Efficient Solid-Phase Synthesis of FK228 Analogues as Potent Antitumoral Agents

Salvatore Di Maro,[†,‡] Rey-Chen Pong,[§] Jer-Tsong Hsieh,[§] and Jung-Mo Ahn*[,†]

Department of Chemistry, University of Texas at Dallas, Richardson, Texas 75080, and Department of Urology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390

Received July 29, 2008

Abstract: Novel structural analogues of a HDAC inhibitor FK228 have been synthesized by modifying the most synthetically challenging unit, (3*S*,4*E*)-3-hydroxy-7-mercaptoheptenoic acid, with simple isosteric substitutions. These changes did not alter the backbone structure from FK228 but enabled facile and rapid synthesis by using readily available starting materials and high-yielding reactions. FK228 analogues were examined for their antitumoral activity on a variety of human cancer cells and led to the identification of new potent compounds.

Histone deacetylases (HDACs) are involved in a post-translational modification of histones by removing acetyl groups at the ε-amino groups of histone tails, which results in changing chromosomal DNA conformation and which then influences gene expression, often referred as epigenetic gene regulation.[1] Epigenetic gene regulation is known to play a critical role in embryo development, cell proliferation, and differentiation. In contrast, altered epigenetic regulation has been noticed in various cancers. Thus, it is considered as an attractive target for the treatment of cancers.[2] Until now, a number of HDAC inhibitors have been identified including TSA,[a] SAHA, and FK228.[2]

A natural product, FK228 (also known as FR901228) is a highly potent HDAC inhibitor that is isolated from *Chromobacterium violaceum* (Figure 1A).[3] Structurally unrelated to other HDAC inhibitors, it is a unique bicyclic depsipeptide[4] and a stable prodrug that becomes activated by the reduction of its disulfide bond after uptake into cells.[5] As Furumai et al. have demonstrated, the released sulfhydryl group appears to be important for the interaction with the zinc cation localized at the active site of HDACs. In addition, it shows high selectivity toward class I HDACs,[6] which have been reported to be more relevant for therapeutic intervention in oncology.[7]

Its first total synthesis was reported by Simon and co-workers in 1996 and achieved by following a rather laborious synthetic route giving a moderate yield (18% overall yield over 16 steps).[8] Three principal challenges noticed are (1) the asymmetric synthesis of the (3*S*,4*E*)-3-hydroxy-7-mercapto-4-heptenoic acid, (2) the macrolactonization to form the 16-membered depsipeptide, and (3) the oxidation of thiols to create the 15-membered ring. An improved synthesis was recently reported to prepare the heptenoic acid using an asymmetric Noyori hydrogen transfer reaction; however, no increase in overall yield was achieved (13%).[9]

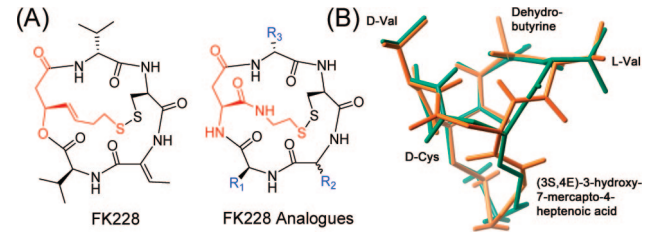


Figure 1. Structures of a novel FK228 analogue. (A) Analogue design and (B) conformation (orange) overlaid on FK228 (green).

Despite its promising biological activity, these difficulties appear to deter the synthesis of a series of FK228 analogues that would facilitate structure−activity relationship studies and reveal the requirements to achieve higher potency and specificity. Thus, we report herein novel FK228 analogues that were designed by employing simple isosteric substitutions, and its efficient solid-phase synthesis to produce a large number of FK228 analogues.

To overcome the synthetic challenges identified from the previous synthesis,[8,9] (3*S*,4*E*)-3-hydroxy-7-mercapto-4-heptenoic acid in FK228 was modified into a structure that can be easily assembled using readily available starting materials, yet still has a capability to retain the same conformation required for the biological activity. First, the trans double bond in the heptenoic acid was replaced by an isosteric amide bond. To develop peptidomimetics, an amide bond in peptides has been changed to a trans double bond because of its structural rigidity and capability to present two alkyl chains on opposite sides.[10] Second, the ester bond to form the depsipeptide was replaced by another amide bond for facile ring closure that can provide higher synthetic yield and increased in vivo stability. These two simple modifications transformed the synthetically challenging heptenoic acid into a structure that can be easily assembled with an L-aspartic acid and a cysteamine (Figure 1A). In addition, another unnatural amino acid in FK228, (*Z*)-dehydrobutyrine (Dhb), was substituted with various L- and D-amino acids for easy construction of FK228 analogues. All of these changes allowed to achieve highly efficient synthesis (vide infra).

