Molecular tools that block maturation of the nuclear lamin A and decelerate cancer cell migration

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Keywords:
Prelamin A
Zinc metalloprotease STE24
ZMPSTE24
Lamin A

\textbf{A B S T R A C T}

Lamin A contributes to the structure of nuclei in all mammalian cells and plays an important role in cell division and migration. Mature lamin A is derived from a farnesylated precursor protein, known as prelamin A, which undergoes post-translational cleavage catalyzed by the zinc metalloprotease STE24 (ZMPSTE24). Accumulation of farnesylated prelamin A in the nuclear envelope compromises cell division, impairs mitosis and induces an increased expression of inflammatory gene products. ZMPSTE24 has been proposed as a potential therapeutic target in oncology. A library of peptidomimetic compounds were synthesized and screened for their ability to induce accumulation of prelamin A in cancer cells and block cell migration in pancreatic ductal adenocarcinoma cells. The results of this study suggest that inhibitors of lamin A maturation may interfere with cell migration, the biological process required for cancer metastasis.

1. Introduction

The nuclear lamina is composed of several lamin-type proteins that include lamin A as a key component. Lamin A is derived from a precursor protein, known as prelamin A, which undergoes four post-translational modifications before releasing the mature lamin A (Fig. 1).\textsuperscript{1} First prelamin A is farnesylated at the C-terminal cysteine residue of the CaaX motif (Fig. 1; intermediate I); a modification which contributes to its localization at the nuclear membrane. Second, the three C-terminal amino acids (i.e. -aaX) are cleaved-off by the human zinc metalloprotease STE24 (ZMPSTE24; also known as FACE1) leading to intermediate II. However, a biochemical redundancy mechanism exists for this second step that can allow alternative processing of intermediate I to II by the Ras converting endopeptidase 1 (RCE1). Third, the farnesyl cysteine residue of intermediate II is converted to the methyl ester intermediate III, under the catalytic activity of isoprenylcysteine carboxyl methyltransferase (ICMT). Finally, the last 15 amino acids (containing the farnesylated cysteine residue) are cleaved-off by ZMPSTE24, releasing the mature lamin A protein. Currently, ZMPSET24 is the only known enzyme that can perform this last critical endoproteolytic cleavage and prelamin A is the only known mammalian substrate of this enzyme. Therefore, ZMPSTE24 plays a key role in the maturation of the nuclear envelope filament lamin A, contributing to the structure of nuclei in all mammalian cells.\textsuperscript{7} Inhibition of ZMPSTE24 leads to accumulation of the farnesylated prelamin A and premature cell senescence. Unlike apoptosis, cellular senescence is a permanent cell cycle arrest that halts tumorigenesis and simultaneously activates antitumor immune responses.\textsuperscript{3} Consequently, it has been proposed that inhibitors of ZMPSTE24 may act as senescence agonists.\textsuperscript{4} Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare genetic disease, which mimics some aspects of human aging.\textsuperscript{5} HGPS cells accumulate a variant of prelamin A, known as progerin, which cannot be cleaved by ZMPSTE24 and presumed to be responsible for the disease symptoms. Cells expressing progerin have genomic instability,\textsuperscript{6} telomere dysfunction,\textsuperscript{7} altered epigenetic modifications of histones,\textsuperscript{8} abnormal chromosome segregation, binucleation\textsuperscript{9} and changes in nuclear architecture.\textsuperscript{10} However, the exact molecular mechanism(s) explaining all these alterations and their connection to cellular senescence is still unknown. Therapeutic agents that block the biosynthesis of farnesylated prelamin A in cells provide some in vivo benefits, both in humans and animal models of HGPS. For example, a combination treatment with pravastatin (1) and zoledronic acid (2), was found to extend longevity in a mouse model of human premature aging (Fig. 2).\textsuperscript{11} Pravastatin (1) and zoledronic acid (2), are clinically validated drugs...
that specifically target the mevalonate pathway, inhibiting HMG-CoA reductase and farnesyl pyrophosphate synthase (fFPPS), respectively. These drugs block isoprenoid biosynthesis and consequently, farnesylation of prelamin A. Similarly, inhibition of the farnesyl transferase enzyme (FTPase) with lonafarnib (3) in monotherapy,12 or in combination with ZMPSTE24, leading to the accumulation of prelamin A in cellular assays4–6). It is noteworthy that clinical observations from HIV infected patients treated with these drugs are inconsistent or contradictory to these findings.22 However, this discrepancy may be due to the low affinity of these compounds for ZMPSTE24, their high protein binding and the differences between protein concentrations in cultured cells versus human plasma. Inhibition of ZMPSTE24 with the only non-peptidic HIV PI, tipranavir18 and accumulation of prelamin A in human fibroblasts treated with geranylgeranyl transferase I (GGTase I) inhibitor have also been reported.25 However, the structural features of these latter compounds deviate significantly from those of the peptidomimetics 4–6.

