5), 1.36 (s, 9H, Boc), 1.48 (m, 2H, δ-K5), 1.52 (m, 1H, β-K3), 1.55 (d, *J* = 3.5, 2H, γ2-I4), 1.55 (m, 1H, β-R3), 1.59 (m, 2H, γ-R4), 1.59 (m, 1H, β-R3), 1.68 (m, 1H, β-K3), 1.82 (m, 1H, β-P3), 2.02 (m, 2H, γ-P4), 2.06 (m, 1H, β-P3), 3.03 (m, 2H, ε-K6), 3.08 (m, 2H, δ-R5), 3.50 (m, 1H, δ-P5), 3.61 (m, 1H, δ-P5), 3.85 (s, 3H, T-$COOCH_3$), 3.99 (m, 1H, α-I2), 4.24 (m, 1H, α-K2), 4.29 (m, 1H, β-T3), 4.34 (m, 1H, α-R2), 4.86 (d, *J* = 3.0 Hz, *J* = 8.3 Hz, α-T2), 4.87 (dd, *J* = 4.5 Hz, *J* = 8.9 Hz, 1H, α-P2), 5.02 (s, 1H, T3-OH), 6.44 (d, *J* = 7.8 Hz, 1H, α-RaN), 7.39 (m, 1H, δ-KeN), 7.65 (d, *J* = 8.4 Hz, 1H, α-IN), 7.74 (d, *J* = 7.6 Hz, 1H, α-TN), 7.78 (d, *J* = 8.8 Hz, 1H, α-TN), 7.98 (d, *J* = 6.9 Hz, 1H, α-KaN), 8.10 (bs, 1H, δ-RdN), 8.10 (t, *J* = 5.0 Hz, ε-K6), 8.11 (d, *J* = 7.4 Hz, 1H, α-KN), 8.44 (bs, 1H, δ-R5). MS $[M+H]^+$ *m/z* calcd for $C_{38}H_{68}N_{10}O_{13}$ 872.50, found 873.40; R_f = 0.56 (B). $[\alpha]_D^{20}$ −42.5 (c 0.5, $CHCl_3$).

*4.3. General procedure for synthesis of BAT-tuftsin/retro-tuftsin analogues **4a**–**l***

To a mixture of BAT **1** (0.125 mmol) and tuftsin or retro-tuftsin derivatives **3a**–**l** (0.125 mmol) in anhydrous DMF (500 μL) was added anhydrous pyridine (3.09 mmol) followed by T3P (0.31 mmol, 50% solution in DMF) in drops. The mixture was cooled to −15 °C under nitrogen atmosphere for 4 h then for 24 h at room temperature. After evaporating the solvent *in vacuo* the residue was taken up in ethyl acetate. The solution was washed successively with 10% citric acid, water, 5% $NaHCO_3$ solution and water (3 times in each case). The product was purified by TLC.

*4.3.1. Boc-Thr-Lys(Boc)-Pro-Arg(NO_2)-BAT **4a***

Yield 41%, mp. 192–195 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.22 (d, *J* = 8.6 Hz, 3H, γ-T4), 1.32 (s, 9H, Boc), 1.49 (m, 2H, γ-K4), 1.49 (m, 2H, δ-K5), 1.81 (m, 1H, γ-R4a), 1.79 (m, 1H, β-K3a), 1.89 (m, 1H, β-K3b), 1.90 (m, 1H, γ-R4b), 2.06 (m, 2H, γ-P4), 2.8 (m, 2H, β-P3), 2.34 (m, 2H, β-R3), 3.17 (m, 1H, ε-K6a), 3.23 (m, 1H, ε-K6b), 3.42 (m, 2H, δ-R5), 3.86 (d, *J* = 6.3 Hz, 1H, α-P2), 4.34 (d, *J* = 7.6 Hz, 1H, α-T2), 4.36 (m, 1H, β-T3), 4.51 (m, 1H, α-K2), 4.55 (m, 2H, δ-P5), 4.78 (m, 1H, α-R2), 4.91 (s, 2H, 10), 4.99 (s, 1H, T3-OH), 6.39 (d, *J* = 7.8 Hz, 1H, α-TN), 7.35 (bdb, *J* = 8.0 Hz, 1H, 6), 7.37 (m, 1H, δ-KeN), 7.54 (bd, *J* = 8.0 Hz, 1H, 7), 7.58 (bs, 1H, 9), 7.73 (t, *J* = 7.0 Hz, 1H, 3), 7.78 (t, *J* = 7.0 Hz, 1H, 2), 7.80 (d, *J* = 7.3 Hz, 1H, α-RaN), 7.87 (d, *J* = 7.0 Hz, 1H, 4), 7.92 (d, *J* = 7.3 Hz, 1H, α-KaN), 7.98 (d, *J* = 7.0 Hz, 1H, 1), 8.18 (bs, 1H, δ-RdN), 10.12 (s, 1H, 8″-CONH); ^{13}C NMR (DMSO, 125 MHz, 30.0 °C: 177.55 (C-P1), 172.73 (C-T1), 172.03 (C-R1), 171.55 (C-K1), 167.04 (C-12), 160.70 (C-R6), 156.86 (C-B1), 156. 68 (C-BK1), 148.65 (C-4b), 139.37 (C-5a), 135.33 (C-4a), 135.37 (C-8), 133.65 (C-2), 132.73 (C-3), 131.20 (C-12a), 128.88 (C-6), 123.46 (C-4), 123.19 (C-9a), 122.53 (C-1), 120.05 (C-7), 118.67 (C-9), 79.47 (C-B2), 79.45 (C-BK2), 68.32 (C-T2), 61.71 (C-P2), 60.52 (C-T3), 60.34 (C-R2), 57.69

(C-K2), 48.32 (C-P5), 47.75 (C-R5), 41.25 (C-10), 40.73 (C-K6), 32.56 (C-K3), 31.51 (C-P3), 30.41 (C-R3), 30.02 (C-B5), 29.95 (C-BK5), 29.80 (C-B4), 29.64 (C-R4), 29.49 (C-K5), 29.33 (C-BK4), 28.63 (C-B3), 28.48 (C-BK3), 25.93 (C-P4), 23.38 (C-K4), 22.98 (C-T4). MS $[M+H]^+$ *m/z* calcd for $C_{46}H_{64}N_{12}O_{12}$ 977.07, found 978.50. $R_f = 0.45$ (C).

