Bioconjugate Chemistry

Article

pubs.acs.org/bc

Enhanced Cellular Uptake of a New, *in Silico* Identified Antitubercular Candidate by Peptide Conjugation

Kata Horváti,[†] Bernadett Bacsa,[†] Nóra Szabó,[‡] Sándor Dávid,[†,‡] Gábor Mező,[†] Vince Grolmusz,[§,#] Beáta Vértessy,[∥,⊥] Ferenc Hudecz,[†,○] and Szilvia Bősze*[,†]

[†]Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest, Hungary

[‡]Laboratory of Bacteriology, Korányi National Institute for Tuberculosis and Respiratory Medicine, Budapest, Hungary

[§]Protein Information Technology Group, Eötvös L. University, Budapest, Hungary

[∥]Institute of Enzimology, Hungarian Academy of Science, Budapest, Hungary

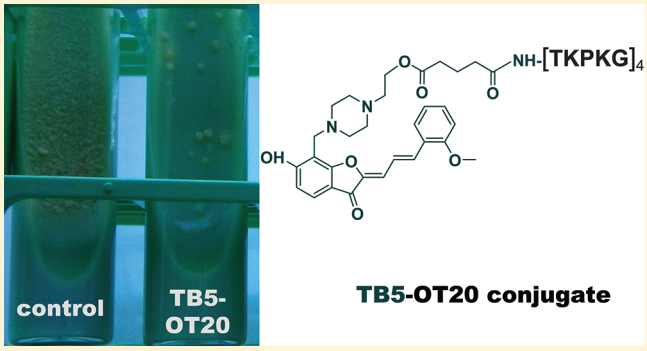
[⊥]Department of Applied Biotechnology, Budapest University of Technology and Economics, Budapest, Hungary

[#]Uratim Ltd., Budapest, Hungary

[○]Department of Organic Chemistry, Eötvös L. University, Budapest, Hungary

Supporting Information

ABSTRACT: *Mycobacterium tuberculosis* is a successful pathogen, and it can survive in infected macrophages in dormant phase for years and decades. The therapy of tuberculosis takes at least six months, and the slow-growing bacterium is resistant to many antibiotics. The development of novel antimicrobials to counter the emergence of bacteria resistant to current therapies is urgently needed. *In silico* docking methods and structure-based drug design are useful bioinformatics tools for identifying new agents. A docking experiment to *M. tuberculosis* dUTPase enzyme, which plays a key role in the bacterial metabolism, has resulted in 10 new antitubercular drug candidates. The uptake of antituberculars by infected macrophages is limited by extracellular diffusion. The optimization of the cellular uptake by drug delivery systems can decrease the used dosages and the length of the therapy, and it can also enhance the bioavailability of the drug molecule. In this study, improved *in vitro* efficacy was achieved by attaching the TB5 antitubercular drug candidate to peptide carriers. As drug delivery components, (i) an antimicrobial granulysin peptide and (ii) a receptor specific tuftsin peptide were used. An efficient synthetic approach was developed to conjugate the *in silico* identified TB5 coumarone derivative to the carrier peptides. The compounds were effective on *M. tuberculosis* $H_{37}Rv$ culture *in vitro*; the chemical linkage did not affect the antimycobacterial activity. Here, we show that the OT20 tuftsin and GranF2 granulysin peptide conjugates have dramatically enhanced uptake into human MonoMac6 cells. The TB5−OT20 tuftsin conjugate exhibited significant antimycobacterial activity on *M. tuberculosis* $H_{37}Rv$ infected MonoMac6 cells and inhibited intracellular bacteria.



INTRODUCTION

It is estimated that more than one-third of the world's population are infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). Latent TB is an asymptomatic phase of the disease during which bacilli do not multiply but persist within their host cells. Individuals with latent TB are assumed to harbor viable tubercle bacilli in their body. These bacilli have the potential to reactivate and cause disease. Around 10% of infected individuals will become sick with active TB during their lifetime. The risk of active TB is 20 to 40 times higher among patients living with HIV/AIDS, those with diabetes, cancer patients, organ transplant recipients, and those undergoing treatment for autoimmune diseases.[1] Among all TB cases, 5% are multidrug-resistant TB (MDR-TB), and in 2010, MDR-TB caused at least 150 000 deaths. MDR-TB strains are resistant to at least isoniazid and rifampicin, the two most effective first-line antituberculars. Additionally, a growing number of XDR-TB (extensively drug-resistant TB; resistant to isoniazid, rifampicin, any fluoroquinolones, and any of the second-line anti-TB injectable drugs) was reported.[2] The therapy of resistant TB can take up to two years with drugs that are less effective, more expensive, and more toxic.

The alarming number of bacterial strains resistant to current therapies led to the development of antituberculotics with novel mechanisms of action. *M. tuberculosis* dUTPase enzyme, which is required for mycobacterial growth, is one of the potential

Received: April 30, 2011
Revised: January 19, 2012
Published: April 19, 2012

target enzymes.[3−5] The dUTPase enzyme (EC 3.6.1.23; Rv2697) plays an important role in preventive DNA repair mechanism and thymidilate biosynthesis.[5] The X-ray crystal structure and the catalytic mechanism of *Mycobacterial* dUTPase was published previously.[6] It has been proposed that binding to the species-specific loop of dUTPase protein might be an effective tool to inhibit the growth of *M. tuberculosis*.[7,8]

Small molecular ligands which are capable of binding to and perturbing the function of an essential enzyme of *M. tuberculosis* can be identified using a high-throughput *in silico* docking method. *In silico* docking is a promising approach for fast screening of high numbers of drug-like compounds. Inputs for this process require three key components: (i) 3D structure of target proteins, (ii) a small molecular ligand database containing drug-like compounds, and (iii) an adequately performing docking protocol.

The commercial protein−ligand docking programs were quite secretive about their *exact* optimization methods and *exact* scoring functions, and fine-tuning for the specific needs of the application were not possible since the users cannot interfere with the inner settings of the program. This problem led us to develop our own energy based protein−ligand docking algorithm: the FRIGATE. This program was used to (i) analyze the structure of crucial bacterial enzymes for possible binding sites, (ii) generate candidate molecule conformations, (iii) dock these molecules with the target, and (iv) rank them according to their binding affinities. The software uses different strategies for local and global energy optimization in the typically 10−22 dimensional conformational space by a state-of-the art method combining continuous and discrete optimization techniques.

As a ligand library, we used the free ZINC database[9] of the Shoichet laboratory of the University of California at San Francisco (http://zinc.docking.org/). The database contains the three-dimensional description (SDF and mol2 files) of more than 12 million compounds collected and updated regularly from the catalogues of chemical compound manufacturers worldwide. For the ligand search, the subset generated according to the Lipinski's rule of five[10] was used; this subset contains approximately 2 million drug-like molecules.