We initiated our medicinal chemistry efforts with the knowledge that aspartate proteases (e.g. HIV protease) and the zinc metalloproteases (e.g. ZMPSTE24, thermolysin, angioteisin converting enzyme and others) share a common mechanistic action. Both families of enzymes use a water molecule as the nucleophile that attacks the scissile amide bond. This is in contrast to the serine/ cysteine proteases, which use the side chain of a nucleophilic amino acid residue to initiate amide bond hydrolysis. Based on the mechanistic similarities of aspartate proteases and the zinc metalloprotease, we designed a minimal con-
The hydroxyethylene moiety of compounds such as 4, 5 and 6, whereas, inhibitors of zinc metalloproteases often have a phosphinic acid pharmacophore, which serves as a bioisostere of the metal chelating transition state (Fig. 3). Interestingly, HIV PIs that have been reported to inhibit ZMPSTE24 (i.e. 4–6) are characterized by aromatic moieties at both the P1 and the P1' residues (i.e. binding to the S1 and S1'...
moieties were introduced, usually from an acid chloride, such as the 2-pyridone; Fig. 3), whereas the structurally related compound darunavir, a dual inhibitor of the endothelin converting enzyme (ECE) and the endothelin converting enzyme (ECE-1). Accordingly, the minimal consensus scaffold representing the general structures 7a, a dual inhibitor of the endothelin converting enzyme (ECE) and neutral endopeptidase (NEP) and 7b, a dual inhibitor of angiotensin converting enzyme (ACE) C-domain and the endothelin converting enzyme-1 (ECE-1). Accordingly, the minimal consensus scaffold represented by the general structures 8 and 13 (Fig. 3) was designed and the synthesis of a 65-member library was initiated in search of hits creating a catalytically relevant mutant) or by suspending them in high membrane-bound enzymes are typically rendered soluble in buffer by either turnover of ZMPSTE24 remains a significant challenge. Highly membrane-bound enzymes are typically rendered soluble in buffer by either cleaving a “tail” peptide that anchors them into a membrane (i.e., fragment f23). Subsequently, deprotection of the N-Boc group of the valine moiety under standard TFA conditions and coupling of various carboxylic acids, using standard peptide coupling chemistry, introduced various -R1 groups to give analogs 8 (Scheme 1); specific examples from this library are shown in Figure 5.

2.2. Synthesis of phosphinic acid-based analogs 13

In spite of the significant similarities, in both structure and function, between the bacterial zinc metalloprotease thermolysin (TLN) and ZMPSTE24, neither inhibitors of TLN nor inhibitors of any other zinc metalloprotease have been reported to inhibit ZMPSTE24 or block prelamin A accumulation in cells. Interestingly, TLN was used to model a tetrapeptide ligand bound to the human ZMPSTE24 and the resemblance between the active sites of ZMPSTE24 and TLN was highlighted. We decided to probe the ability of phosphinyl peptides to block prelamin A processing and consequently, we synthesized derivatives 13, based again on the minimal consensus model shown in Fig. 3. In a previous study, screening of hydrophobic amino acids at the C-terminal of tripeptide ligands (i.e. residue presumed to bind in the S2′ sub-pocket of the active site) identified alanine as the residue providing the most potent phosphonamidic acid inhibitors of TLN (e.g. compound 14, Fig. 6). We decided to incorporate this finding in the design of our phosphinic acid-based analogs 13, as well as maintained the Cα and Cβ benzyl substituents, which presumably could bind in the S1 and S1′ sub-pockets of the active site (Fig. 3).

