Understanding the molecular mechanism associated with reversal of oral submucous fibrosis targeting hydroxylysine aldehyde-derived collagen cross-links

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Abstract:
Fibrosis is a pathological state characterized by excessive deposition of the extracellular matrix components leading to impaired tissue function in the affected organ. It results in scarring of the affected tissue akin to an over-healing wound as a consequence of chronic inflammation and repair in response to injury. Persistent trauma of susceptible oral mucosa due to habitual chewing of betel quid resulting in zealous healing of the mucosal tissue is one plausible explanation for the onset of oral submucous fibrosis (OSF). The irreversibility and resistance of collagen to degradation and its high potential to undergo malignant change are a major reason for morbidity in OSF. Hence, early diagnosis and timely treatment are crucial to prevent the progression of OSF to malignancy. This review focuses on the mechanistic insight into the role of collagen cross-links in advancing fibrosis and possible therapeutic targets that bring about a reversal of fibrosis. These options may be beneficial if attempted as a specific therapeutic modality in OSF as is in organ fibrosis. The upregulation of lysyl oxidase and lysyl hydroxylase has been shown to exhibit the higher levels of the hydroxylysine aldehyde-derived cross-links in fibrosis and tumor stroma promoting the tumor cell survival, resistance, and invasion. The in silico analysis highlights the potential drugs that may target the genes regulating collagen crosslinking.

Keywords:
Anti-fibrotic targets, collagen cross-links, fibrosis, oral submucous fibrosis, repair, treatment, wound healing

Introduction
Fibrosis is associated with a chronic inflammatory state as a result of an immune response, tissue remodeling, and repair mechanisms. The release of pro-fibrotic cytokines and growth factors results in the excessive deposition of extracellular components. A similar mechanism is antecedent in oral submucous fibrosis (OSF), a chronic debilitating condition of the oral cavity, oropharynx, hypopharynx, and upper two-thirds of the esophagus, characterized by generalized submucosal fibrosis, marked rigidity, and inability to open the mouth. Reportedly, 7%-13% of OSF has the potential to undergo malignant transformation. The existing evidence suggests that the deregulation in collagen remodeling results in OSF. Therefore, an increase in collagen synthesis in association with decreased collagen degradation is one of the plausible causes for...
the onset of this condition. Many biological pathways and molecular mechanisms are implicated in the initiation of fibrosis. A wide range of treatment modalities such as surgical therapy, drug management, and physiotherapy has been attempted with varying degrees of benefit but none can halt or reverse the fibrosis completely. The early diagnosis and sequentially early treatment of OSF are crucial to prevent its progression to malignancy.

Recent studies underscore the significance of a specific type of collagen cross-links that dictates irrevocable changes associated with a fibrotic disease. The therapies largely target the enzymes involved in the cross-linking of collagen as it determines the stability of the extracellular matrix (ECM). Understanding the pathophysiology of collagen cross-link would be important specifically target its progression and reverse fibrosis to prevent the interference of anti-fibrotic drugs in normal tissue repair, but this literature survey brings to light the potential approaches to identify the molecular targets to reverse fibrosis in OSF paving the way for evidence-based therapeutic protocols for the management of OSF.

Mechanism of Collagen Cross-Linking

Collagen is composed of three polypeptide alpha chains coiled around each other to form a triple helix configuration rich in proline and glycine. Each of the three \( \alpha \)-chains within the molecule forms an extended left-handed helix with 18 amino acids per turn. Glycine is found at every third position of the polypeptide chain and forms the part of the repeating sequence Gly-X-Y, where X and Y can be any amino acid but are usually occupied by proline and hydroxyproline respectively. Thus, all types of collagen have this basic structure forming triple helix of three polypeptide chains; however, their size, function, and tissue distribution vary considerably.

An understanding of collagen cross-linking is important as it determines the physical and mechanical properties and stability of the ECM. The cross-linking of the fibril-forming collagens, type I, II, III, V, XI, XXIV, and XXVII involves the posttranslational modifications of the procollagen molecules. The collagen cross-linking involves two pathways, one based on Allysine, the lysine-derived aldehyde (Lys\(_{\text{ald}}\)), and the other, Hydroxyallysine, the hydroxylysine aldehyde derived (Hyl\(_{\text{ald}}\)).