Considering that *M. tuberculosis* can survive in host cells, the elimination from infected phagocytes could be more efficient with cell directed delivery of antituberculars. The optimization of the uptake rate using peptide based drug delivery systems can increase the efficacy of the compounds. We describe new peptidic conjugates of the *in silico* identified TB5 coumarone derivative: (i) an antimicrobial peptide (AMP) and (ii) a tuftsin receptor specific conjugate.

However, in many cases, the exact mechanism of bacterial killing of the AMPs is not known; these evolutionarily conserved peptides can interact with the lipid membrane, induce cell lysis, and provoke a broad spectrum of antimicrobial activity against bacteria, viruses, and fungi.[11] Granulysin, a 9 kDa protein found in granules of cytotoxic T lymphocytes and natural killer cells, lyses a variety of tumor and bacterial cells *in vitro* and directly kills *M. tuberculosis*, altering the membrane integrity.[12,13] GranF2, a 23-mer peptide analogue of granulysin, represents a helix−loop−helix region, which is postulated to be the membrane-docking part of the protein.[14] At a relatively high concentration (400 μg/mL, 132 μM), GranF2 inhibits the *in vitro* growth of multidrug resistant cultures. In the presence of a pore forming protein, such as perforin, GranF2 was found to be active against intracellular *M. tuberculosis*.[15,16] The cytolytic and ion channel-forming property of perforin is located at the N-terminus;[17] thus, the peptide represents this part of the protein that can be used in combination with granulysin. Conjugation of the TB5 molecule to the GranF2 peptide is proposed to intensify the antimycobacterial effect by a double mode of action: binding and inhibiting a crucial bacterial enzyme and causing bacterial cell lysis.

Receptor mediated endocytosis can also enhance the intracellular killing of *M. tuberculosis*.[18,19] More efficient inhibition of *M. tuberculosis* from infected phagocytes would be based on the internalization through receptors which are expressed mainly on macrophages. On the basis of the literature, the tuftsin receptor is used for targeted drug delivery.[20−22] Recently, the relevance of tuftsin conjugates as macrophage specific imaging biomarkers was also demonstrated.[23,24] Tuftsin is a natural phagocytosis stimulating peptide produced by enzymatic cleavage of the Fc-domain of the heavy chain of immunoglobulin G. Binding of tuftsin to its receptor causes macrophage activation. An activated macrophage has the ability to enhance phagocytosis and lysis of intracellular parasites.[25] During the past decade, a new group of sequential oligopeptide carriers with discrete molecular masses and defined sequences has been developed in our laboratory: oligotuftsin derivatives consisting of tandem pentapeptide repeat unit $[TKPKG]_n$ (n = 2, 4, 6, and 8) based on the canine tuftsin sequence TKPK.[26,27] These compounds are nontoxic and nonimmunogenic and exhibit tuftsin-like biological properties, e.g., receptor binding, immunostimulatory activity, and chemotactic activity on monocytes and macrophages. In this study, a tetramer tuftsin derivative $[TKPKG]_4$ (OT20) was used as a carrier peptide for the TB5 molecule.

Here, we report the synthesis, characterization, *in vitro* antimycobacterial activity, cytotoxicity, cellular uptake, and intracellular antitubercular efficacy of TB5−peptide conjugates. As the carrier/targeting moiety, a granulysin derived peptide (GranF2) and a tetra-tuftsin derivative (OT20) were used. Our primary aim was to enhance the cellular uptake and intracellular antimycobacterial efficacy of the TB5 *in silico* identified drug candidate.

EXPERIMENTAL PROCEDURES

Materials. Glutaric anhydride, *N,N*-diisopropylethylamine (DIEA), *N,N′*-diisopropylcarbodiimide (DIC), triisopropylsilane (TIS), and phenol were purchased from Fluka (Buchs, Schwitzerland). The amino acid derivatives were obtained from Reanal (Budapest, Hungary) and IRIS Biotech (Marktredwitz, Germany). 1-Hydroxybenzotriazole (HOBt) and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) were also from IRIS Biotech. Fmoc-Rink Amide MBHA resin was purchased from NovaBiochem (Läufelfingen, Schwitzerland). Acetonitrile, trifluoroacetic acid (TFA), *N*-methylpyrrolidone (NMP), and dimethyl sulfoxide (DMSO) were from Merck (Darmstadt, Germany). Ninhydrin, RPMI-1640 medium, Löwenstein−Jensen medium base and the components of the Sula medium was from Sigma-Aldrich (St. Louis, MO, USA). *N,N*-Dimethylformamide (DMF) and dichloromethane (DCM) were from Reanal (Budapest, Hungary). The TB5 molecule was purchased from Ubichem (Budapest, Hungary). HPMI buffer contains 9 mM glucose, 10 mM $NaHCO_3$, 119 mM NaCl, 9 mM HEPES, 5 mM KCl, 0.85 mM $MgCl_2$, 0.053 mM $CaCl_2$, and 5 mM $Na_2HPO_4 \times 2H_2O$ (pH 7.4). ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 300 MHz instrument. Chemical shifts (δ) are expressed in ppm downfield from TMS as an internal standard.

dx.doi.org/10.1021/bc200221t | *Bioconjugate Chem.* 2012, 23, 900−907

***In Silico* Docking Experiment.** The FRIGATE docking program unites discretization approaches of the energy field around the target protein, similar to that of AUTODOCK[28] with optimization techniques, completely different from AUTODOCK. The FRIGATE program stores the precomputed energy field around the target protein in a grid format. That grid needs to be placed into the RAM during the computation phase. Flexible small molecules are evaluated in that grid, but naturally, the atoms of the small molecules almost never coincide with the point of the grid. Therefore, the forces for the atoms of small molecules between grid points need to be approximated from the grid. FRIGATE uses continuously differentiable B-spline approximation. The main advantage of the B-splines is that for the energy functions we can apply highly developed continuous optimization techniques, namely, the conjugate gradient method, together with stochastic global optimization techniques. The hybrid of the discrete and continuous approaches retains the best from both worlds: discrete storage of the precomputed energy data on a grid makes possible storage at reasonable cost, while the conjugate gradient method yields fast and reliable local energy optimization, lacking in most of the docking programs used today.

Evaluation of Antimycobacterial Activity. *In vitro* antimycobacterial activity of the compounds was determined on *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) by serial dilution in Sula semisynthetic medium, which was prepared in-house (pH 6.5).[29−31] Compounds were added to the medium as DMSO solutions at 10 various doses (range of final concentration was between 0.5 and 100 μg/mL). MIC was determined after incubation at 37 °C for 28 days. MIC was the lowest concentration of a compound at which the visible inhibition of the growth of *M. tuberculosis* $H_{37}Rv$ occurred. In order to confirm growth inhibition, colony forming unit (CFU) was determined by subculturing from the Sula medium onto drug-free Löwensten−Jensen solid medium. Samples were incubated for 28 days. Experiments were repeated at least two times.