Synthesis of compounds 13a,b was initiated with the three-component condensation of diphenylmethanamine hydrochloride, phenyl acetic aldehyde and hypophosphorous acid to give N-protected phosphinic acid 15, as previously reported (Scheme 2). Deprotection of the amine moiety under acidic conditions, followed by coupling with benzyl chloroformate produced the racemic Cbz-protected aminophosphinic acid-based analogs 16, which was subsequently converted to its diastereomeric salt with R(-)-methyl benzylamine. The desired R-enantiomer of 16 was isolated after two recrystallizations in moderate yield; all characterization data were consistent with those previously reported, including the high optical purity, which was based on the reported optical rotation. Intermediate R-16 was condensed with the acrylate 17; the latter reagent was prepared in two steps from diethyl 2-benzylmalonate, which was converted to the acrylate via a Mannich-type reaction and simultaneous in situ decarboxylation. The trivalent phosphorus species, generated by heating 16 with neat hexamethyldisilazane (HMDS), condensed with 17 via a phospha-Michael addition to give the phosphinate 18 in good yield. The 31P NMR of 18 indicated that a single set of diastereomers was formed, confirming the high enantiomeric purity of R-16. However, for the purpose of this study, authentic standards were not synthesized and consequently, the exact %ee was not determined. After saponification of 18 and coupling with the methyl ester of l-alanine under standard peptide coupling conditions, the methyl ester 13a was obtained, which was first purified by C-18 reversed phase HPLC and then saponified to give the free C-terminal carboxylic acid 13b (Scheme 2).

2.3. Biological evaluation of key analogs from general structure 8 and 13

In spite of several previous efforts, the development of a high-throughput and reliable in vitro assay for measuring the catalytic turnover of ZMPSTE24 remains a significant challenge. Highly membrane-bound enzymes are typically rendered soluble in buffer by either cleaving a “tail” peptide that anchors them into a membrane (i.e., creating a catalytically relevant mutant) or by suspending them in high concentrations of protein-detergents complexes or micelle preparations; in the case of ZMPSTE24 only the latter approach is feasible. However, this approach often leads to artifacts that can derail SAR studies in medicinal chemistry. Previously, detecting ligand binding to ZMPSTE24

Fig. 5. Select examples of compounds designed to explore prelamin A accumulation, plausibly by inhibiting ZMPSTE24. The exact structures of all the fragments used for this library (i.e. f1 to f35) are shown in Fig. 4.

2.1. Synthesis of peptidomimetic analogs 8

Enantioselective synthesis of the hydroxyethylene dipeptide biosaestre 11 was previously reported (Scheme 1). In brief, the formation of enantiomerically enriched intermediate 11 was dictated by the chirality of L-phenylalanine and intramolecular hydrogen bonding between the -NH2 group and the keto of the enaminone 10, leading to the formation of an ~18:1 diastereomeric ratio of ketones (after the first reduction step). Additionally, the presence of the two bulky protecting groups on the α-amino of phenylalanine is assumed to bias the reduction of the keto 10 to the desired β-alcohol in high enantiomeric purity (i.e. in the second reduction step). For the preparation of our library, the doubly reduced product 11 (desired diastereomer isolated in 55% yield) was condensed with S-4-isopropoxyloxazolidine-2,5-dion (prepared as previously reported50) and the free amine of the valine moiety was Boc-protected. After debenzylolation of the phenylalanine to obtain intermediate 12, various -R2 moieties were introduced, usually from an acid chloride, such as the 2-(2,6-dimethylphenoxy)acetyl group found in lopinavir (i.e. fragment f23). Subsequently, deprotection of the N-Boc group of the valine moiety under standard TFA conditions and coupling of various carboxylic acids, using standard peptide coupling chemistry, introduced various -R1 groups to give analogs 8 (Scheme 1); specific examples from this library are shown in Figure 5.