4.3.2. Boc-Thr-Lys(BocGly)-Pro-Arg(NO_2)-BAT ***4b***

Yield 38%, mp. 187–190 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.28 (d, *J* = 9.0 Hz, 3H, γ-T4), 1.37 (s, 9H, Boc), 1.42 (m, 2H, γ-K4), 1.42 (m, 2H, δ-K5), 1.88 (m, 1H, γ-R4a), 1.92 (m, 1H, β-K3a), 1.93 (m, 1H, β-K3b), 1.97 (m, 1H, γ-R4b), 2.14 (m, 2H, γ-P4), 2.29 (m, 2H, β-P3), 2.32 (m, 2H, β-R3), 3.20 (m, 1H, ε-K6a), 3.37 (m, 1H, ε-K6b), 3.45 (m, 2H, δ-R5), 3.62 (m, 2H, α-G2), 3.89 (d, *J* = 6.5 Hz, 1H, α-P2), 4.28 (d, *J* = 7.8 Hz, 1H, α-T2), 4.29 (m, 1H, β-T3), 4.52 (m, 1H, α-K2), 4.53 (m, 2H, δ-P5), 4.90 (m, 1H, α-R2), 4.93 (s, 2H, 10), 5.00 (s, 1H, T3-OH), 7.35 (bdb, *J* = 8.0 Hz, 1H, 6), 7.37 (m, 1H, δ-KeN), 7.39 (d, *J* = 7.6 Hz, 1H, α-GN), 7.67 (bd, *J* = 8.0 Hz, 1H, 7), 7.68 (bs, 1H, 9), 7.74 (d, *J* = 7.6 Hz, 1H, α-TN), 7.76 (t, *J* = 7.4 Hz, 1H, 3), 7.79 (t, *J* = 7.5 Hz, 1H, 2), 7.80 (d, *J* = 7.4 Hz, 1H, α-RaN), 7.86 (d, *J* = 7.4 Hz, 1H, 4), 7.91 (d, *J* = 7.3 Hz, 1H, α-KaN), 7.99 (d, *J* = 7.5 Hz, 1H, 1), 8.15 (bs, 1H, δ-RdN), 9.54 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for $C_{48}H_{67}N_{13}O_{13}$ 1034.12, found 1035.70. $R_f = 0.43$ (C).

4.3.3. Boc-Thr-Lys(BocAla)-Pro-Arg(NO_2)-BAT ***4c***

Yield 29%, mp. 195–198 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.22 (d, *J* = 8.4 Hz, 3H, γ-T4), 1.31 (m, 3H, β-A3), 1.39 (s, 9H, Boc), 1.48 (m, 2H, γ-K4), 1.48 (m, 2H, δ-K5), 1.83 (m, 1H, γ-R4a), 1.82 (m, 1H, β-K3a), 1.89 (m, 1H, β-K3b), 1.89 (m, 1H, γ-R4b), 2.10 (m, 2H, γ-P4), 2.34 (m, 2H, β-R3), 2.46 (m, 2H, β-P3), 3.18 (m, 1H, ε-K6a), 3.44 (m, 1H, ε-K6b), 3.49 (m, 2H, δ-R5), 3.86 (d, *J* = 6.3, 1H, α-P2), 4.25 (m, 1H, α-A2), 4.34 (d, *J* = 7.5 Hz, 1H, α-T2), 4.36 (m, 1H, β-T3), 4.52 (m, 1H, α-K2), 4.55 (m, 2H, δ-P5), 4.71 (m, 1H, α-R2), 4.92 (s, 2H, 10), 4.99 (s, 1H, T3-OH), 6.49 (d, *J* = 7.0 Hz, 1H, α-AN), 7.36 (bdb, *J* = 8.0 Hz, 1H, 6), 7.37 (m, 1H, δ-KeN), 7.57 (bd, *J* = 8.0 Hz, 1H, 7), 7.59 (bs, 1H, 9), 7.69 (d, *J* = 7.3 Hz, 1H, α-TN), 7.74 (t, *J* = 7.0 Hz, 1H, 3), 7.79 (t, *J* = 7.0 Hz, 1H, 2), 7.80 (d, *J* = 7.3 Hz, 1H, α-RaN), 7.87 (d, *J* = 7.0 Hz, 1H, 4), 7.92 (d, *J* = 7.3 Hz, 1H, α-KaN), 7.98 (d, *J* = 7.0 Hz, 1H, 1), 8.17 (bs, 1H, δ-RdN), 10.15 (s, 1H, 8″-CONH). ^{13}C NMR (DMSO, 125 MHz, 30.0 °C: 178.48 (C-A1), 177.33 (C-P1), 177.29 (C-T1), 175.67 (C-R1), 172.29 (C-K1), 166.87 (C-12), 160.56 (C-R6), 156.89 (C-B1), 155.99 (C-BA1), 148.10 (C-4b), 139.37 (C-5a), 134.18 (C-4a), 135.96 (C-8), 133.14 (C-2), 132.20 (C-3), 130.82 (C-12a), 128.43 (C-6), 123.78 (C-4), 122,94 (C-9a), 122.25 (C-1), 119.41 (C-7), 118.17 (C-9), 80.86 (C-B2), 78.68 (C-BA2), 70.37 (C-T2), 67.78 (C-P2), 61.34 (C-T3), 59.64 (C-R2), 59.64 (C-K2), 50.40 (C-A2), 47.72 (C-P5), 40.73 (C-R5), 40.74 (C-10), 40.72 (C-K6), 32.64 (C-K3), 38.30 (C-P3), 29.74 (C-R3), 29.64 (C-B5), 29.49 (C-BA5), 29.33 (C-B4), 28.87 (C-B3), 28.72 (C-BA3), 27.93 (C-P4), 25.35 (C-K4), 22.22 (C-T4), 18.39 (C-A3). MS $[M+H]^+$ *m/z* calcd for $C_{49}H_{69}N_{13}O_{13}$ 1048.15, found 1048.90. $R_f = 0.43$ (C).