Derivatization of TB5 with Glutaric Anhydride. Fifty milligrams (0.115 mmol) of TB5 was dissolved in 2 mL of DCM, and in the presence of 5 equiv of DIEA (98 μL, 0.575 mmol), it was reacted with 3 equiv of glutaric anhydride (39 mg, 0.345 mmol). After 2 h of stirring, the solvent was removed *in vacuo*, and the resulting product was subjected to flash chromatography to afford the desired compound as a bright yellow oil. The synthesized TB5−glutaric acid derivative (pentanedioic acid mono-[2-(4-{6-hydroxy-2-[3-(2-methoxy-phenyl)-allylidene]-3-oxo-2,3-dihydro-benzofuran-7-ylmethyl}-piperazin-1-yl)-ethyl] ester) was purified using an automated chromatography system (SP1, Biotage) using cartridges packed with KP-SIL, 60 Å (40−63 μm particle size), and a chloroform/methanol mixture (gradient from 0 to 10% methanol) as eluent (43 mg, 68%). ^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): δ = 1.88−2.00 (m, 6H), 2.34−2.43 (m, 8H), 2.79 (t, J = 4.3 Hz, 2H), 2.85 (br s, 1H), 3.88 (s, 3H), 4.00 (s, 2H), 4.26 (t, J = 4.8 Hz, 2H), 6.61 (dd, J = 7.8 and 3.1 Hz, 1H), 6.75 (dd, J = 11.3 and 3.4 Hz, 1H), 6.85−7.00 (m, 2H), 7.20−7.42 (m, 3H), 7.58 (dd, J = 8.4 and 3.1 Hz, 1H), 7.66 (dd, J = 7.7 and 1.2 Hz, 1H), 8.26 (br s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): δ = 20.0, 33.1, 33.2, 51.6, 52.3, 53.0, 55.6, 56.1, 60.3, 103.6, 111.2, 113.5, 114.3, 114.5, 120.7, 120.8, 125.3, 125.4, 127.1, 130.5, 135.7, 148.1, 157.5, 165.1, 166.9, 172.9, 173.6, 178.0, 182.1. MS (pos. ESI): calcd 550.2 (M_{mo}); found, 550.2. R_t (RP-HPLC): 26.3 min.

To ease further handling, the product was next prepared in larger amounts as a TFA-salt as follows: after 2 h of stirring, 10 mL of 0.1% TFA/H2O (v/v) was added to the solution, and the yellow precipitate was filtered, washed with water, and dried over P_2O_5 under vacuum. The isolated salt was obtained as a yellow solid and used without further purification.

Synthesis of Tuftsin and Granulysin Derived Carrier Peptides. The tetramer analogue of the canine tuftsin sequence (TKPKGTKPKGTKPKGTKPKG) and GranF2, a granulysin derivative (^{33}VCRTGRSRWRDVCRNFMRRYQSR^{55}) were produced on Fmoc-Rink Amide MBHA resin in an automated peptide synthesizer (Syro-I, Biotage, Uppsala, Sweeden) using Fmoc/^{t}Bu strategy. The protocol of the synthesis was the following: (i) Fmoc deprotection with 40% piperidine/DMF (v/v), 2 min; 20% piperidine/DMF (v/v), 20 min; (ii) washing with DMF (5 × 1 min); (iii) coupling twice with 4 equiv of Fmoc-amino acid derivative−DIC−HOBt dissolved in NMP (2 × 60 min); and (iv) washing with DMF (5 × 1 min). Peptides were cleaved from the resin with the TFA/H_2O/TIS (9.5:2.5:2.5 v/v) mixture (2 h, RT). After filtration, compounds were precipitated in cold diethyl ether, centrifuged (4000 rpm, 5 min), and freeze-dried in water. Crude products were purified by semipreparative RP-HPLC as described below. Purified peptides were analyzed by analytical RP-HPLC, ESI MS, and amino acid analysis.

Conjugation of TB5−glut to Tuftsin and Granulysin Derived Carrier Peptides. TB5−glut compound, which contains a free carboxylic group, was conjugated to the *N*-terminus of the previously synthesized peptides on solid phase. Two equiv of TB5−glut−DIC−HOBt−DIEA dissolved in NMP was mixed with the peptide−resin. After 60−120 min, the efficacy of the coupling was monitored by ninhydrin assay. TB5−tuftsin and TB5−granulysin conjugates were cleaved from the resin with TFA in the presence of H_2O and TIS (9.5:2.5:2.5 v/v; 2 h, RT). After filtration, conjugates were precipitated in cold diethyl ether, centrifuged (4000 rpm, 5 min), and freeze-dried. Crude conjugates were purified by semipreparative RP-HPLC and characterized by analytical RP-HPLC and ESI MS.

Cell Culturing. MonoMac6 human monocytic cell line[32] (DSMZ no.: ACC 124, Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH, Braunschweig, Germany) was maintained in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamine, and 160 μg/mL gentamycin at 37 °C in 5% CO_2 atmosphere.

Cell Viability and Uptake Studies by Flow Cytometry. The measurement of cell viability and cellular uptake of the compounds were evaluated by flow cytometry (BD LSR II, BD Biosciences, San Jose, CA, USA) and fluorescent microscopy (Olympus CKX41, Hamburg, Germany) on MonoMac6 human monocytic cell line. Cells were harvested in the logarithmic phase of growth and plated on a 24-well tissue culture plate (10^5 cells/1 mL medium/well) 24 h prior to the experiment. Compounds were dissolved in serum free RPMI-1640 medium, and running dilutions were prepared. The highest concentration of the compounds on the cells was 300 μM. Cells were incubated with compounds TB5, TB5−glut, TB5−OT20, TB5−GranF2, GranF2, and OT20 for 60 min (37 °C, 5% CO_2 atmosphere). After centrifugation (1000 rpm, 5 min), the supernatant was removed, and 100 μL of 1 mM trypsin was added to the cells. After 5 min of incubation at 37 °C, 1 mL of 10% FCS/RPMI-1640 medium was added, and then cells were washed two times with medium and resuspended in 0.5 mL

dx.doi.org/10.1021/bc200221t | *Bioconjugate Chem.* 2012, 23, 900−907

HPMI. Cell viability and cellular uptake were determined by BD LSR II using a 488 nm (Coherent Sapphire, 22 mW) laser. The cell viability was assessed using propidium iodide (PI). The cells in suspension were mixed 1:1 with 10 μg/mL PI solution. Viable cells did not stain significantly with PI, whereas the nonviable cell population demonstrated significant PI staining (see Supporting Information, Figure S1). The intracellular fluorescence intensity of the cells was measured on channel PE LP550 (emission at λ = 550 nm). Data were analyzed with FACSDiva 5.0 software (BD Biosciences, San Jose, CA, USA). All measurements were performed in duplicate. In parallel with flow cytometry measurements, microscopic images of the cells were captured with an Olympus CKX41 microscope.