Figure 5. Select examples of compounds designed to explore prelamin A accumulation, plausibly by inhibiting ZMPSTE24. The exact structures of all the fragments used for this library (i.e. f1 to f35) are shown in Fig. 4.
relied on high-resolution mass spectrometry of the protein-inhibitor complex,\textsuperscript{34} or indirect in vitro inhibition assays that were coupled to the methylation step catalyzed by ICMT (\textit{i.e.} detecting $^{14}$C-incorporation from S-adenosyl-l-[methyl-$^{14}$C]methionine).\textsuperscript{33,35}

We decided to initiate efforts towards the discovery of novel hits targeting ZMPSTE24 using a phenotypic, cell-based assay that measures the accumulation of farnesylated prelamin A in cells treated with a compound. Although cell-based assays do not usually support the development of a reliable SAR model, they have the advantage of providing hits with better drug-like properties (\textit{e.g.} cell membrane permeability). Based on the minimal consensus scaffold of analogs 8 and 13, designed to resemble the key structural motifs binding to the S1 and S1’ sub-pockets of known aspartic protease\textsuperscript{36} and zinc metalloproteases, a 65-member library was synthesized. All members of this library were initially screened at a fixed concentration of 10 µM in human osteosarcoma cells (U-2 OS) for their ability to induce prelamin A accumulation. U-2 OS cells were incubated with each compound for 24 h and then their cell lysates were analyzed by Western Blots using a prelamin A-specific antibody; the results from select compounds are shown in Fig. 7. The levels of prelamin A were compared to tubulin,
since tubulin expression should not be altered by biochemical events associated with the biosynthesis or processing of prelamin A. To adjust for the normal variability between assays, lopinavir (4) was tested in parallel as the reference control. Several analogs were identified that induced greater accumulation of prelamin A than lopinavir (albeit only modestly greater), including compounds 8f, 8k, 8l, 8n, 8g (Fig. 7), as well as the phosphinyl analog 13a (Fig. 8a). Interestingly, the carboxylic acid analog 13b was inactive at the concentration tested (10µM), possibly due to lower cell permeability than its corresponding methyl ester 13a (Fig. 8a). Compound 13b was previously explored as an inhibitor of the neutral endopeptidase (NEP) and the endothelin converting enzyme (ECE), as well as a dual inhibitor of nephrilysin (neutral endopeptidase) and aminopeptidase N that are associated with the degradation of enkephalins. Additionally, 13b was also previously explored as a potential angiotensin converting enzyme 2 inhibitor, but found to be essentially inactive (Ki > 10µM).

According to microarray studies available in Oncomine, ZMPSTE24 is overexpressed in many human cancers, including glioma, melanoma, ovarian, prostate and pancreatic cancers. To further probe the effects of our compounds, we tested one of our best analogs, compound 13a, in pancreatic adenocarcinoma cells (HPAF II, SW1990 and KP-4), and human colon cancer cells (HCT-116). In general, higher intracellular accumulation of the farnesylated prelamin A was observed with 13a compared to lopinavir in all cases (Fig. 8).