The initial step involves the oxidative deamination of lysine and hydroxylysine residues into aldehydes at the terminal sequence of the collagen or the telopeptide by either of the family of five lysyl oxidases (LOX and LOX-like [LOXL] 1-4), the copper metalloenzymes. Further Lys\(_{\text{ald}}\) and Hyl\(_{\text{ald}}\) undergo intramolecular and intermolecular condensation with lysine, hydroxylysine, or histidine in the triple helix to form difunctional, trifunctional, or tetrafunctional cross-links. In the allysine (Lys\(_{\text{ald}}\)) pathway, the Lys\(_{\text{ald}}\) in the telopeptides of the collagen molecule forms an intramolecular cross-link, aldol condensation product that matures as dehydrohistidinoxylysinomerodesmosine (deH-HHMD). Lys\(_{\text{ald}}\) also undergoes intermolecular condensation to form histidinoxylysinonorleucine (HHL) [Figure 1a and b]. In the hydroxyallysine (Hyl\(_{\text{ald}}\)) pathway, the Hyl\(_{\text{ald}}\) in the telopeptide undergoes intermolecular condensation to form intermediate divergent cross-link, dehydro-hydroxylysinonorleucine and dehydro-dihydroxylysinonorleucine (deH-DHLNL), which further matures into stable pyridinoline, hydroxylysyl pyridinoline (HP), lysyl pyridinoline (LP) and pyrrole (d-PRL = deoxypyrrole; PRL = pyrrole) cross-links [Figure 1c, d and e]. In the skin, the telopeptide lysines are not hydroxylated, while in bone and cartilage, the telopeptide lysines are highly hydroxylated. However, there can be a switch from Lys\(_{\text{ald}}\) to Hyl\(_{\text{ald}}\) pathways by hydroxylation of lysine at the triple helix or the telopeptide by lysyl hydroxylase enzyme (LH) and its three isoforms (LH1, LH2, and LH3) [Figure 2]. The hydroxylated lysine at the telopeptide has a different amino acid sequence, to that hydroxylated in the triple helix, due to different types of LH enzymes involved in hydroxylation.

The hydroxylation occurs only in the helical sequence Gly-X-Lys, but not when the helical sequence is Gly-Lys-Y, where lysine is in X position. The Lys\(_{\text{ald}}\) pathway is noticed in the skin and cornea, whereas the...
HyL ald pathway outweighs in bone, cartilage, tendon, dentin, and ligaments.[6,13] The HyL ald pathway usually predominates in the ECM due to the mechanical, thermal, and chemical stability of the cross-links formed.[11,15,16]

**Collagen Cross-Link and Reversibility of Fibrosis**

Fibrosis is the result of increased deposition of collagen accompanied by decreased proteolytic degradation of collagen, resulting due to an imbalance between the matrix metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases.[3] The reversibility of fibrosis may depend on the type of cross-linking of collagen as several fibrotic disorders are associated with the increased HyL ald-mediated cross-links.[17,18] The lysyl residue in the telopeptide of the collagen in the skin is not hydroxylated; hence, HHL and deH-HHMD are generally found, while the hydroxyllysyl residues, the pyridinolines (HP and LP) are seen in traces.[13] In skeletal tissue, the lysyl residues are hydroxylated forming stable mature cross-links like HP, LP, and pyrrole, required for mechanical stability.[11] Reduction in collagen degradation is associated with the increased amounts of collagen cross-links derived from both enzymatic[9] and nonenzymatic (glycation) pathways.[20,21]

The accumulation of Hyl ald-mediated cross-links is indicative of irreversible collagen deposition and decreased susceptibility to proteolytic enzymes. An increase of ~0.1 Schiff-base cross-links per collagen molecule results in a 2–3-fold increase in resistance to human collagenase compared with uncross-linked collagen.[19] However, it is not just the amount but also the type of cross-link that determines the digestibility of the collagen in fibrosis. Furthermore, a switch from the Lys ald to the HyL ald pathway may be a significant event in soft-tissue fibrosis, rather than just the increase in the number of cross-links.[23]

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**Hydroxylysine Aldehyde-Derived Cross-Links in Self-Limiting versus Progressive Fibrosis**

