More than genes and cells: drug discovery in the ECM

Drug discovery in the last few decades has focused on the cellular and genetic mechanisms of disease. This has been very successful in cancer, which is a disease of somatic genetics, and moderately successful elsewhere. But the declining productivity of pharmaceutical and biotechnology investment in drug discovery and development[1] suggests that we should be alert to other approaches. One is to look outside the cell, at the extracellular superstructure of the body. Once viewed as an inert structure that is just the biological equivalent of a petri dish, the extracellular milieu is now being seen as a therapeutic target, especially for diseases of old age. Importantly, targeting the scaffold of the body might be a much faster route to treatment for some conditions than attempting to find, and fix, underlying cellular or genetic aetiology of disease.

By Dr William Bains

It is a truism that we become less flexible as we age. This is literally true of many tissues, which become more mechanically rigid and less amenable to tissue remodelling with age. Loss of flexibility is due at least in part to chemical changes in the Extracellular Matrix (ECM) in the tissue, converting a flexible network of molecules into a rigid mesh. Stiffness increases have been recorded in lung, major arteries, bone, muscle, tendon and lens of the eye among other tissues in older compared to younger people. However, this remodelling with age is more important to disability and disease than just piling on the wrinkles or reducing our score in tennis. In a range of contexts it has been linked to disease, and research in the last decade has suggested how targeting ECM proteins could be blocked or reversed to open a new route to treat those diseases.

The ECM makes up around one-third of our bodies by dry mass. It is a complex meshwork of proteins, proteoglycans and other molecules constructed on a scaffold of collagen. There are two broad types of ECM: basement membrane and interstitial ECM. The basement membrane directly underlies endothelial and epithelial cells, and is composed of primarily of type IV collagen, laminins, entactin, nidogen and heparan sulfate proteoglycans such as perlecan. The interstitial ECM, which makes up most of the extracellular mass of the body, consists of many different types of collagen, tenascin, proteoglycans and elastin in elastic tissues such as skin or tendon. The ECM can also bind a variety of soluble proteins such as cytokines and growth factors[2].

Chemical cross-linking of the proteins in the ECM is central to its construction: human mutations or mouse knock-outs of cross-linking enzymes are usually severely disabling or lethal. During