Further biological evaluation of compound 13a in our most highly tumorigenic pancreatic cancer cell lines (HPAF-II and KP-4) revealed that despite accumulation of prelamin A, proliferation was not significantly affected in these cells (Fig. 9a). Consistent with this observation, treatment of HPAF-II and KP-4 cells with 13a did not lead to accumulation of the proliferation inhibitor p21 nor reduction in the expression levels of the proliferation biomarker MCM6 (Fig. 9b). However, in a transmigration potential assay, analog 13a was found to inhibit cell migration, whereas lopinavir (4) did not have any apparent effect (Fig. 9c). Metastasis remains a leading cause of mortality from cancer. Cancer cell migration through connective tissues requires that cells undergo large distortions in order to crawl through constrained spaces of tissue matrix. It has been shown that intracellular levels of lamin A modulate the elasticity/stiffness of solid tumors and consequently, the nuclear plasticity of cancer cells plays a key role in the 3D migration of these cells. Even a very modest increase in intracellular prelamin A levels (10%) has been reported to inhibit the migration of lung cancer cells A549 by 90%. These findings suggest that although modest increase in intracellular levels of prelamin A accumulation may not lead to any cell toxicity, it may still have therapeutic value in deterring cancer metastasis. Therefore, optimization of compounds such as 8f and 13a is worth pursuing as potential...
therapeutics in oncology.

3. Conclusion

Lamins are an integral part of the nuclear envelope proteins and play a critical role in nuclear stability, as well as in numerous other biochemical processes that are incompletely understood at this time. Recent studies have shown that accumulation of high levels of the farnesylated prelamin A protein around the nuclear membrane induces abnormalities to the nuclear shape, resulting in polyploid cells and exhibiting features of cellular senescence that trigger an antitumor response. The nucleoskeletal properties of farnesylated prelamin A confer cell stiffness and downregulate cancer cell migration/metastasis. This precursor protein must undergo four post-translational modifications, including two proteolytic steps catalyzed by the zinc metalloprotease STE24 (ZMPSTE24), to produce the mature lamin A. A number of recent biochemical studies suggest that inhibition of ZMPSTE24 leads to prelamin A accumulation at the nuclear envelope, thus compromising cell division, impairing mitosis and inducing cell senescence. The biochemical consequences of ZMPSTE24 inhibition suggest that this enzyme may be a valuable therapeutic target for both the treatment of cancer and the prevention of metastasis.

Although the development of an in vitro assay that is reliable for guiding medicinal chemistry efforts remains a challenge for this enzyme, phenotypic drug discovery can be an alternative approach, which can provide hits with better initial drug-like properties (e.g. cell membrane permeability). Phenotypic screening is a complimentary approach to the more traditional target-based screening with the potential to allow interrogation of yet incompletely understood biochemical processes that are associated with a disease. A library of compounds was synthesized based on the consensus tripeptide-like core of known aspartate protease and zinc metalloprotease inhibitors. All analogs were screened using Western Blot analysis of prelamin A accumulation in cancer cells and compared to lopinavir, a previously reported inhibitor of ZMPSTE24. Several analogs were identified with sufficient cell permeability to induce intracellular accumulation of prelamin A in osteosarcoma (U-2 OS), pancreatic adenocarcinoma (HPAFII, SW1990, KP-4) and colon carcinoma (HCT-116) cells. Some of these compounds were more potent than lopinavir in blocking prelamin A processing, without exhibiting any overt toxicity to cells. However, even when the level of toxicity was nearly negligible, as in the case of the highly tumorigenic pancreatic cancer cells KP-4, treatment of these cells with the most potent analog 13a led to simultaneous accumulation of prelamin A and inhibition in cell migration. Cell migration requires the deformation of the nucleus, as previously demonstrated in neutrophils that possess a multi-lobulated nucleus and express low levels of mature lamin A. Furthermore, cell migration is a necessary biological event for tumor cell dissemination and consequently, our data suggests that analog 13a represents a valuable hit for optimization into inhibitors of prelamin A processing, which even at sub-toxic concentration can have anti-metastatic effect. Potency optimization and target engagement studies that will confirm binding of our inhibitors to ZMPSTE24 in cells are currently in progress.

Acknowledgments

Financial support for this work was provided from research grants from the Canadian Institute of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC) to G. Ferreyre and Y.S. Tsantrizos, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2018.10.001.

References

1. (a) Young SG, Fong LG, Michaelis S. Prelamin A, Zmpste24, mishapen cell nuclei, and Progeria - new evidence suggesting that protein farnesylation could be important for disease pathogenesis. J. Lipid Res. 2005;46:2531–2558;