Infection of MonoMac6 Monolayers with *M. tuberculosis* H_{37}Rv and Determination of Efficacy against Intracellular Bacteria (Modified Method Based On Refs 33 and 34). Briefly, MonoMac6 monocytes (2×10^5 cells/1 mL medium/well) were cultured with RPMI-1640 medium containing 10% FCS in a 24-well plate 24 h prior to the experiment. Adherent cells were infected with *M. tuberculosis* H_{37}Rv at a multiplicity of infection (MOI) of 10 for 4 h. Nonphagocytosed extracellular bacteria were removed, and the culture was washed three times with serum free RPMI-1640. The infected monolayer was incubated for 1 day before antitubercular treatment. Infected cells were then treated with TB5 conjugates at 50 μM final concentration with or without 100 μM perforin peptide. After 3 days, the treatment was repeated with fresh solution of the compounds for an additional 3 days. Untreated cells were considered as the negative control. As the control compound, isoniazid (INH) was used at 50 μg/mL (365 μM) concentration. After washing steps, in order to remove the antituberculars, infected cells were lysed with 2.5% sodium dodecyl sulfate solution. The CFU of *M. tuberculosis* was enumerated on Löwenstein–Jensen solid media after 4 weeks of incubation.

RESULTS

Results of *in Silico* Docking. The FRIGATE program generated docking energies for each of the more than 2 million small molecules from the ZINC database. The best 1000 scored molecules were filtered according to their log *P* and predicted solubility properties. We also checked for their drug likeness and ranked them according to their binding affinities.[35] In the first run of the experiments, we ordered the 20 best molecules from compound manufacturers, using the ZINC database. The *in vitro* antimycobacterial activity of the compounds was characterized by the determination of the minimal inhibitory concentration (MIC) on *M. tuberculosis* H_{37}Rv culture with 4-week exposure period (Table 1). One compound was not soluble at the used concentration. Fifty-three percent (10/19) of the tested molecules showed relevant MIC values (lower than 100 μg/mL). Chemical structure of the effective compounds is given in Figure 1. For peptide conjugation, a fluorescent compound, TB5 coumarone derivative, was chosen. The fluorescent property of the TB5 compound (Figure 2) can be used conveniently to determine the cellular uptake rate by flow cytometry and fluorescent microscopy.

Conjugation of the TB5 Molecule to Carrier Peptides. Carrier peptides were synthesized using the standard Fmoc/^{t}Bu strategy. All of the peptides were obtained with amide *C*-termini after final cleavage. These peptides were also used as control compounds in further experiments. Data concerning analytical characterization of peptides are presented in Table 2.

Table 1. Antimycobacterial Effect of the *in Silico* Identified Compounds

compd	MIC[a] (μg/mL)	MIC (μM)	CFU[b]
control	no inhibition		+++[c]
isoniazid (INH)[d]	0.16	1.2	12
norfloxacin[d]	5	15	1
TB1	25	51	n.d.
TB2	5	12	5
TB3	15	31	42
TB4	30	63	n.d.
TB5	20	46	2
TB6	45	100	7
TB7	25	53	20
TB8	1	3.7	8
TB9	45	93	70
TB10	45	110	2

[a]MIC (minimal inhibitory concentration) was determined on the *M. tuberculosis* H_{37}Rv strain in Sula semisynthetic media, pH 6.5 (4 weeks). [b]To confirm the growth inhibition, CFU (colony forming unit) was determined on Löwenstein–Jensen solid media (4 weeks). [c]+++: confluent colonies. [d]As positive control, bacteria were inoculated in the absence of drugs. Izoniazid (a first line antitubercular drug) and norfloxacin (a fluoroquinolone) were used to compare the MIC results.

All purified peptides demonstrated a single peak on analytical RP-HPLC with a mass coinciding with the theoretically calculated molecular mass. The amino acid composition of peptides was proved by amino acid analysis where the accuracy was less than 5%.

In order to prepare TB5–peptide conjugates, the TB5 molecule was first esterificated with glutaric anhydride (Figure 3). Through the free carboxylic group, TB5–glut was conjugated to tuftsin and granulysin derived peptides on solid phase using the standard DIC/HOBt coupling method. After the final cleavage with TFA, TB5–tuftsin and TB5–granulysin conjugates were purified and carefully characterized (Table 2).

Antitubercular Effect of TB5 and TB5–glut. The *in vitro* antimycobacterial activity of the new compounds was characterized by the determination of the minimal inhibitory concentration (MIC) using *M. tuberculosis* H_{37}Rv strain with a 4-week exposure period. TB5–glut was effective against the bacteria and exhibited almost the same MIC value as free TB5. MIC(TB5) = 20 μg/mL; 46 μM) and MIC(TB5–glut) = 40 μg/mL; 73 μM. These results suggested that chemical modification did not influence the antitubercular effect of TB5 molecule.

Cell Viability. Viability of MonoMac6 human monocytic cells was measured by flow cytometry after 1 h of treatment with TB5, TB5–glut, TB5–GranF2, TB5–OT20 conjugates, GranF2, and OT20 carrier peptides. Relative percentage of living cells was determined compared to that of the untreated control (Figure 4). The most cytotoxic compound was TB5–GranF2, where the IC_{50} value is close to that of the free GranF2 carrier peptide (42 and 85 μM). The microscopic images (Figure 5C and E) clearly demonstrate that the GranF2 peptide and TB5–GranF2 conjugate disrupted the membrane integrity. The tuftsin conjugated TB5–OT20 compound is less cytotoxic on MonoMac6 cells; the IC_{50} value (201 μM) is 5 times higher than the IC_{50} of TB5–GranF2. Treatment with TB5, TB5–glut compounds, and OT20 peptide did not influence the cell viability up to 300 μM concentration.