4.3.4. Boc-Thr-Lys(BocVal)-Pro-Arg(NO_2)-BAT ***4d***

Yield 25%, mp. 183–187 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.97 (d, *J* = 4.8 Hz, 6H, δ1-V4, δ2-V5), 1.02 (d, *J* = 4.5 Hz, 1H, β-V3), 1.17 (d, *J* = 8.0 Hz, 3H, γ-T4), 1.37 (s, 9H, Boc), 1.47 (m, 2H, γ-K4), 1.47 (m, 2H, δ-K5), 1.80 (m, 1H, γ-R4a), 1.82 (m, 1H, β-K3a), 1.89 (m, 1H, β-K3b), 1.96 (m, 1H, γ-R4b), 2.16 (m, 2H, γ-P4), 2.32 (m, 2H, β-P3), 2.34 (m, 2H, β-R3), 3.17 (m, 1H, ε-K6a), 3.45 (m, 1H, ε-K6b), 3.41 (m, 2H, δ-R5), 3.86 (d, *J* = 6.3 Hz, 1H, α-P2), 4.35 (d, *J* = 7.3 Hz, 1H, α-T2), 4.36 (m, 1H, β-T3), 4.37 (m, 1H, α-K2), 4.61 (m, 2H, δ-P5), 4.90 (m, 1H, α-R2), 4.92 (s, 2H, 10), 4.94 (m, 1H, α-V2), 4.99 (s, 1H, T3-OH), 7.04 (d, *J* = 7.0 Hz, 1H, α-VN), 7.36 (bdb, *J* = 8.0 Hz, 1H, 6), 7.37 (m, 1H, δ-KeN), 7.57 (bd, *J* = 9.0 Hz, 1H, 7), 7.59 (bs, 1H, 9), 7.74 (d, *J* = 7.8 Hz, 1H, α-TN), 7.75 (t, *J* = 7.0 Hz, 1H, 3), 7.78 (t, *J* = 7.0 Hz, 1H, 2), 7.80 (d, *J* = 7.3 Hz, 1H, α-RaN), 7.88 (d, *J* = 7.0 Hz, 1H, 4), 7.91 (d, *J* = 7.3 Hz, 1H, α-KaN), 7.98 (d, *J* = 7.0 Hz, 1H, 1), 8.16 (bs, 1H, δ-RdN), 10.07 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for $C_{51}H_{73}N_{13}O_{13}$ 1076.20, found 1077.60. $R_f = 0.45$ (C).

4.3.5. Boc-Thr-Lys(BocLeu)-Pro-Arg(NO_2)-BAT ***4e***

Yield 39%, mp. 185–188 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.82 (d, *J* = 2.9 Hz, 3H, δ1-L5), 0.83 (d, *J* = 3.9 Hz, 3H, δ2-L6), 1.28 (d, *J* = 7.6 Hz, 3H, γ-T4), 1.33 (d, *J* = 4.5 Hz, 2H, β-L3) 1.37 (s, 9H, Boc), 1.47 (m, 2H, γ-K4), 1.47 (m, 2H, δ-K5), 1.55 (d, *J* = 4.6 Hz, 1H, γ-L4), 1.79 (m, 1H, γ-R4a), 1.81 (m, 1H, β-K3a), 1.86 (m, 1H, β-K3b), 1.97 (m, 1H, γ-R4b), 2.05 (m, 2H, γ-P4), 2.28 (m, 2H, β-P3), 2.35 (m, 2H, β-R3), 3.14 (m, 1H, ε-K6a), 3.42 (m, 1H, ε-K6b), 3.44 (m, 2H, δ-R5), 3.90 (d, *J* = 6.5 Hz, 1H, α-P2), 4.08 (m, 1H, α-L2), 4.35 (d, *J* = 7.0 Hz, 1H, α-T2), 4.36 (m, 1H, β-T3), 4.39 (m, 1H, α-K2), 4.49 (m, 2H, δ-P5), 4.92 (m, 1H, α-R2), 4.92 (s, 2H, 10), 4.99 (s, 1H, T3-OH), 7.36 (bdb, *J* = 8.3 Hz, 1H, 6), 7.37 (m, 1H, δ-KeN), 7.57 (bd, *J* = 8.5 Hz, 1H, 7), 7.58 (bs, 1H, 9), 7.60 (d, *J* = 7.0 Hz, 1H, α-LN), 7.68 (d, *J* = 7.7 Hz, 1H, α-TN), 7.74 (t, *J* = 7.5 Hz, 1H, 3), 7.79 (t, *J* = 7.0 Hz, 1H, 2), 7.80 (d, *J* = 7.3 Hz, 1H, α-RaN), 7.88 (d, *J* = 7.8 Hz, 1H, 4), 7.89 (d, *J* = 7.3 Hz, 1H, α-KaN), 7.99 (d, *J* = 7.0 Hz, 1H, 1), 8.17 (bs, 1H, δ-RdN), 10.05 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for $C_{52}H_{75}N_{13}O_{13}$ 1090.23, found 1091.10. $R_f = 0.44$ (C).