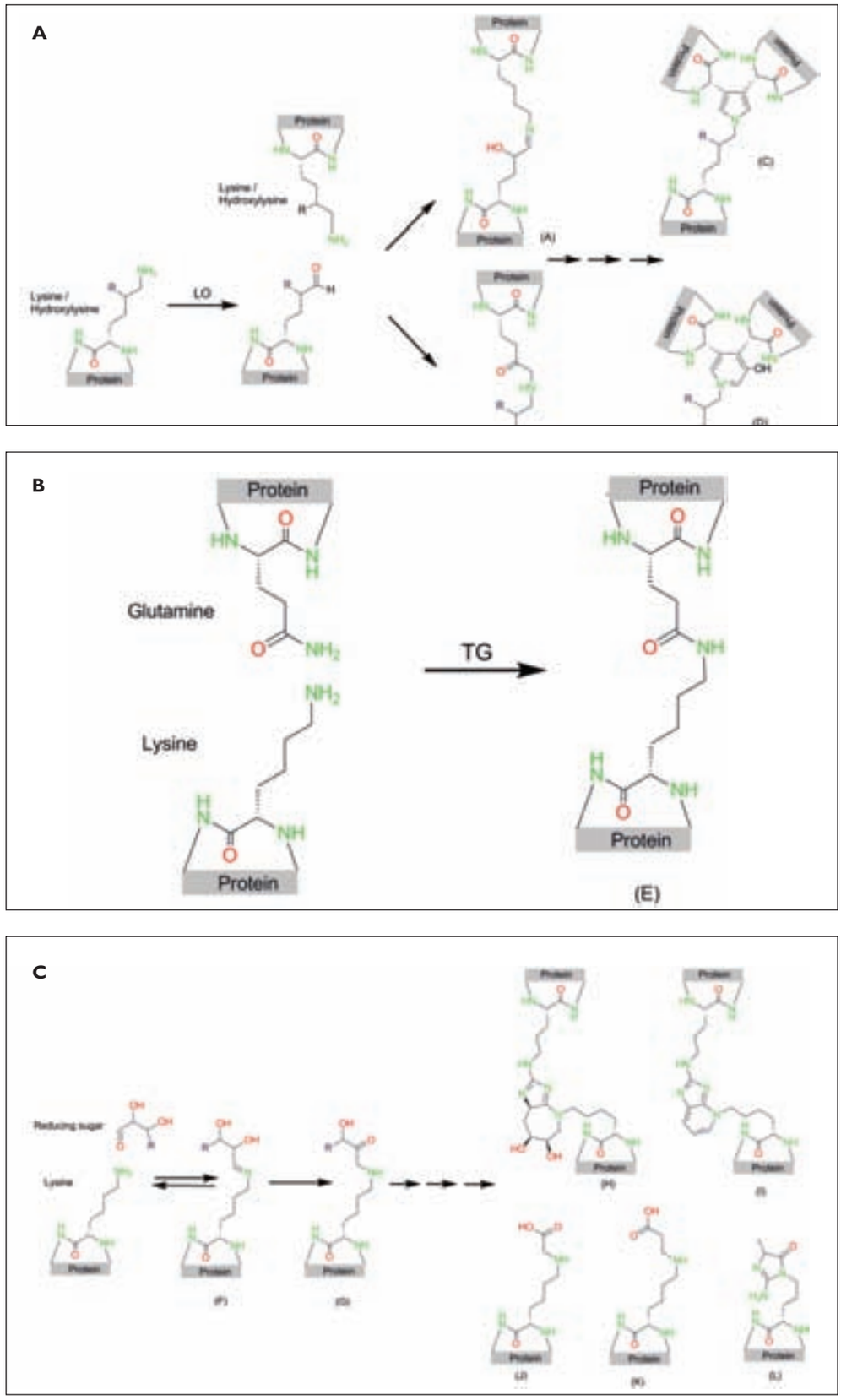

Figure 1
Cross-links and their chemistries. Square boxes represent the rest of the protein chain
A Lysyl Oxidase. Loysyl Oxidase (LO) acts on lysine (R=H) and hydroxylysine (R=OH) to form allysyl peptides which cross-link to form (hydroxy)lysino-D-norleucine (Compound A) and (hydroxy)lysinoketonorleucine (Compound B). These then can add to further cross-links to form Lysyl Pyrrole (compound C) and Lysyl Pyridinoline (Compound D) end products
B Transglutaminase. Glutamine and Lysine are cross-linked by transglutaminases (TG) to form an e-(g-glutamyl)-lysine (EGGL) cross-link (Compound E)
C Glycation. Amine side-chains (here just illustrated with lysine) react with reducing sugars (a polyol aldehyde, typically glucose or fructose, where R is a polyol of the form $(CHOH)_nCH_2OH$) to form a Schiffs base (Compound F), which undergoes Amadori rearrangement to form a stable fuctosamine (Compound G). Oxidation and additional cross-links and rearrangement result in a complex mix of products, of which only Glucosepane (Compound H), Pentosidine (Compound I), Carboxymethyl Lysine (CML – compound J), Carboxyethyl Lysine (CEL – Compound K) and one of the Methylglyoxal hydroimidazolones (MGH-3 – Compounds L) are shown

growth and development the principal cross-linking chemistry is catalysed by Lysyl Oxidase (LO) (**Figure 1A**), a family of at least four enzymes in man that oxidise lysine to an aldehyde which can then react with other nitrogen-containing amino acid side-chains to form cross-links that are very stable under physiological conditions. The amount and chemical sites of LO-derived cross-links vary substantially between tissues and depend, at least in part, on the proteins being cross-linked.