dx.doi.org/10.1021/bc200221t | *Bioconjugate Chem.* 2012, 23, 900–907

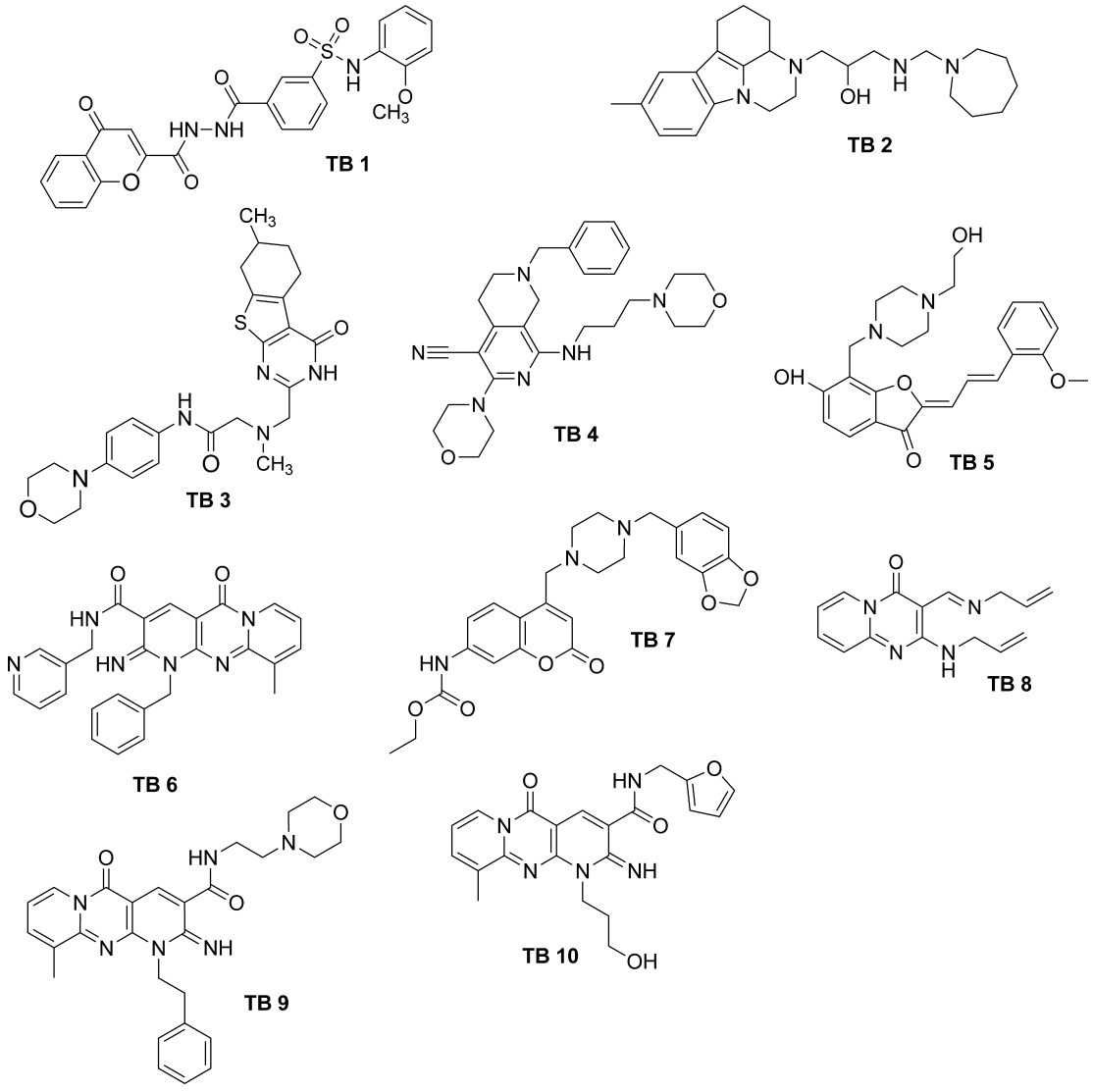


Figure 1. Chemical structure of the *in silico* identified antitubercular drug candidates.

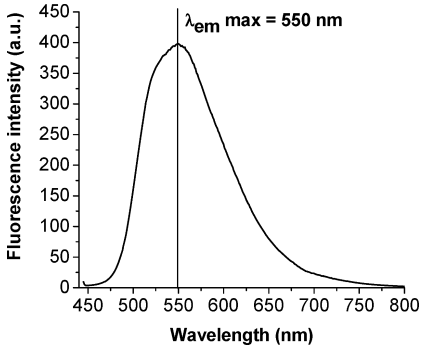


Figure 2. Fluorescent emission spectra of the TB5 compound. The sample was measured on a VARIAN Cary Eclipse fluorescence spectrophotometer in HPMI media at 10 μM concentration. The emission spectra indicate the use of TB5 and its peptide conjugates in flow cytometry and fluorescent microscopy.

Cellular Uptake of TB-5 Conjugates. Cellular uptake was evaluated on the MonoMac6 human monocytic cell line using flow cytometry (BD LSRII, Figure 6) and fluorescent microscopy (Olympus CKX41, Figure 5) after trypsinization. The treatment of MonoMac6 monocytic cells with TB5 and TB5−glut compounds has resulted in the same intracellular fluorescent intensity as that of the untreated control. In contrast, the internalization rates of TB5−OT20 and TB5−GranF2 conjugates were more than ten-times higher than the rates of TB5 compounds.

Intracellular Killing of *M. tuberculosis*. Intracellular efficacy of antitubercular drug candidates was evaluated on MonoMac6 human monocytes using a modified previously described method.[33,34] At 100 μM or higher concentration, TB5−GranF2 and GranF2 are cytotoxic for the monocytes (IC_{50} = 42 and 85 μM), and false intracellular inhibition can be detected. Therefore, 50 μM final concentration was used in this experiment. When infected, MonoMac6 cells were treated with only carrier peptides (GranF2, OT20), no antitubercular activity was observed on intracellular bacteria. In the case of TB5−GranF2 conjugate, significantly reduced CFU was enumerated compared to CFU of the lysed untreated control cells. Applying the combination of GranF2 and perforin resulted in 10 times higher activity, but in the case of the TB5−GranF2 conjugate in the presence of perforin, no further improvement in efficacy was detected. Isoniazid (INH), a first-line

dx.doi.org/10.1021/bc200221t | *Bioconjugate Chem.* 2012, 23, 900−907

Table 2. Analytical Characterization of Peptide–Drug Conjugates and Free Carrier Peptides[a]

compd	composition	M_{av}[b] calcd/found	R_t[d] (min)
TB5		436.4/436.4[c]	24.5
TB5–glut		550.5/550.5[c]	26.3
OT20	$[TKPKG]_4$	2063.5/2063.5	14.8[e]
TB5–OT20	TB5–$[TKPKG]_4$	2596.0/2596.0	28.4[e]
GranF2	^{33}VCRTGRSRWRDVCRNFMRRYQSR^{55}	2988.5/2988.7	21.6
TB5–GranF2	TB5–^{33}VCRTGRSRWRDVCRNFMRRYQSR^{55}	3521.0/3520.8	30.1

[a]The *C*-termini of the peptides and peptide conjugates were in amidated form. [b]Measured average molecular mass by Bruker Esquire 3000+ ESI-MS. Samples were dissolved in a mixture of acetonitrile/water = 1/1 (v/v) containing 0.1% acetic acid and introduced by a syringe pump with a flow rate of 10 μL/min. [c]Monoisotopic molecular mass. [d]RP-HPLC, Knauer, Eurospher-100, C18, 5 μm, 250 × 4 mm column; 1 mL/min flow rate; detection at λ = 214 nm. A eluent: 0.1% TFA/H_2O. B eluent: 0.1% TFA/acetonitrile/H_2O = 80:20 (v/v). Gradient: 10% B, 5 min; 10–80% B, 35 min. [e]Gradient: 5% B, 5 min; 5–60% B, 35 min.