4.3.6. Boc-Thr-Lys(BocIle)-Pro-Arg(NO_2)-BAT ***4f***

Yield 32%, mp. 191–195 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.90 (brs, 6H, γ1-IMe3, δ-IMe5), 1.16 (d, J = 3.9 Hz, 2H, γ2-I4), 1.20 (d, J = 7.8 Hz, 3H, γ-T4), 1.37 (s, 9H, Boc), 1.48 (m, 2H, γ-K4), 1.50 (m, 2H, δ-K5), 1.77 (m, 1H, γ-R4a), 1.79 (m, 1H, β-K3a), 1.91 (m, 1H, β-K3b), 1.91 (m, 1H, γ-R4b), 2.03 (m, 2H, γ-P4), 2.25 (m, 2H, β-P3), 2.25 (m, 2H, β-R3), 3.12 (m, 1H, ε-K6a), 3.25 (m, 1H, ε-K6b), 3.38 (m, 2H, δ-R5), 3.83 (m, 1H, β-I3), 3.89 (m, 1H, α-I2), 4.29 (d, J = 6.0 Hz, 1H, α-P2), 4.35 (d, J = 7.5 Hz, 1H, α-T2), 4.53 (m, 1H, β-T3), 4.55 (m, 1H, α-K2), 4.64 (m, 2H, δ-P5), 4.66 (m, 1H, α-R2), 4.94 (s, 2H, 10), 5.01 (s, 1H, T3-OH), 6.06 (d, J = 7.5 Hz, 1H, α-TN), 6.18 (d, J = 9.0 Hz, 1H, α-IN), 7.35 (bdb, J = 8.0 Hz, 1H, 6), 7.45 (m, 1H, δ-KeN), 7.57 (bd, J = 8.6 Hz, 1H, 7), 7.59 (bs, 1H, 9), 7.74 (t, J = 7.0 Hz, 1H, 3), 7.79 (t, J = 7.0 Hz, 1H, 2), 7.80 (d, J = 7.0 Hz, 1H, α-RaN), 7.86 (d, J = 7.0 Hz, 1H, 4), 7.92 (d, J = 7.5 Hz, 1H, α-KaN), 7.97 (d, J = 7.0 Hz, 1H, 1), 8.02 (bs, 1H, δ-RdN), 9.50 (s, 1H, 8″-CONH). MS $[M+H]^+$ m/z calcd for $C_{52}H_{75}N_{13}O_{13}$ 1090.23, found 1091.80. $R_f = 0.47$ (C).

4.3.7. Boc-Arg(NO_2)-Pro-Lys(Boc)-Thr-BAT ***4g***

Yield 41%, mp. 198–204 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.22 (d, *J* = 7.8 Hz, 3H, γ-T4), 1.33 (s, 9H, Boc), 1.45 (m, 2H, γ-K4), 1.89 (m, 2H, γ-R4), 1.97 (m, 1H, γ-P4a), 2.06 (m, 1H, γ-P4b), 2.07 (m, 2H, β-R3), 2.35 (m, 2H, β-P3), 2.98 (m, 2H, β-K3a, β-K3b), 3.01 (m, 2H, δ-K5), 3.14 (m, 1H, ε-K6a), 3.15 (m, 1H, ε-K6b), 3.41 (m, 2H, δ-R5), 3.56 (d, *J* = 7.7 Hz, 1H, α-P2), 4.12 (d, *J* = 7.7 Hz, 1H, α-T2), 4.15 (m, 1H, β-T3), 4.45 (m, 1H, α-K2), 4.64 (m, 2H, δ-P5), 4.67 (m, 1H, α-R2), 4.92 (s, 2H, 10), 5.00 (s, 1H, T3-OH), 7.11 (d, *J* = 7.0 Hz, 1H, α-RaN), 7.37 (bdb, *J* = 7.9 Hz, 1H, 6), 7.38 (m, 1H, δ-KeN), 7.52 (bd, *J* = 8.0 Hz, 1H, 7), 7.54 (bs, 1H, 9), 7.74 (t, *J* = 7.3 Hz, 1H, 3), 7.76 (d, *J* = 7.5 Hz, 1H, α-TN), 7.80 (t, *J* = 7.0 Hz, 1H, 2), 7.88 (d, *J* = 7.3 Hz, 1H, 4), 7.89 (d, *J* = 7.3 Hz, 1H, α-KaN), 7.99 (d, *J* = 6.8 Hz, 1H, 1), 8.28 (bs, 1H, δ-RdN), 9.92 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for $C_{46}H_{64}N_{12}O_{12}$ 977.07, found 978.40. $R_f = 0.45$ (C).