Collagen is the most widespread LO substrate. There are at least 27 different collagen types, arranged in many different architectures[3]. Different collagens are cross-linked to different degrees; for example, collagen III is present in bone fibrils but not detectable in extracts, probably because high levels of cross-linking render it completely insoluble[4]. LO also links elastin into the ECM, although it forms different crosslinks between elastin chains.

LO is not only essential for the construction of ECM during growth and development, but also during wound healing. After mechanical damage or inflammation new ECM is laid down and cross-linked with LO family members and transglutaminases (discussed below). With chronic damage, especially chronic inflammation, this wound healing response becomes dysregulated and excessive masses of poorly organised ECM are laid down, resulting in fibrosis. Many fibrotic conditions are associated with increase in collagen cross-linking density as well as increase in collagen mass[5]. Changed mechanical tension in the ECM itself in such fibrotic tissue enhances fibroblast's secretion of inflammatory cytokines, driving further inflammation and fibrosis.

Collagen is turned over very slowly: some measures of ECM turnover in tendons and bone suggest protein half-lives of years to decades in man. Partly for this reason, fibrosis has primarily been seen as a pathology of excess ECM production, and especially production of collagen fibres that are poorly ordered compared to healthy tissue, because once in place the collagen is seen as 'there for life'. However, cross-linking is key to this, both because it locks collagen into disordered networks and because it renders it harder to turn over and remodel into healthier material. Thus LO inhibitors are being seen as an important therapeutic approach to a wide range of fibrotic disease. In particular, Barry-Hamilton et al[6] recently showed that inhibiting Lysyl Oxidase homologue LOXL2 with an enzyme-specific monoclonal antibody reduces fibrosis and cross-linking in mice with liver fibrosis induced with carbon tetrachloride. Although the inflammation was no less severe in the treated mice, they lived longer than control animals. By targeting the fibrotic damage effectively, this approach modifies disease without delving into the complexities either of inflammation or of hepatic metabolism.

The body does turn over collagen through wholesale degradation of the ECM, probably by metalloproteases and subsequent excretion of cross-links. An ideal treatment for chronic fibrotic disease might therefore be to supplement this turnover with therapeutic enzymes that break just the cross-links, allowing less radical remodelling of fibrotic tissue *in situ*. This would be a specific example of an approach termed 'Medical Bioremediation'[7] (by analogy with environmental bioremediation) more usually discussed in terms of clearing accumulated intracellular material using exogenously supplied enzymes. Applying such an approach to fibrotic disease is precedented with the approval of injectable *Clostridium* collagenase as a treatment for Dupuytren's Contracture[8], and is being pursued at a preclinical level as a therapy for macular degeneration and atherosclerosis among other diseases. However, no suitable enzyme for cross-link clearance is known, and targeting would have to be quite specific to avoid large-scale unlinking of ECM collagen.

While LO is a major cross-link of collagen, a second class of enzyme cross-links a wider variety of ECM proteins during development, inflammation and wound-healing. Transglutaminases (TGs) are a group of enzymes that catalyse the formation of an isopeptide bond between the side-chains of glutamine and lysine residues (**Figure 1B**). Unlike LOs, TGs have been widely discussed as a drug target. In particular, the ubiquitously expressed TG2 has been researched as a drug target for fibrosis and for cancer[9].

TG2 is a multifunctional protein; as well as its transglutaminase activity, it is active as a G protein to GPCRs, a GTPase, a disulfide isomerase, a protein kinase and it binds directly to fibronectin and integrin, thus mediating cell:ECM interactions. It is found in the cell nucleus, cytoplasm and plasma membrane as well as in the ECM, has at least two spice variants, is modulated by Ca^{2+}, NO, GTP and redox state, and has several roles in inflammation and apoptosis as well as ECM structural modification[10]. Its biology is therefore complex, but its transglutamination role can be well elucidated by looking at the levels of the cross-link it forms (EGGL – see **Figure 1B**) in the ECM. Alas, this is rarely done, as protein and mRNA levels are easier to measure, so those are what most research examines.