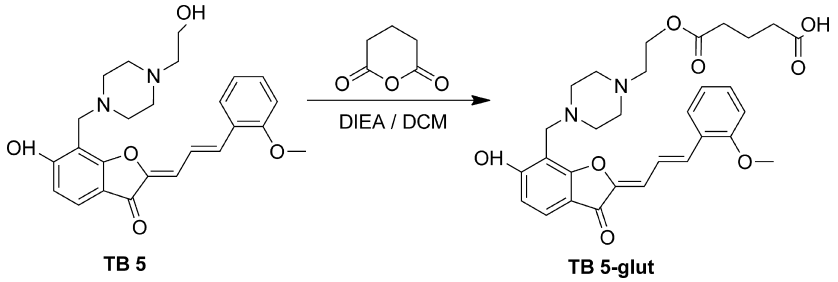


Figure 3. Outline of the esterification of the TB5 molecule with glutaric anhydride (TB5–glut).

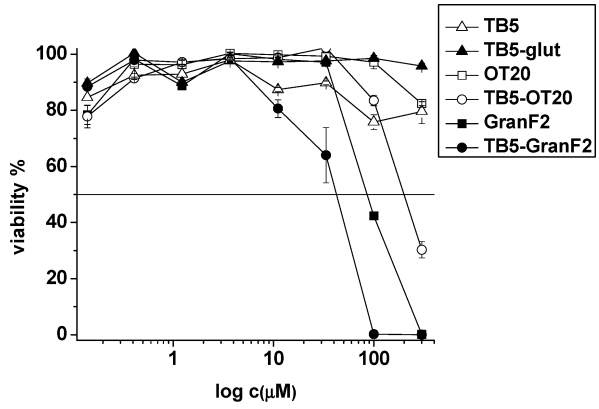


Figure 4. Viability of MonoMac6 human monocytic cells after 1 h of treatment with TB5, TB5–glut, TB5–GranF2, TB5–OT20 conjugates, GranF2, and OT20 carrier peptides. Relative percentage of viable cells was determined compared to that of the untreated control. Each point represents the mean ± SD of triplicate measurements. GranF2 and TB5–GranF2 are the most cytotoxic compounds *in vitro*. IC_{50}(GranF2) = 85 μM; IC_{50}(TB5–GranF2) = 42 μM.

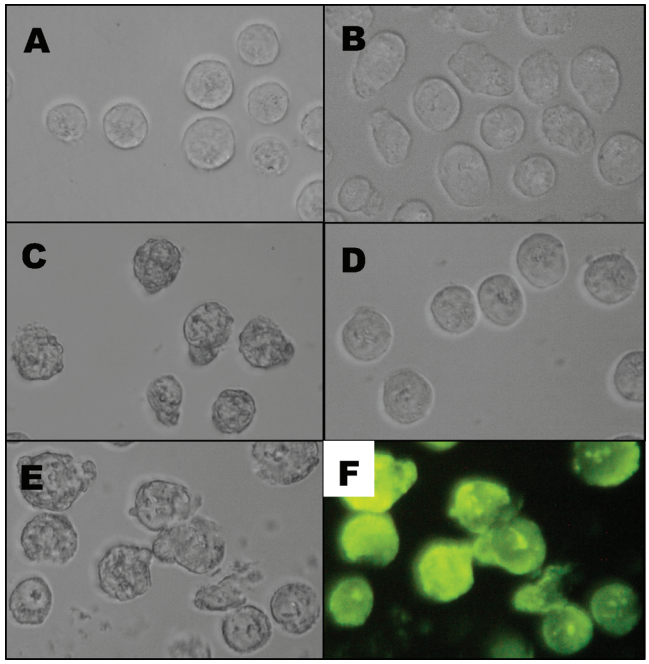


Figure 5. Microscopic images of MonoMac6 human monocytic cells treated with TB5 compounds. Untreated control (A), TB5 (B), GranF2 (C), OT20 (D), and TB5–GranF2 (E), fluorescent image of TB5–GranF2 (F). Treatment with TB5 and the OT20 peptide did not change the morphology of the cells, while GranF2 and TB5–GranF2 induced cell lysis and membrane disruption.

antitubercular drug, was also tested and did not show antitubercular activity even at 365 μM concentration against intracellular bacteria. Treatment with TB5–OT20 dramatically reduced the intracellular growth of *M. tuberculosis* (Figure 7).

DISCUSSION

An *in silico* docking experiment to *M. tuberculosis* dUTPase enzyme has resulted in 10 new antimycobacterial compounds (TB1-TB10). Considering MIC values, solubility features, and chemical structure, the TB5 compound was chosen for conjugation to oligotuftsin and granulysin derived carrier peptides. Furthermore, the fluorescent property of TB5 molecule gives us the possibility to study cellular uptake and cell viability by flow cytometry and fluorescence microscopy. Efficient synthetic pathway was introduced to conjugate the TB5 compound to peptidic carriers. The antitubercular activity of TB5 coumarone derivative was preserved after chemical modification.

The main goal of our study was to improve the antimycobacterial efficacy of the compound against intracellular

dx.doi.org/10.1021/bc200221t | *Bioconjugate Chem.* 2012, 23, 900–907

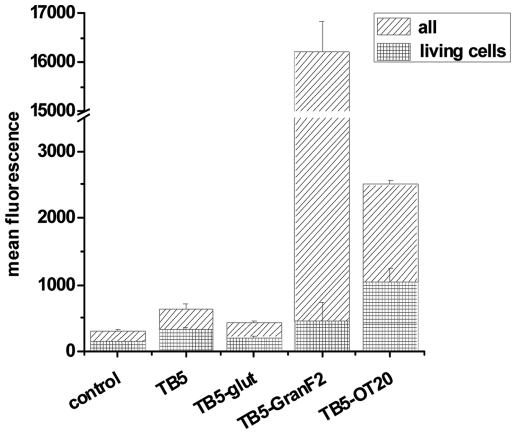


Figure 6. MonoMac6 human monocytic cells were treated with the TB5 compound and conjugates at 100 μM concentration. The cellular uptake was determined after trypsination by flow cytometry (BD LSRII) measuring the intracellular fluorescent intensity. No internalization was detected for TB5 and TB5−glut compare to that of the untreated control. Conjugation to GranF2 and the OT20 peptide significantly increased cellular uptake. TB5−OT20 treatment has resulted in the highest intracellular fluorescence intensity of viable cells.

