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Suppression of Tumor Growth in Mice by Rationally Designed Pseudopeptide Inhibitors of Fibroblast Activation Protein and Prolyl Oligopeptidase[1]

Kenneth W. Jackson*, Victoria J. Christiansen*, Vivek R. Yadav†, Robert Silasi-Mansat§, Florea Lupu§, Vibhudutta Awasthi†, Roy R. Zhang‡ and Patrick A. McKee*

*William K. Warren Medical Research Center, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA; †College of Pharmacy, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA; ‡Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA; §Cardiovascular Biology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA

Abstract

Tumor microenvironments (TMEs) are composed of cancer cells, fibroblasts, extracellular matrix, microvessels, and endothelial cells. Two prolyl endopeptidases, fibroblast activation protein (FAP) and prolyl oligopeptidase (POP), are commonly overexpressed by epithelial-derived malignancies, with the specificity of FAP expression by cancer stromal fibroblasts suggesting FAP as a possible therapeutic target. Despite overexpression in most cancers and having a role in angiogenesis, inhibition of POP activity has received little attention as an approach to quench tumor growth. We developed two specific and highly effective pseudopeptide inhibitors, M83, which inhibits FAP and POP proteinase activities, and J94, which inhibits only POP. Both suppressed human colon cancer xenograft growth >90% in mice. By immunohistochemical stains, M83- and J94-treated tumors had fewer microvessels, and apoptotic areas were apparent in both. In response to M83, but not J94, disordered collagen accumulations were observed. Neither M83- nor J94-treated mice manifested changes in behavior, weight, or gastrointestinal function. Tumor growth suppression was more extensive than noted with recently reported efforts by others to inhibit FAP proteinase function or reduce FAP expression. Diminished angiogenesis and the accompanying profound reduction in tumor growth suggest that inhibition of either FAP or POP may offer new therapeutic approaches that directly target TMEs.

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Introduction

Tumor microenvironments (TMEs) as small as 1 to 2 mm^3 contain parenchymal-derived cancer cells within stroma that is composed of activated fibroblasts, developing microvasculature, and extracellular matrix (ECM); stroma may account for ~90% of tumor weight [1,2]. Fibroblast activation protein (FAP), a type II integral membrane protein and prolyl-specific serine proteinase, is overexpressed on cell membranes of fibroblasts in more than 90% of epithelial cell–derived malignancies, i.e., lung, breast, colon, and so on [3,4]. FAP is rarely found on adult normal tissues and is essentially absent on benign tumors, features that make it an attractive diagnostic and therapeutic target [5–10]. It is believed that 1) FAP engages in proteolysis of ECM during tissue invasion [11–15], 2) FAP-expressing cells appear

Abbreviations: FAP, fibroblast activation protein; POP, prolyl oligopeptidase; TME, tumor microenvironment; DPPIV, dipeptidyl peptidase IV; ECM, extracellular matrix; IHC, immunohistochemistry; CAFs, cancer-associated fibroblasts; MMPs, matrix metalloproteinases

Address all correspondence to: Kenneth W. Jackson, PhD, 975 NE 10th St, Room 1217, Oklahoma City, OK 73104.
E-mail: ken-jackson@ouhsc.edu

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to foster immune tolerance within TME [16–22], and 3) FAP/FAP-expressing cells support angiogenesis [9,10,23–26]. Efforts to limit FAP activities that might enhance tumor growth have focused on inhibiting its proteolytic properties [5,24,27] or blocking putative FAP^+ cell–induced immunotolerance of growing cancer [16,17,20–22].

Commanding less attention has been another prolyl oligopeptidase (POP) normally found in many tissues but commonly overexpressed along with the ubiquitous protein thymosin β4 in a number of malignancies [28–36]. Following partial cleavage of Tβ4 by an unknown enzyme, its degraded form is digested further by POP to yield the potent angiogenic peptide, Ac-SDKP [37,38]. POP proteinase activity clearly has a role in angiogenesis [38–41], but unlike FAP, it resides on cells throughout the tumor and not just on stroma. While inhibition of POP proteolytic activity is reported to arrest the growth of gastric cancer cells in culture [42], *in vivo* studies of POP inhibition in tumor models are lacking. The individual contribution of either POP or FAP to tumor expansion is difficult to decipher, given their overlapping proteolytic activities for cleaving Z-Gly-Pro-AMC, succinyl-Gly-Pro-AMC, and similar non-specific substrates; in addition, the lack of highly efficient aqueous soluble specific inhibitors of FAP or POP adds to the problem.