To ensure that the isosteric substitutions do not alter the backbone structure from FK228, the novel FK228 analogue was examined by molecular modeling. A Monte Carlo conformational search using MacroModel[11] (version 9.1, Schrödinger) and united atom AMBER force field showed an almost identical structure compared to FK228 (rmsd = 0.20 Å; Figure 1B).

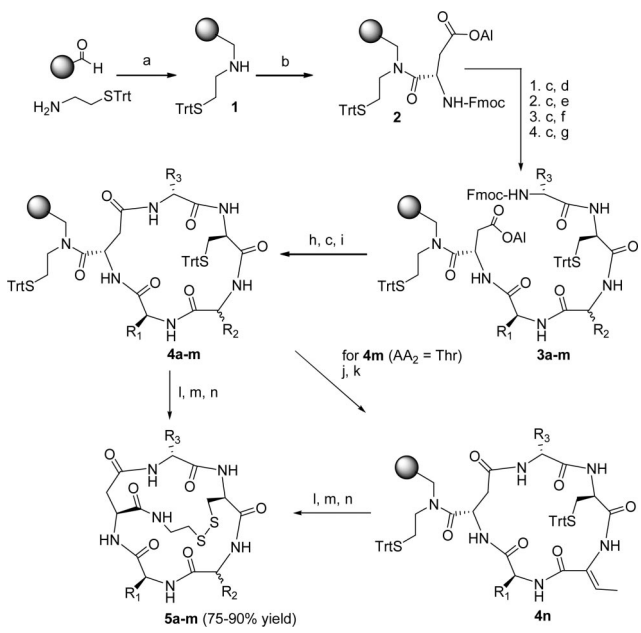
For high synthetic efficiency, we have focused on developing a solid-phase strategy, since this platform would ultimately allow the production of a large number of FK228 analogues. As shown in Scheme 1, a backbone amide linker (BAL)[12] was coupled to aminomethylpolystyrene resin, and a subsequent reductive amination anchored a cysteamine on the resin. To the resulting secondary amine (**1**), the first amino acid, Fmoc-L-Asp(OAl), was coupled with HBTU[13] for 12 h (80% yield). However, when several coupling methods were screened, TFFH[14] was found to provide a higher yield (95%). Also, coupling the amino acid as a symmetric anhydride by treating with DIC was effective (98% yield). The aspartylcysteamine (**2**) was constructed by one simple coupling reaction and is a surrogate for the challenging

* To whom correspondence should be addressed. Phone: +1-972-883-2917. Fax: +1-972-883-2925. E-mail: jungmo@utdallas.edu.
† University of Texas at Dallas.
‡ Present address: Department of Pharmacological and Toxicological Chemistry, University of Naples, "Federico II", Naples 80100, Italy.
§ University of Texas Southwestern Medical Center at Dallas.

[a] Abbreviations: TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; BAL, 5-(4-formyl-3,5-dimethoxyphenoxy)butyric acid; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; DIC, diisopropyl carbodiimide; TFA, trifluoroacetic acid; L-1-Nal, L-1-naphthylalanine.

10.1021/jm800959f CCC: $40.75
Published on Web 10/08/2008

Scheme 1. Synthesis of FK228 analogues[a]



[a] Reagents and conditions: (a) $NaBH_3CN$, rt, 12 h; (b) Fmoc-Asp(OAl), DIC; (c) Piperidine; (d) Fmoc-AA_1, HBTU; (e) Fmoc-AA_2, HBTU; (f) Fmoc-*D*-Cys(Trt), HBTU; (g) Fmoc-AA_3, HBTU; (h) $Pd(PPh_3)_4$, DMBA; (i) HBTU; (j) TsCl, pyridine; (k) DBU; (l) 1% TFA/DCM; (m) I_2; (n) TFA (95%), triisopropylsilane.

heptenoic acid that was synthesized over five steps (51% yield) in the previously reported synthesis.[8]

Using standard *N*-Fmoc/^{t}Bu solid-phase peptide synthesis strategy, the remaining four amino acids were introduced to build the linear pentapeptides (**3a**−**m**). This strategy facilitated exploring each position with a variety of amino acids in L- and D-configurations besides the synthesis of a FK228 analogue containing a *Z*-Dhb (**5m**). To ensure backbone conformations close to FK228, each residue was initially substituted with an amino acid in the same configuration. In other words, the L-Val was replaced by an L-amino acid, whereas the D-Val was substituted by a D-amino acid. However, both L- and D-amino acids were used to replace *Z*-Dhb because of the absence of C^{α}-chirality. For the construction of the bicyclic structure, the D-Cys was conserved in all FK228 analogues. All reactions used to prepare linear pentapeptides (**3a**−**m**) proceeded with high yields providing high purity (>95%).