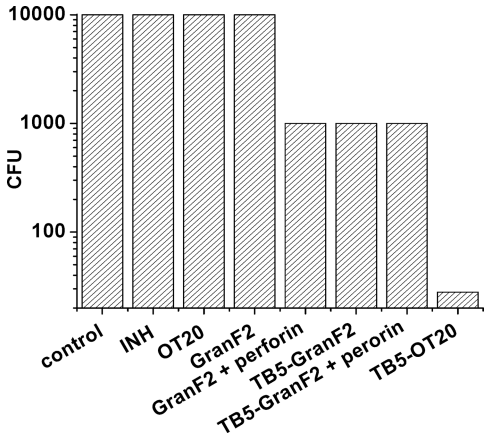


Figure 7. Inhibition of intracellular *M. tuberculosis* by TB5−peptide conjugates. Cultured MonoMac6 cells were infected with *M. tuberculosis* H_{37}Rv and treated with compounds at 50 μM final concentration. As the control, untreated cells were used. In the case of the GranF2 peptide, perforin peptide was added at 100 μM concentration. After SDS lysis, the colony forming units (CFU) of *M. tuberculosis* was enumerated on Löwenstein−Jensen solid media.

M. tuberculosis. To determine the efficacy against intracellular bacteria, MonoMac6 human monocytes were infected with *M. tuberculosis* H_{37}Rv and treated with the new compounds. MonoMac6 cells are phagocytic and express the CR3 receptor, which is important for the entry of *M. tuberculosis* bacteria. MonoMac6 was established as a cell line, which appears to have phenotypic and functional characteristics of mature blood monocytes; therefore, it is frequently used as a host cell model to measure activity against phagocytosed intracellular bacteria.

In order to adjust the experimental parameters on infected MonoMac6, first the cellular uptake of the compounds was determined. The treatment of MonoMac6 cells with the TB5 compound evoked almost the same intracellular fluorescent intensity as that of the untreated control, which means that the uptake rate of the free drug candidates is limited. In contrast, the mean fluorescent intensity was significantly higher after the incubation with the tuftsin and granulysin conjugated TB5 compound. The most efficient internalization was measured for TB5−granF2, but the antimicrobial granulysin derived peptide provoked membrane disruption and lysis of MonoMac6 cells. Coupling TB5 to OT20 peptide has resulted in a less cytotoxic conjugate, and the intracellular fluorescent intensity of TB5−OT20 treated cells was five-times higher than that of TB5 treated cells. The TB5−OT20 conjugate was remarkably effective against phagocytised intracellular *M. tuberculosis bacteria*: a significant decrease in CFU was enumerated at 50 μM concentration. At this concentration, GranF2 and TB5−GranF2 conjugates resulted in 35-times higher CFU even in the presence of a pore forming perforin.

We can conclude that conjugation of antitubercular agents to a peptidic carrier is a promising approach to enhance cellular uptake and *in vitro* selectivity. In this study, we showed that the OT20 tuftsin conjugate efficiently inhibited the intracellular *M. tuberculosis* bacteria.

■ ASSOCIATED CONTENT

Supporting Information

Gating strategy in flow cytometry. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Pázmány P. stny. 1/A, Budapest, Hungary 1117. Tel: +36-1-372-2500 ext. 1736. Fax: +36-1-372-2620. E-mail: bosze@chem.elte.hu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by NKFP_07_1-TB_INTER-HU; Hungarian Research Fund (T68358, T68258, CK78646, NK-84008). New Hungary Development Plan (TAMOP-4.2.1/B-09/1/KMR-2010-0002, 0003); Baross program of the New Hungary Development Plan (3DSTRUCT, OMFB-00266/2010 REG-KM-09-1-2009-0050). We thank Dr. Hedvig Medzihradszky-Schweiger for the amino acid analysis and Dr. Toma N. Glasnov for the characterization of the TB5−glut compound.

■ REFERENCES

(1) Dyer, C. M. (2010) *Tuberculosis*, pp 89−121, Chapters 7 and 8, Greenwood Press, Santa Barbara, CA

(2) Wright, A., and Zignol, M. (2008) *Anti-Tuberculosis Drug Resistance in the World: Fourth Global Report (WHO/HTM/TB/2008.394)*, WHO Press, Geneva, Switzerland

(3) Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003) Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol. 48*, 77−84.

(4) Mészáros, B., Tóth, J., Vértessy, B. G., Dosztányi, Z., and Simon, I. (2011) Proteins with complex architecture as potential targets for drug design: a case study of *Mycobacterium tuberculosis*. *PLoS Comput. Biol.* 7 (7), e1002118.

(5) Vértessy, B. G, and Tóth, J. (2009) Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. *Acc. Chem. Res. 42*, 97−106.

(6) Chan, S., Segelke, B., Lekin, T., Krupka, H., Cho, U. S., Kim, M. Y., So, M., Kim, C. Y., Naranjo, C. M., Rogers, Y. C., Park, M. S., Waldo, G. S., Pashkov, I., Cascio, D., Perry, J. L., and Sawaya, M. R.