Despite lacking specificity, PT-100 (valyl-proline boronic acid; Val-boroPro) and PT-630 (glutamyl-proline boronic acid; Glu-boroPro) have been used to study the effects of FAP proteinase inhibition on cancer growth [24,43–47]. Both PT-100 and PT-630, however, also inhibit dipeptidyl peptidase IV (DPPIV) and, to a lesser extent, POP in purified solution. Moreover, PT-100 and PT-630 both rapidly cyclize in physiologic media and lose inhibitory activity [48,49]. Narra et al. [45] and Santos et al. [24] showed that PT-630 inhibited endogenous lung cancer growth in immunodeficient mice and in syngeneic colon cancer grafts in mice. In both studies, inhibition of FAP or DPPIV by PT-100 or PT-630 appeared to suppress tumor growth [24,43,50]. Huang et al. [51,52] reported that human breast cancer cells transfected with proteolytically inactive recombinant FAP, or breast cancer cells transfected to express wild-type proteolytically active FAP that is inhibitable by PT-630, still formed rapidly growing breast tumors in severe combined immunodeficiency mice. As a consequence, they suggested that FAP proteolytic activity has little or no impact on cancer growth; however, since transfected cancer cells served as FAP^+ cells instead of stromal fibroblasts as in human breast cancers, their model differed from established biology of such cancers [51].

In a mouse syngeneic 4T1 mammary carcinoma model, when short hairpin inhibitory RNA (shRNA) targeting FAP was injected intratumorally and peritumorally, FAP expression was knocked down by ~50%, tumor growth was reduced, angiogenesis was suppressed, collagen accumulation increased within the tumor, and tumor apoptosis was promoted; apparent side effects were not noted [53]. FAP gene silencing for 17 days did not induce paraneoplastic features such as cachexia, anemia, and lethal bone toxicities that were noted with tumor growth inhibition by immunologic depletion of FAP^+ cells within TME [18–20]. Given the reduction in FAP protein, FAP proteinase activity should also have been significantly reduced. Interestingly, the FAP-knockdown results closely mirrored those yielded by studies in which FAP proteinase activity was inhibited [24,45]. The sum of studies to date clearly indicates the need for more efficient and predictable FAP inhibition to determine whether simply inhibiting FAP proteolytic activity will circumvent FAP^+ cell destruction and thereby avoid perturbing potential FAP^+ cell functions that might cause adverse constitutional effects. Moreover, the suggested therapeutic potential for targeted POP inhibition to diminish angiogenesis and reduce tumor growth [40,54] has not been explored as far as we are aware and deserves direct evaluation. To examine these issues, we designed and synthesized a more stable, specific, and soluble FAP and POP inhibitor that we termed M83 and a highly specific, soluble inhibitor of POP only that we designated as J94 [10,49].

We used the primary structure surrounding the scissile bond of the only established physiologic substrate for FAP, namely, $alpha_2$-antiplasmin, as a template for designing M83 [49,55]; similarly, the scissile bond region of POP substrates was used to design J94 [49,56]. Extensive characterization showed that both inhibitors possessed similar features, i.e., excellent aqueous solubility at neutral pH, low molecular weights [529 (M83) and 554 (J94)], absence of cyclization in aqueous solution, and retention of inhibitory function after prolonged exposure to human plasma. Both are charged and hydrophilic, thereby minimizing intracellular entry; moreover, both M83 and J94 have low nanomolar K_i values for inhibiting FAP or POP or only POP, respectively. J94 does not inhibit FAP and neither M83 nor J94 significantly inhibits DPPIV [49]. When on live cells characteristic of TME, the membrane-associated form of either enzyme can be rapidly and completely inhibited, suggesting easy accessibility to the active site [10]. We now report analyses of substantial growth suppression of human colon cancer xenografts by M83 or J94 in immunodeficient mice without apparent adverse effects.

Methods

Cell Culture

HCT116, a human colon cancer cell line, and H441, a human lung cancer cell line, were obtained from ATCC (Manassas, Virginia) and grown as monolayer cultures in RPMI or Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were maintained in a humid 5% CO_2 atmosphere at 37°C.

Drug Design and Synthesis

The FAP/POP dual inhibitor, M83, was synthesized in two steps as previously reported [49]. Briefly, a structure consisting of *acetyl-Arg(Pbf)-peg*-D-*Ala*, based on the sequence surrounding the P1-P1′ scissile bond in Met-α_2AP, where *peg* is defined as 8-amino-3,6-dioxaoctanoic acid and Pbf represents N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, was synthesized and purified. The second step of the synthesis was linking this protected peptide-like construct to L-boroPro pinanediol ester and subsequently purifying the protected product. Finally, the pinanediol and Pbf groups were chemically removed and M83 was purified by reversed-phase HPLC.

The POP specific inhibitor, J94, was prepared using the equivalent strategy as for M83. The design of the precursive molecule was based on the reported sequence N-terminal to the scissile bond of optimal peptide substrates determined for POP [56]. The protected tripeptide *acetyl-Lys(Boc)-Leu-Arg(Pbf)*-OH was synthesized and purified, where Boc represents *N-tert*-butyloxycarbonyl. The second step was identical to that for M83, where the precursor peptide was linked to L-boroPro pinanediol ester and then HPLC purified.

Animals

Five- to six-week-old male athymic nude Foxn1nu mice were purchased from Harlan Laboratories (Houston, Texas). Animals were allowed to acclimate for ~5 days before any procedures. All animal protocols used in