4.3.8. Boc-Arg(NO_2)-Pro-Lys(BocGly)-Thr-BAT ***4h***

Yield 30%, mp. 202–206 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.17 (d, *J* = 8.0 Hz, 3H, γ-T4), 1.37 (s, 9H, Boc), 1.44 (m, 2H, γ-K4), 1.85 (m, 2H, γ-R4), 2.02 (m, 1H, γ-P4a), 2.04 (m, 1H, γ-P4b), 2.05 (m, 2H, β-R3), 2.34 (m, 2H, β-P3), 2.62 (m, 2H, β-K3a, β-K3b), 2.68 (m, 2H, δ-K5), 3.17 (m, 1H, ε-K6a), 3.23 (m, 1H, ε-K6b), 3.39 (m, 2H, δ-R5), 3.64 (m, 2H, α-G2), 3.97 (d, *J* = 6.0 Hz, 1H, α-P2), 4.34 (d, *J* = 6.5 Hz, 1H, α-T2), 4.35 (m, 2H, δ-P5), 4.35 (m, 1H, α-R2), 4.39 (m, 1H, β-T3), 4.45

(m, 1H, α-K2), 4.91 (s, 2H, 10), 5.05 (s, 1H, T3-OH), 6.48 (d, $J = 7.3$ Hz, 1H, α-RaN), 7.35 (bdb, $J = 8.2$ Hz, 1H, 6), 7.37 (m, 1H, δ-KeN), 7.43 (d, $J = 7.3$ Hz, 1H, α-GN), 7.57 (bd, $J = 8.0$ Hz, 1H, 7), 7.59 (bs, 1H, 9), 7.69 (d, $J = 7.0$ Hz, 1H, α-TN), 7.74 (t, $J = 7.2$ Hz, 1H, 3), 7.81 (t, $J = 6.9$ Hz, 1H, 2), 7.87 (d, $J = 7.3$ Hz, 1H, 4), 7.89 (d, $J = 7.0$ Hz, 1H, α-KaN), 7.98 (d, $J = 7.2$ Hz, 1H, 1), 8.18 (bs, 1H, δ-RdN), 10.10 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for $C_{48}H_{67}N_{13}O_{13}$ 1034.12, found 1035.50. $R_f = 0.45$ (C).

4.3.9. Boc-Arg(NO$_2$)-Pro-Lys(BocAla)-Thr-BAT ***4i***

Yield 28%, mp. 213–217 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 1.20 (d, $J = 8.4$ Hz, 3H, γ-T4), 1.34 (m, 3H, β-A3), 1.37 (s, 9H, Boc), 1.48 (m, 2H, γ-K4), 1.80 (m, 2H, γ-R4), 2.01 (m, 1H, γ-P4a), 2.04 (m, 1H, γ-P4b), 2.11 (m, 2H, β-R3), 2.30 (m, 2H, β-P3), 2.85 (m, 2H, β-K3a, β-K3b), 2.87 (m, 2H, δ-K5), 3.18 (m, 1H, ε-K6a), 3.25 (m, 1H, ε-K6b), 3.38 (m, 2H, δ-R5), 3.92 (d, $J = 6.0$, 1H, α-P2), 4.28 (m, 2H, α-A2), 4.30 (m, 1H, α-K2), 4.38 (d, $J = 6.5$ Hz, 1H, α-T2), 4.55 (m, 2H, δ-P5), 4.55 (m, 1H, α-R2), 4.42 (m, 1H, β-T3), 4.92 (s, 2H, 10), 5.00 (s, 1H, T3-OH), 6.33 (d, $J = 7.6$ Hz, 1H, α-RaN), 6.79 (d, $J = 7.9$ Hz, 1H, α-AN), 7.36 (bdb, $J = 8.0$ Hz, 1H, 6), 7.50 (m, 1H, δ-KeN),7.56 (bd, $J = 7.9$ Hz, 1H, 7), 7.58 (bs, 1H, 9), 7.68 (d, $J = 7.5$ Hz, 1H, α-TN), 7.74 (t, $J = 7.2$ Hz, 1H, 3), 7.79 (t, $J = 7.4$ Hz, 1H, 2), 7.88 (d, $J = 7.6$ Hz, 1H, 4), 7.93 (d, $J = 7.0$ Hz, 1H, α-KaN), 7.99 (d, $J = 7.5$ Hz, 1H, 1), 8.18 (bs, 1H, δ-RdN), 9.92 (s, 1H, 8″-CONH). ^{13}C NMR (125 MHz, DMSO, 30.0 °C: 173.52 (C-A1), 172.16 (C-P1), 169.50 (C-T1), 163.52 (C-R1), 169,30 (C-K1), 166.57 (C-12), 160.51 (C-R6), 155.99 (C-B1), 155.97 (C-BA1), 148.06 (C-4b), 138.70 (C-5a), 136.58 (C-4a), 135.04 (C-8), 133.16 (C-2), 132.21 (C-3), 130.84 (C-12a), 128.35 (C-6), 122.97 (C-4), 122,63 (C-9a), 122.64 (C-1), 119.48 (C-7), 118.09 (C-9), 78.72 (C-B2), 78.55 (C-BA2), 67.33 (C-T2), 67.32 (C-P2), 61.34 (C-T3), 59.97 (C-R2), 59.20 (C-K2), 50.55 (C-P5), 47.79 (C-R5), 40.78 (C-10), 38.51 (C-K6), 30.69 (C-K3), 30.61 (C-P3), 29.74 (C-R3), 29.43 (C-B5), 29.34 (C-BA5), 29.18 (C-B4), 28.88 (C-B3), 28.72 (C-BA3), 27.91 (C-P4), 23.03 (C-K4), 20.06 (C-T4). MS $[M+H]^+$ *m/z* calcd for $C_{51}H_{73}N_{13}O_{13}$ 1076.20, found 1077.50. $R_f = 0.46$ (C).