The irreversibility of fibrosis is the result of resistance exhibited by Hyl ald-mediated cross-links to proteinases, and hence, the quantity of Hyl ald cross-links may be an important factor in assessing the irreversibility of fibrosis. The changes in the topology of cross-links in self-limiting and progressive forms of fibrosis may aid in strengthening these hypotheses.[7] Both the self-limiting fibrosis (wound healing) and progressive form of fibrosis initially respond with the deposition of Hyl ald-cross-link deH-DHLNL. However, eventually, the self-limiting form of fibrosis replaces the Hyl ald-cross-links with Lys ald-cross-links, while the hypertrophic scars retain a 1:1 ratio of both the types of cross-links and hence are resistant to degradation.[7] Further, in fibrotic conditions such as lipodermatosclerosis, there is over hydroxylation of lysine residues accompanied by increased enzymatic glycosylation of hydroxylysine residues. Glycosylation modulates the physicochemical properties of the cross-link by decreasing the susceptibility to proteolysis.[24] However, in normal skin, glycosylation occurs only to a smaller extent.[25]

**Collagen Cross-Linking in Tumor Stroma**

The upregulation of LOX and LOXL2 levels has been reported in numerous cancer types[2,26,27] and is known to initiate the collagen cross-linking and stiffening of the tumor stroma. LOX portents metastasis by increasing the number of collagen cross-links in the tumor stroma.[28] LOX secreted by tumor cells facilitates the collagen crosslinking and thus stiffening of the stroma leading to the integrin-mediated formation of focal adhesions that initiate the tumor invasion.[28] LOX plays a critical role in establishing a
microenvironment that is growth-permissive and is capable of promoting metastatic invasion by enhancing tumor cell survival and persistence.\[25\] Similar to fibrosis, there is hydroxylation of lysine residues in tumor stroma by LH2, eventually leading to the higher levels of Hy[14d] cross-links and lower levels of Lys[14d] cross-links. The change in tumor stroma promotes ECM stiffening, tumor invasion, and metastasis.\[16\] The tumor invasion and metastasis enhanced by LH are mediated through the upregulation of hypoxia-inducible factor-1α (HIF-1α). Both LOX and LH are upregulated in response to hypoxia and are the targets of HIF-1α; hence, hypoxia determines the type and quantity of collagen cross-links formation.\[26\] HIF-1α promotes tumor progression by activating the transcription of genes involved in angiogenesis, energy metabolism, and adaptive survival.\[30\] Therefore, the therapeutics targeting LOX and LH will abrogate not only the progression of fibrosis but also will prevent fibrosis-induced tumor invasion and metastasis.\[29\]

**Upregulation of Lysyl Oxidases in Oral Submucous Fibrosis and Oral Squamous Cell Carcinoma**

A strong correlation of activation of the TGF-β pathway in the pathogenesis of OSF has been reported suggesting the role of TGF-β as a principal regulator of fibrosis.\[31\] Arecoline, the principle constituent of betel quid, has been shown to upregulate the TGF-β and Thrombospondin 1 an activator of latent TGF-β in OSF.\[32\] While TGF-β promotes the expression of LOX both at the mRNA and protein levels in various cell lines.\[33\] The LOX upregulation is also facilitated with increased copper levels in OSF as LOX is copper dependent for its functional activity.\[34\] This suggests the excessive deposition of highly cross-linked collagen in OSF may be due to the upregulated activity of LOX [Figure 3].\[2\] The upregulation of LOX activity is observed in the fibroblasts cultured from OSF patients, which suggests its role in the formation of fibrotic bands in OSF.\[15\]

An upregulated LOX expression pattern is also seen in the invasive front of oral squamous cell carcinoma (OSCC) arising from OSF, postulating the stromal response.\[2\] Previous studies suggest a multifunctional role of LOX at various stages of tumorigenesis.\[36\] Several single nucleotide polymorphism sites have been identified in the LOX coding region such as C225G, G409C, G473A, C476A, G816A, T924G, and A1135G, where G473A shows a higher frequency of polymorphism.\[33\] Recently, Bhanu et al.\[37\] reported that elevated LOX in OSCC patients favored tumor growth and lymph node metastasis (LNM). The stiffened matrix due to LOX-led matrix cross-linking compressed the vasculature resulting in tissue hypoxia. The ensuing hypoxia then promoted the Rho-GTPase-dependent cytoskeletal tension leading to aberrant tumor morphogenesis, which successively augmented cellular motility resulting in metastasis. The higher expression of LOX at the invasive tumor front (ITF) resulted in a greater propensity to invade deeper structures.\[37\] Yu et al.\[38\] have reported a stronger LOX expression at the ITF when compared to the tumor center. Furthermore, LOX expression in basal cells of the nontumor epithelium was insignificant when compared to ITF. Saito et al.\[39\] have shown that LH2, LOX, and LOXL2 are considerably upregulated in late-stage OSCC, associated with LNM, and were allied to poor prognosis. In these tumors, augmentation of LH was particularly related to metastatic propensity as LH promoted the more stable Hy[14d] crosslink. Moreover, the elevation of the stromal Hy[14d]/Lys[14d] cross-link ratio played a crucial role in the LNM of OSCC. This indicates that the quality of collagen cross-linking played a more significant role in cancer metastasis.\[39\]