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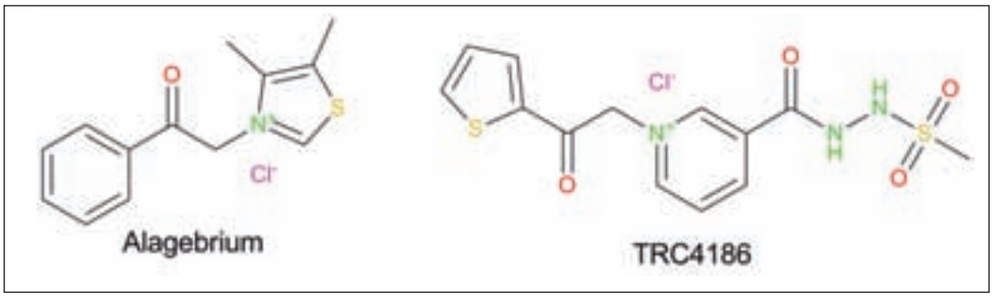

Figure 2: AGE-breaker chemistry. Structure of compounds ALT711, TRC4186

Like LO, TG2 is relatively promiscuous in its substrate, acting on amino acids on the surface of proteins that are in inherently disordered regions of structure and which approach near enough in the ECM to be cross-linked. Unlike LO, knocking out TG2 in mice results in animals that are nearly normal, a bizarre result for a multifunctional, ubiquitously expressed enzyme. It is likely that other enzymes, especially blood clotting factor XIIIa (which is also a transglutaminase) can replace TG2's function in some roles in development.

TG2's transglutaminase activity is seen in inflammatory disease, and especially fibrotic disease, so this may be another target for fibrosis. There has been a lot of work on inhibitors of TG2, primarily aimed at its role in cancer biology[11]. Small molecule TG2 inhibitors reduce fibrotic scarring in rodent models of diabetic nephropathy, and a TG2 knock-out mouse has reduced fibrosis in a lung fibrosis model[12]. However, these have not yet had quite as dramatic a therapeutic effect as the LO inhibitor described above.

As with LO cross-links, it would be useful not just to block the formation of EGGL in the ECM but to clear EGGL embedded in long-lived proteins such as tendon and bone collagen. Here we have more reason to be optimistic, because enzymes that cleave the EGGL isopeptide bond specifically are known, for example from leeches[13] and the brown moth[14]. The technical barrier to getting even a preclinical candidate therapeutic will be to find an enzyme with minimum activity against the peptide bonds in proteins, and hence with an acceptable safety profile.

There are many other chemical changes in the ECM with disease and age, but the last we will consider here is glycation. Glycation is an entirely spontaneous and non-enzymatic chemical change to proteins (as opposed to glycosylation, which is a different, enzyme-controlled process). Reducing sugars react spontaneously with amines to form Schiffs bases, which can rearrange to Amadori products. The Schiff's bases are readily hydrolysed back to the free amines and sugars, but the Amadori products are relatively stable and can undergo further, complex oxidative chemistry to form a complex soup of compounds collectively called Advanced Glycation Endproducts (AGEs) (**Figure 1C**). AGEs are stable under physiological conditions, and so accumulate in long-lived proteins. Some are fluorescent, and so tissue fluorescence is sometimes used as a measure of AGEs. The most common AGEs are CML and CEL, and many studies examine these (they are the immunodominant target of the few commercial anti-AGE antibodies that show any specificity for an AGE). However, recent research shows that other, non-fluorescent AGEs are more physiologically important, and particularly the cross-link glucosepane[15].