(2004) Crystal structure of the *Mycobacterium tuberculosis* dUTPase: insights into the catalytic mechanism. *J. Mol. Biol. 341*, 503−517.
(7) Varga, B., Barabás, O., Takács, E., Nagy, N., Nagy, P., and Vértessy, B. G. (2008) Active site of mycobacterial dUTPase: structural characteristics and a built-in sensor. *Biochem. Biophys. Res. Commun. 373*, 8−13.
(8) Takács, E., Nagy, G., Leveles, I., Harmat, V., Lopata, A., Tóth, J., and Vértessy, B. G. (2010) Direct contacts between conserved motifs of different subunits provide major contribution to active site organization in human and mycobacterial dUTPases. *FEBS Lett. 584*, 3047−3054.
(9) Irwin, J. J., and Shoichet, B. K. (2005) ZINC - a free database of commercially available compounds for virtual screening. *J. Chem. Inf. Model. 45*, 177−182.
(10) Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev. 46*, 3−26.
(11) Reddy, K. V., Yedery, R. D., and Aranha, C. (2004) Antimicrobial peptides: premises and promises. *Int. J. Antimicrob. Agents 24*, 536−547.
(12) Stenger, S., Hanson, D. A., Teitelbaum, R., Dewan, P., Niazi, K. R., Froelich, C. J., Ganz, T., Thoma-Uszynski, S., Melián, A., Bogdan, C., Porcelli, S. A., Bloom, B. R., Krensky, A. M., and Modlin, R. L. (1998) An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science 282*, 121−125.
(13) Clayberger, C., and Krensky, A. M. (2003) Granulysin. *Curr. Opin. Immunol. 15*, 560−565.
(14) Andreu, D., Carreño, C., Linde, C., Boman, H. G., and Andersson, M. (1999) Identification of an anti-mycobacterial domain in NK-lysin and granulysin. *Biochem. J. 344*, 845−849.
(15) Toro, J. C., Hoffner, S., Linde, C., Andersson, M., Andersson, J., and Grundström, S. (2006) Enhanced susceptibility of multidrug resistant strains of *Mycobacterium tuberculosis* to granulysin peptides correlates with a reduced fitness phenotype. *Microbes Infect. 8*, 1985−1993.
(16) Walch, M., Latinovic-Golic, S., Velic, A., Sundstrom, H., Dumrese, C., Wagner, C. A., Groscurth, P., and Ziegler, U. (2007) Perforin enhances the granulysin-induced lysis of Listeria innocua in human dendritic cells. *BMC Immunol. 8*, 14.
(17) Ojcius, D. M., Persechini, P. M., Zheng, L. M., Notaroberto, P. C., Adeodato, S. C., and Young, J. D. (1991) Cytolytic and ion channel-forming properties of the N terminus of lymphocyte perforin. *Proc. Natl. Acad. Sci. U.S.A. 88*, 4621−4625.
(18) Majumdar, S., and Basu, S. K. (1991) Killing of intracellular *Mycobacterium tuberculosis* by receptor-mediated drug delivery. *Antimicrob. Agents Chemother. 35*, 135−140.
(19) Horváti, K., Mező, G., Szabó, N., Hudecz, F., and Bősze, Sz. (2009) Peptide conjugates of therapeutically used antitubercular isoniazid-design, synthesis and antimycobacterial effect. *J. Pept. Sci. 15*, 385−391.
(20) Amoscato, A. A., Davies, P. J., Babcock, G. F., and Nishioka, K. (1983) Receptor-mediated internalization of tuftsin. *Ann. N.Y. Acad. Sci. 419*, 114−134.
(21) Gottlieb, P., Hazum, E., Tzehoval, E., Feldman, M., Segal, S., and Fridkin, M. (1984) Receptor-mediated endocytosis of tuftsin by macrophage cells. *Biochem. Biophys. Res. Commun. 119*, 203−211.
(22) Agrawal, A. K., and Gupta, C. M. (2000) Tuftsin-bearing liposomes in treatment of macrophage-based infections. *Adv. Drug Delivery Rev. 41*, 135−146.
(23) Feng, J., Meloni, M. M., Allan, S. M., Faulkner, S., Narvainen, J., Vidyasagar, R., and Kauppinen, R. (2010) Tuftsin derivatives of FITC, Tb-DOTA or Gd-DOTA as potential macrophage-specific imaging biomarkers. *Contrast Media Mol. Imaging 5*, 223−230.
(24) Wong, E., Bennett, S., Lawrence, B., Fauconnier, T., Lu, L. F., Bell, R. A., Thornback, J. R., and Eshima, D. (2001) Tuftsin receptor-binding peptide labeled with technetium: chemistry and preliminary in vitro receptor-binding study. *Inorg. Chem. 40*, 5695−5700.
(25) Najjar, V. A. (1983) Tuftsin, a natural activator of phagocyte cells: an overview. *Ann. N.Y. Acad. Sci. 419*, 1−11.
(26) Mező, G., Kalászi, A., Reményi, J., Majer, Zs., Hilbert, A., Láng, O., Kőhidai, L., Barna, K., Gaál, D., and Hudecz, F. (2004) Synthesis, conformation, and immunoreactivity of new carrier molecules based on repeated tuftsin-like sequence. *Biopolymers 73*, 645−656.
(27) Bai, K. B., Láng, O., Orbán, E., Szabó, R., Kőhidai, L., Hudecz, F., and Mező, G. (2008) Design, synthesis, and *in vitro* activity of novel drug delivery systems containing tuftsin derivatives and methotrexate. *Bioconjug. Chem. 19*, 2260−2269.
(28) Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem. 19*, 1639−1662.
(29) Sula, L. (1963) WHO Co-operative studies on a simple culture technique for the isolation of mycobacteria. 1. Preparation, lyophilization and reconstitution of a simple semi-synthetic concentrated liquid medium; culture technique; growth pattern of different mycobacteria. *Bull. W. H. O. 29*, 589−606.
(30) Sula, L. (1963) WHO cooperative studies on a simple culture technique for the isolation of mycobacteria: 2. Comparison of the efficacy of lyophilized liquid medium with that of Lowenstein-Jensen (L-J) medium. *Bull. W. H. O. 29*, 607−625.
(31) Vinsova, J., Cermakova, K., Tomeckova, A., Ceckova, M., Jampilek, J., Cermak, P., Kunes, J., Dolezal, M., and Staud, F. (2006) Synthesis and antimicrobial evaluation of new 2-substituted 5,7-di-tert-butylbenzoxazoles. *Bioorg. Med. Chem. 14*, 5850−5865.
(32) Ziegler-Heitbrock, H. W. L., Thiel, E., Fütterer, A., Herzog, V., Wirtz, A., and Riethmüller, G. (1988) Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer 41*, 456−461.
(33) Wright, E. L., Quenelle, D. C., Suling, W. J., and Barrow, W. W. (1996) Use of Mono Mac 6 human monocytic cell line and J774 murine macrophage cell line in parallel antimycobacterial drug studies. *Antimicrob. Agents Chemother. 40*, 2206−2208.
(34) Tomioka, H., Sato, K., Kajitani, H., Akaki, T., and Shishido, S. (2000) Comparative antimicrobial activities of the newly synthesized quinolone WQ-3034, levofloxacin, sparfloxacin, and ciprofloxacin against *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *Antimicrob. Agents Chemother. 44*, 283−286.
(35) Schnöller, D., Pénzes, Cs. B., Horváti, K., Bősze, Sz., Hudecz, F., and Kiss, É. (2011) Membrane affinity of new antitubercular drug candidates using a phospholipid Langmuir monolayer model and LB technique. *Prog. Colloid Polym. Sci. 138*, 131−137.