4.3.10. Boc-Arg(NO$_2$)-Pro-Lys(BocVal)-Thr-BAT ***4j***

Yield 25%, mp. 203–206 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.85 (d, $J = 3.9$ Hz, 6H, δ1-V4, δ2-V5), 1.01 (m, 3H, β-V3), 1.22 (d, $J = 8.4$ Hz, 3H, γ-T4), 1.35 (s, 9H, Boc), 1.49 (m, 2H, γ-K4), 1.85 (m, 2H, γ-R4), 2.02 (m, 1H, γ-P4a), 2.04 (m, 1H, γ-P4b), 2.07 (m, 2H, β-R3), 2.34 (m, 2H, β-P3), 2.85 (m, 2H, β-K3a, β-K3b), 2.85 (m, 2H, δ-K5), 3.06 (m, 1H, ε-K6a), 3.10 (m, 1H, ε-K6b), 3.39 (m, 2H, δ-R5), 4.06 (d, $J = 6.0$ Hz, 1H, α-P2), 4.30 (m, 1H, α-K2), 4.35 (d, $J = 6.5$ Hz, 1H, α-T2), 4.37 (m, 2H, δ-P5), 4.38 (m, 1H, α-R2), 4.40 (m, 1H, β-T3), 4.92 (s, 2H, 10), 4.98 (s, 1H, T3-OH), 5.02 (m, 1H, α-V2), 6.55 (d, $J = 7.6$ Hz, 1H, α-RaN), 6.96 (d, $J = 8.4$ Hz, 1H, α-VN), 7.35 (bdb, $J = 7.9$ Hz, 1H, 6), 7.37 (m, 1H, δ-KeN), 7.56 (bd, $J = 7.9$ Hz, 1H, 7), 7.58 (bs, 1H, 9), 7.68 (d, $J = 7.8$ Hz, 1H, α-TN), 7.74 (t, $J = 7.0$ Hz, 1H, 3), 7.80 (t, $J = 7.1$ Hz, 1H, 2), 7.88 (d, $J = 7.2$ Hz, 1H, 4), 7.89 (d, $J = 7.0$ Hz, 1H, α-KaN), 7.98 (d, $J = 7.3$ Hz, 1H, 1), 8.16 (bs, 1H, δ-RdN), 9.90 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for 1076.20, found 1077.50. $R_f = 0.46$ (C).

4.3.11. Boc-Arg(NO$_2$)-Pro-Lys(BocLeu)-Thr-BAT ***4k***

Yield 30%, mp. 210–214 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.83 (d, $J = 2.9$ Hz, 3H, δ1-L5), 0.84 (d, $J = 3.8$ Hz, 3H, δ2-L6), 1.15 (d, $J = 8.4$ Hz, 3H, γ-T4), 1.34 (m, $J = 6.4$ Hz, 3H, β-L3), 1.35 (s, 9H, Boc), 1.48 (m, 2H, γ-K4), 1.85 (m, 2H, γ-R4), 2.01 (m, 1H, γ-P4a), 2.06 (m, 1H, γ-P4b), 2.10 (m, 2H, β-R3), 2.34 (m, 2H, β-P3), 2.87 (m, 2H, β-K3a, β-K3b), 2.85 (m, 2H, δ-K5), 3.00 (m, 1H, ε-K6a), 3.11 (m, 1H, ε-K6b), 3.38 (m, 2H, δ-R5), 4.06 (m, 1H, α-L2), 4.08 (d, $J = 6.0$ Hz, 1H, α-P2), 4.30 (m, 1H, α-K2), 4.38 (d, $J = 6.5$ Hz, 1H, α-T2), 4.55 (m, 2H, δ-P5), 4.55 (m, 1H, α-R2), 4.44 (m, 1H, β-T3), 4.91 (s, 2H, 10), 4.98 (s, 1H, T3-OH), 6.46 (d, $J = 7.6$ Hz, 1H, α-RaN), 7.35 (bdb, $J = 7.9$ Hz, 1H, 6), 7.44 (m, 1H, δ-KeN),7.56 (bd, $J = 7.9$ Hz, 1H, 7), 7.58 (bs, 1H, 9), 7.59 (d, $J = 8.4$ Hz, 1H, α-LN), 7.68 (d, $J = 7.8$ Hz, 1H, α-TN), 7.74 (t, $J = 7.0$ Hz, 1H, 3), 7.80 (t, $J = 7.1$ Hz, 1H, 2), 7.87 (d, $J = 7.2$ Hz, 1H, 4), 7.92 (d, $J = 7.0$, 1H, α-KaN), 7.98 (d, $J = 7.3$ Hz, 1H, 1), 8.17 (bs, 1H, δ-RdN), 10.03 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for 1090.23, found 1090.70. $R_f = 0.41$ (C).