**Targets for Reversibility of Fibrosis**

The LOX enzyme has seemingly been the main target to inhibit the covalent cross-linking of collagen as it leads to a stable, insoluble ECM due to decreased degradation by MMPs.\[40\] LOX inhibition affects the cross-linking of collagen in liver fibrosis and also brings about widening and splitting of fibrotic bands with disorganization and disappearance of collagen bundles over time.\[40,41\] Molecules targeting LOX, LOXL1, and LOXL2 have proven to be beneficial in vivo liver fibrosis.\[40,43\] The LOXL2 has been specifically targeted in phase 2 clinical trials for the treatment of myelofibrosis,\[43\] idiopathic pulmonary fibrosis,\[44\] and HIV-induced liver fibrosis\[45\] using the newly developed humanized monoclonal antibody, Simtuzumab.\[8\] Clinical trials on Simtuzumab have also been conducted for the treatment of pancreatic adenocarcinoma,\[8\] nonalcoholic steatohepatitis-induced fibrosis,\[47\] primary sclerosing cholangitis,\[48\] and colorectal adenocarcinoma.\[49\] Although Simtuzumab has shown anti-fibrotic properties in rodent fibrotic liver models by preventing and reversing fibrosis,\[50,51\] the results have not been beneficial in human trials both in the treatment of fibrosis and carcinoma possibly due to its high specificity and failure to inhibit other isoforms or the target site [Table 1].\[8\]

The copper-binding motif and lysyl tyrosyl quinone domain of LOX enzymes are other structural targets for its inhibition. The copper chelating agents such as D-penicillamine and tetrathiomolybdate used in in vivo experiments have reasonable evidence in potentially reversing fibrosis by LOX inhibition.\[52,53\] Clinical trials of D-penicillamine indicate its role in the reversal of
childhood cirrhosis bringing about a significant decrease in fibrosis.\cite{51,52} However, D-penicillamine failed to prevent the progressive course of Prednisone-treated idiopathic pulmonary fibrosis.\cite{53} \(\beta\)-aminopropionitrile (BAPN) is another extensively studied LQT domain inhibitor of LOX, which forms an irreversible covalent complex.\cite{57} BAPN has been widely studied \textit{in vivo} to reverse liver, peritoneal, and myocardial fibrosis.\cite{58,59} Clinical trials of BAPN have been conducted on scleroderma patients with limited therapeutic effects.\cite{54} The other important target known to attenuate irreversible fibrosis is the LH enzyme, which is crucial for the formation of the stable

**Table 1: Drug targets against lysyl oxidase tested in human clinical trials for fibrosis**

| Studies | LOX isoform | Drug | Class | Mechanism | Condition | Phase |
|---------|-------------|------|-------|-----------|-----------|-------|
| Raghu \textit{et al.}, 2017\cite{44} | LOXL2 | Simtuzumab | Monoclonal antibody | Inhibitor | Idiopathic pulmonary fibrosis | Phase 2 |
| Verstovsek \textit{et al.}, 2017\cite{43} | | | | | Thrombocythaemia myelofibrosis | Phase 2 |
| Meissner \textit{et al.}, 2016\cite{45} | | | | | HIV-induced liver fibrosis | Phase 2 |
| Harrison \textit{et al.}, 2018\cite{46} | | | | | Nonalcoholic steatohepatitis-induced fibrosis | Phase 2 |
| Muir \textit{et al.}, 2019\cite{48} | | | | | Primary sclerosing cholangitis | Phase 2 |
| Benson \textit{et al.}, 2017\cite{49} | | | | | Pancreatic adenocarcinoma | Phase 2 |
| Hecht \textit{et al.}, 2017\cite{50} | | | | | Metastatic KRAS mutant colorectal adenocarcinoma | Phase 2 |
| Bhusnurmath \textit{et al.}, 1991\cite{51} | Copper binding motif | D-penicillamine | Copper chelating agents | Inhibitor | Childhood cirrhosis-associated fibrosis | - |
| Pradhan \textit{et al.}, 1995\cite{52} | | | | | Childhood cirrhosis-associated fibrosis | |
| Selman \textit{et al.}, 1998\cite{53} | | | | | Idiopathic pulmonary fibrosis | |
| Clements \textit{et al.}, 1999\cite{54} | | | | | Diffuse systemic sclerosis | |
| Keiser and Sjoersma, 1967\cite{55} | LTQ domain | \(\beta\)-aminopropionitrile | Small molecule | Inhibitor | Scleroderma | |