The allyl ester of the linear peptide (**3a**−**m**) was selectively removed by Pd^0 and an allyl scavenger, dimethylbarbituric acid (DMBA), followed by the deprotection of the *N*-Fmoc group. Then, a macrolactam (**4a**−**m**) was formed by treating with a coupling reagent like HBTU for 3 h with high purity (>95%). After the selective removal of Trt groups by TFA (1% in DCM), the resulting free thiols were oxidized using iodine to make a disulfide bond. The bicyclic FK228 analogues (**5a**−**m**) were cleaved from the resin by TFA (95%) and characterized by HPLC and ESI-MS. By use of this synthetic route, a number of FK228 analogues were rapidly produced and the advantages of solid-phase reactions provided high overall yield (75−90%) and purity (80−94%). (see Table S1 in Supporting Information).

A FK228 analogue containing a *Z*-Dhb (**5m**) was synthesized using the steps reported in the previous synthesis.[8] A monocyclic peptide bearing L-Thr at the position of the *Z*-Dhb (**4m**) was constructed as described above. Then the β-hydroxyl group of the Thr was tosylated and eliminated by treating with DBU, introducing a *Z*-Dhb (**4n**). The formation of the *Z*-Dhb was

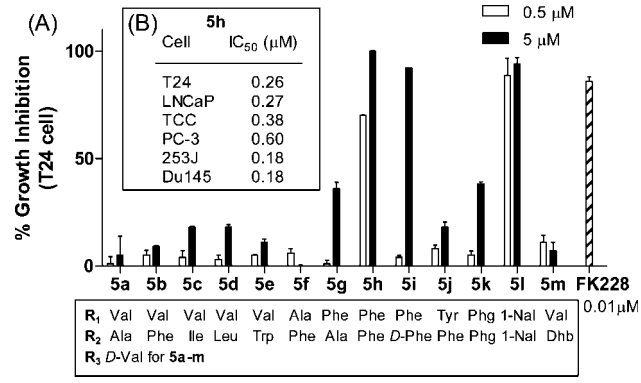


Figure 2. Antitumoral activity of FK228 analogues on a variety of human cancer cells: bladder (T24, TCC, 253J) and prostate (LNCaP, PC-3, Du145).

quantitatively monitored by analytical HPLC, and the subsequent oxidation of thiols produced the compound **5m**.

As Hsieh and co-workers have reported earlier,[15] FK228 inhibits the growth of human cancer cell lines, such as T24 cells derived from urinary bladder carcinoma, with high efficacy. To examine antitumoral activity, the FK228 analogues (**5a**−**m**) were initially screened using T24 cells at 0.5 and 5 μM. As shown in Figure 2A, **5h** and **5l** were identified as the most potent analogues showing 70−86% growth inhibition at 0.5 μM and higher than 95% at 5 μM. One of the lead compounds (**5h**) was then assessed against six human cancer cell lines from either prostate or bladder tumor and demonstrated a strong growth inhibition on all six cancer cells (IC_{50} = 0.18−0.6 μM; Figure 2B). This limited structure−activity study revealed that two aromatic amino acids (L-Phe or L-1-Nal at the positions of the L-Val and the *Z*-Dhb in FK228) yielded high antitumoral activity. It also appears that the bulkier side chain group of the L-1-Nal in **5l** resulted in higher cytotoxicity (IC_{50} = 0.06 μM on T24 cell). In addition, a stereochemical preference was observed at the position of the *Z*-Dhb, as D-Phe in the compound **5i** led to a significant loss in potency.

In summary, we have designed a novel FK228 analogue by simple isosteric replacement, and the modifications enabled rapid and efficient synthesis of a number of FK228 analogues in the solid phase. A limited structure−activity study of the FK228 analogues revealed new compounds with high antitumoral activity on various cancer cells. The approach developed in this study will be of great use to achieve higher potency and to understand the molecular mechanism of FK228.

Acknowledgment. This work was supported in part by University of Texas at Dallas and Welch Foundation (Grant AT-1595, J.-M.A.), NIH Grant CA95730 (J.-T.H.), and University of Naples, "Federico II" (S.D.M).

Supporting Information Available: Experimental details and characterization of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM800959F