4.3.12. Boc-Arg(NO$_2$)-Pro-Lys(BocIle)-Thr-BAT ***4l***

Yield 24%, mp. 207–209 °C; ^{1}H NMR (500 MHz, DMSO) δ ppm: 0.85 (brs, 6H, γ1-IMe3, δ-IMe5), 1.11 (d, $J = 3.9$ Hz, 2H, γ2-I4), 1.22 (d, $J = 8.4$ Hz, 3H, γ-T4), 1.35 (s, 9H, Boc), 1.49 (m, 2H, γ-K4), 1.84 (m, 2H, γ-R4), 1.95 (m, 1H, γ-P4a), 1.97 (m, 1H, γ-P4b), 2.07 (m, 2H, β-R3), 2.35 (m, 2H, β-P3), 2.92 (m, 2H, β-K3a, β-K3b), 2.94 (m, 2H, δ-K5), 3.13 (m, 1H, ε-K6a), 3.15 (m, 1H, ε-K6b), 3.37 (m, 2H, δ-R5), 3.77 (m, $J = 6.4$ Hz, 3H, β-I3), 3.86 (m, 1H, α-I2), 3.86 (d, $J = 6.0$ Hz, 1H, α-P2), 4.28 (m, 1H, α-K2), 4.34 (d, $J = 6.5$ Hz, 1H, α-T2), 4.49 (m, 2H, δ-P5), 4.49 (m, 1H, α-R2), 4.41 (m, 1H, β-T3), 4.92 (s, 2H, 10), 4.99 (s, 1H, T3-OH), 6.38 (d, $J = 8.4$ Hz, 1H, α-IN), 6.39 (d, $J = 7.6$ Hz, 1H, α-RaN), 7.36 (bdb, $J = 7.9$ Hz, 1H, 6), 7.38 (m, 1H, δ-KeN), 7.57 (bd, $J = 7.9$ Hz, 1H, 7), 7.59 (bs, 1H, 9), 7.76 (d, $J = 7.8$ Hz, 1H, α-TN), 7.74 (t, $J = 7.0$ Hz, 1H, 3), 7.80 (t, $J = 7.1$ Hz, 1H, 2), 7.88 (d, $J = 7.2$ Hz, 1H, 4), 7.94 (d, $J = 7.0$ Hz, 1H, α-KaN), 7.98 (d, $J = 7.3$ Hz, 1H, 1), 8.17 (bs, 1H, δ-RdN), 10.08 (s, 1H, 8″-CONH). MS $[M+H]^+$ *m/z* calcd for $C_{52}H_{75}N_{13}O_{13}$ 1090.23, found 1091.50. $R_f = 0.44$ (C).

4.4. General procedure of removal of the Boc-protecting groups

The mixture of analogues **4a-l** in diethyl ether (Et_2O) saturated with hydrochloric acid was stirred in 0 °C for 30 min. The progress of the reaction was monitored with TLC in solvent system C. After evaporating the solvent *in vacuo* the residue was taken up in anhydrous diethyl ether. The solution was decanted, and the residual yellow solid was dried.

4.4.1. 2HClxThr-Lys-Pro-Arg(NO$_2$)-BAT ***5a*** *as a yellow solid*

Yield 87%, mp. 207–210 °C. MS $[M+H]^+$ *m/z* calcd for $C_{36}H_{50}Cl_2N_{12}O_8$ 848.33, found 849.70. $R_f = 0.06$ (C); 92.45% pure by HPLC.

4.4.2. 2HClxThr-Lys(Gly)-Pro-Arg(NO$_2$)-BAT ***5b*** *as a yellow solid*

Yield 82%, mp. 199–204 °C. MS $[M+H]^+$ *m/z* calcd for $C_{38}H_{53}Cl_2N_{13}O_9$ 906.35, found 907.30. $R_f = 0.08$ (C); 98.87% pure by HPLC.

4.4.3. 2HClxThr-Lys(Ala)-Pro-Arg(NO$_2$)-BAT ***5c*** *as a yellow solid*

Yield 85%, mp. 201–205 °C. MS $[M+H]^+$ *m/z* calcd for $C_{39}H_{55}Cl_2N_{13}O_9$ 920.40, found 920.60. $R_f = 0.04$ (C); 95.36% pure by HPLC.

4.4.4. 2HClxThr-Lys(Val)-Pro-Arg(NO$_2$)-BAT ***5d*** *as a yellow solid*

Yield 70%, mp. 195–199 °C. MS $[M+H]^+$ *m/z* calcd for $C_{41}H_{57}Cl_2N_{13}O_9$ 948.36, found 948.80. $R_f = 0.04$ (C); 93.72% pure by HPLC.

4.4.5. 2HClxThr-Lys(Leu)-Pro-Arg(NO$_2$)-BAT ***5e*** *as a yellow solid*

Yield 73%, mp. 194–198 °C. MS $[M+H]^+$ *m/z* calcd for $C_{42}H_{61}Cl_2N_{13}O_9$ 962.38, found 962.70. $R_f = 0.02$ (C); 95.67% pure by HPLC.

4.4.6. 2HClxThr-Lys(Ile)-Pro-Arg(NO$_2$)-BAT ***5f*** *as a yellow solid*

Yield 69%, mp. 203–205 °C. MS $[M+H]^+$ *m/z* calcd for $C_{42}H_{61}Cl_2N_{13}O_9$ 962.38, found 962.90. $R_f = 0.05$ (C); 90.58% pure by HPLC.

*4.4.7. 2HClxArg(NO_2)-Pro-Lys-Thr-BAT **5g** as a yellow solid*

Yield 84%, mp. 207–210 °C. MS $[M+H]^+$ *m/z* calcd for $C_{36}H_{50}Cl_2N_{12}O_8$ 848.33, found 848.70. $R_f = 0.03$ (C); 97.03% pure by HPLC.

*4.4.8. 2HClxArg(NO_2)-Pro-Lys(Gly)-Thr-BAT **5h** as a yellow solid*

Yield 78%, mp. 210–215 °C. MS $[M+H]^+$ *m/z* calcd for $C_{38}H_{53}Cl_2N_{13}O_9$ 906.35, found 907.40. $R_f = 0.08$ (C); 98.04% pure by HPLC.

*4.4.9. 2HClxArg(NO_2)-Pro-Lys(Ala)-Thr-BAT **5i** as a yellow solid*

Yield 76%, mp. 220–223 °C. MS $[M+H]^+$ *m/z* calcd for $C_{39}H_{55}Cl_2N_{13}O_9$ 920.40, found 921.20. $R_f = 0.05$ (C); 96.48% pure by HPLC.