LOX: Lysyl oxidase, LOXL2: Lysyl oxidase-like 2, LTQ: Lysyl tyrosyl quinone, KRAS Gene: Kirsten Rat Sarcoma Gene

**Figure 3:** Schematic diagram illustrating the mechanism of collagen crosslinking in oral submucous fibrosis
In-silico analysis was performed for the genes encoding various enzymes and growth factors favoring Hylald-mediated collagen cross-linking and fibrosis. Gene-Interaction Network was constructed for the genes LOX, LOXL2, PLOD2 (Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2-encoding LH2), TGF-$\beta$ 1, and HIF-1$\alpha$ using GeneMANIA Cytoscape[Figure 4]. Potential drugs targeting these genes were predicted using the drug-gene interaction database [Table 2].

**Conclusion**

The present review provides a mechanistic insight that irreversible fibrosis is not merely due to the abnormal accumulation of collagen accompanied by decreased collagenase degradation but is a result of the deposition of stable mature Hylald-derived cross-links that are resistant to degradation. The upregulation of LOX and LH exhibits higher levels of Hylald-derived cross-links in fibrosis and tumor stroma promoting the tumor cell survival, resistance, and invasion. The LOX and LH enzymes involved in collagen cross-linking are the potential targets to interfere with the fibrotic process and prevent further fibrosis-induced tumor invasion and metastasis. Recent treatment modalities for the reversal of fibrosis have focused on targeting the collagen cross-links along with decreased collagen synthesis. The nature and the type of cross-links or pathways involved in OSF are yet to be explored. A thorough understanding of the collagen cross-links in OSF will determine whether the outcome of the disease is reversible or not. Based on these findings, the decision for an appropriate therapeutic strategy based on evidence is timely and imminent for the treatment of OSF. In-silico analysis revealed the potential drug candidates for future research on anti-fibrotic

**Table 2: Potential drug interactions using the drug-gene interaction database**

| Gene   | Drug              | Interaction type | Sources                                                                 |
|--------|-------------------|-----------------|-------------------------------------------------------------------------|
| HIF1a  | Carvediol        | Modulator       | DrugBank: https://www.dgidb.org/sources/HIF1A#_interactions             |
| HIF1a  | Nitroglycerin     | Inhibitor       | MyCancerGenomeClinicalTrial: https://www.dgidb.org/sources/MyCancerGenomeClinicalTrial |
| HIF1a  | Chembl1080759    | -               | TdgClinicalTrial: https://www.dgidb.org/sources/TdgClinicalTrial         |
| HIF1a  | Deferoxamine      | -               | NCI: https://www.cancer.gov/                                             |
| HIF1a  | Geldanamycin      | -               | NCI: https://www.cancer.gov/                                             |
| HIF1a  | Noscapine         | -               | TdgClinicalTrial: https://www.dgidb.org/sources/TdgClinicalTrial         |
| HIF1a  | Epotin alfa       | -               | NCI: https://www.cancer.gov/                                             |
| HIF1a  | 2-methoxyestradiol| -               | DrugBank: https://go.drugbank.com                                         |
| HIF1a  | Pimonidazole      | -               | NCI: https://www.cancer.gov/                                             |
| HIF1a  | Chembl426560      | -               | DrugBank: https://go.drugbank.com                                         |
| HIF1a  | Chembl299763      | -               | TdgClinicalTrial: https://www.dgidb.org/sources/TdgClinicalTrial         |
| HIF1a  | Sunitinib         | -               | CGI: https://www.cancergenomeneinterpter.org/home                       |
| LOXL2  | Simtuzumab        | Inhibitor       | ChEMBL: https://www.ebi.ac.uk/chembl/                                    |
| PLOD2  | Ascorbate         | Cofactor        | DrugBank: https://go.drugbank.com                                         |

HIF1a: Hypoxia-inducible factor 1-alpha, LOXL2: Lysyl oxidase-like 2, PLOD2: Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2-encoding LH2
therapies that may target the genes regulating collagen cross-linking.

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Conflicts of interest
All the authors of the manuscript hereby state that there is no financial implication or personal relationship with other people or organization that could inappropriately influence the outcome of this work.

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