*4.4.10. 2HClxArg(NO_2)-Pro-Lys(Val)-Thr-BAT **5j** as a yellow solid*

Yield 65%, mp. 208–212 °C. MS $[M+H]^+$ *m/z* calcd for $C_{41}H_{57}Cl_2N_{13}O_9$ 948.36, found 949.00. $R_f = 0.04$ (C). 94.54% pure by HPLC.

*4.4.11. 2HClxArg(NO_2)-Pro-Lys(Leu)-Thr-BAT **5k** as a yellow solid*

Yield 61%, mp. 222–227 °C. MS $[M+H]^+$ *m/z* calcd for $C_{42}H_{61}Cl_2N_{13}O_9$ 962.38, found 962.50. $R_f = 0.03$ (C); 96.89% pure by HPLC.

*4.4.12. 2HClxArg(NO_2)-Pro-Lys(Ile)-Thr-BAT **5l** as a yellow solid*

Yield 68%, mp. 213–219 °C. MS $[M+H]^+$ *m/z* calcd for $C_{42}H_{61}Cl_2N_{13}O_9$ 962.38, found 962.70. $R_f = 0.07$ (C); 95.77% pure by HPLC.

4.5. Cell culture

All cell lines were purchased from American Type Culture Collection (Rockville, MD). The cells A549, HL-60 LNCaP, P388, L1210, WEHI 1640 were maintained in RPMI 1640 medium and HCT-116, HT-29, MCF-7, NIH-3T3 were maintained in DMEM high glucose. Media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and cells were cultivated at 37 °C in 5% (for RPMI 1640) and 10% (for DMEM H/G) CO_2/air atmosphere. All cell lines were screened routinely for Mycoplasma by the PCR method with Mycoplasma Plus PCR primer set (Stratagene, La Jolla, CA).

4.6. Cytotoxicity assay

The cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, exponentially growing cells were continuously exposed to different drug concentrations and the cellular viability was determined after four to five generation times (120 h). Cells were exposed to the MTT tetrazolium salt for 4 h at 37 °C, and the formation of formazan was measured by a microplate reader UVM340 (Biochrom Asys Ltd.). The concentrations required to inhibit cell growth by 50% compared to untreated controls were determined from the curves plotting survival as a function of dose by use of the GraphPad Prism 5 program. All values are averages of at least two independent experiments, each done in duplicate.

4.7. Flow cytometry analysis of cell cycle distribution

Distribution in different phases of the cell cycle was analyzed after treatment of tumor cells with studied drugs. Briefly, following drug treatment with a dose corresponding to IC_{90}, cells were washed in ice-cold phosphate buffered saline (PBS) and fixed in 70% ethanol at −20 °C. Cells were stained in PBS containing 20 μg/mL propidium iodide and 100 μg/mL ribonuclease A for 30 min at room temperature. Samples were analyzed by a Guava EasyCyte™ 8 flow cytometer (Merck-Millipore) equipped with a 488 nm laser, and the distribution of cells in the cell cycle was calculated using MultiCycle software (Phoenix Flow Systems).

4.8. Inhibition of DNA relaxation mediated by topoisomerases

Supercoiled plasmid pBR322 DNA (>95% form I) was purchased from Thermo Scientific, human type I topoisomerase was purchased from MoBiTec and human type IIα topoisomerase was purchased from TopoGen. Positive controls for topoisomerases type I and II activity assays, SN-38 and etoposide, respectively compounds were purchased for Sigma–Aldrich. The reaction mixture contained 200 ng of pBR322 DNA in reaction buffer (20 mM Tris–HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM DTT, 150 mM KCl, 1 mM ATP) as well as studied compounds. The reaction was initiated by the addition of topoisomerase IIα enzyme and allowed to proceed at 37 °C for 30 min. Reactions were terminated by addition of a loading buffer (0.1% SDS, 0.05% bromophenol blue, 2.5 mM EDTA, 10% sucrose, final concentrations). The samples were separated in 1% agarose gels at 0.5 V/cm for 18 h in TBE buffer (90 mM Tris-base, 70 mM boric acid, 1 mM EDTA, pH 8). Gels were stained with 0.5 μg/mL ethidium bromide to visualize DNA and photographed under UV illumination. The conditions for DNA relaxation assay for type I topoisomerase were the same except different reaction buffer was used (10 mM Tris–HCl, pH 7.5, 7.5 mM $MgCl_2$, 60 mM KCl).

4.9. Nuclear and cellular morphology of tumor cells

Cells were attached to cover slides in 35 mm Petri dishes and treated with studied compounds for different time periods. Following drug treatment, cells were stained with 1 μg/mL Hoechst 33342 alone or with 1 μg/mL Hoechst 33342 and 7.5 μg/mL fluorescein diacetate dyes for 15 min and analyzed by Olympus BX-60 fluorescent microscope equipped with respective optical filters. Images were recorded using XC50 digital camera and image acquisition software CellSens.

4.10. β-Galactosidase staining

Cells were treated with studied compounds with dose corresponding to IC_{90} for 120 h and washed in PBS, fixed for 3–5 min (room temperature) in 3.7% formaldehyde, washed, and incubated at 37 °C (no CO_2) with fresh stain solution: 1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (stock 20 mg/ml X-Gal in dimethylformamide)/40 mM citric acid/sodium phosphate, pH 6.0; 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide; 150 mM NaCl; 2 mM $MgCl_2$) for 12–16 h. Images were analyzed by Olympus BX-60 microscope and recorded using XC50 digital camera as well as image acquisition software CellSens.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.10.012.

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