Glucosepane is strongly correlated with diabetic complications in man[16], and has been linked to degradation of the mechanical properties of skin, lung, bone and the arterial wall[17]. The main focus of research in glycation has been the arterial wall, where AGE cross-links have been specifically associated with hypertension[18]. It is believed that this is because AGE cross-links between all the molecules in the elastic ECM of the arterial wall reduce its ability to expand and contract as the systolic pressure wave passes, thus increasing the systolic pressure peak, although other mechanisms are possible. AGEs are also correlated with nephrosis in diabetics. Major causes of morbidity in diabetes are hypertension, kidney damage and associated circulatory damage that leads to ulcers and retinopathy, so AGEs are a good potential target for these diseases.

Alas, AGE formation is purely chemical, and so cannot be blocked by an enzyme inhibitor or a mouse knock-out. AGE formation could be blocked by agents that trap the α-dicarbonyl intermediates on the path to AGE. Aminoguanidine and pyridoxamine have been tested as AGE blockers[19].

Results have not been that impressive in man, however, possibly in part because you have to take the compounds continuously for 10 years to block the accumulation of AGEs in a protein with a 10-year half-life. It is likely that one of the benefits of calorie restriction (among many others) is reduced blood sugar levels and hence reduced glycation, but this is an even harder regime to adhere to.

A better route would be to clear glucosepane from the body, but here again we are stymied for lack of an enzyme or, until very recently, even a good supply of authentic glucosepane to test enzymes on. However one compound – Alagebrium (ALT711 – see **Figure 2**) – has been developed as a chemical AGE-breaker. It was designed to be a reagent that catalytically breaks α-dicarbonyls, which (at the time) were seen as a major form of cross-link *in vivo*[20]. Early trials showed a beneficial effect in man, but a larger follow-up trial showed no benefit[20-22]. There is also controversy over the drug's mode of action. Recent research has confirmed that Alagebrium does catalytically cleave dicarbonyls[23], resolving doubt about this in the past, but it is now believed that dicarbonyls are quantitatively trivial compared to the chemically stable AGE cross-links[16]. So the trials of Alagebrium have generated a drug that does something in rats[24], may do something in man, but it is as yet not clear what or how.

Torrent Pharmaceuticals is taking the compound TRC4186 into clinical trials as an AGE-breaker. Its chemical mode of action is obscure; the compound was identified by randomly reacting highly AGEd serum albumin (made by incubating BSA with 1.67M glucose for 16 weeks) with collagen, a chemistry that bears little relationship to AGE cross-links *in vivo*, and then searching for compounds that released the BSA. TRC4186 appears beneficial in animal hypertension models[25] but no human efficacy data has been released[26].

In the absence of reliable chemical or enzymatic methods of cleaving glucosepane, a supply of pure material to screen, any reliable high-throughput assay methods, and no realistic prospect for stopping its formation, AGE-breaker strategies remain speculative at the moment. Development of technology in any of these areas could open AGE-breaking to be a third approach to ECM cross-link clearance.

Other AGEs accumulate in tissues, but are probably of less physiological importance. The cross-link pentosidine has been the subject of much research because it is easy to detect, but is a minor component compared to glucosepane. Monovalent AGEs such as CML and CEL are also probably of little physiological importance unless they happen to modify a key functional residue, but the MGH AGEs are being seen as physiologically important[27], and may also be a target for future AGE-clearing strategies.

I have focused on fibrotic and inflammatory diseases above because they provide the most obvious conventional target for ECM-targeting therapies. But there is substantial evidence that repairing the ECM could be a valuable approach to cancer therapeutics and will be a central plank of future regenerative medicine treatments.

The ECM environment modulates cancer cell growth and migration[28]. Cells sense mechanical tension as well as chemical environment and respond to changes in the mechanical structure of the ECM: many cancers modulate the ECM around themselves to form a more permissive environment for their own growth. Barry-Hamilton et al[6] showed elevated LOXL2 in some tumours, and that blocking LOXL2 reduced tumour growth in mouse Xenograft models. The role of TG2 in cancer is well known[29]. ECM-targeting strategies have generally not been high on the list of preferred anti-cancer strategies because they can only slow cancer growth, not kill the cells. However, they work through fundamentally different mechanisms from more mainstream approaches, ones unrelated to intracellular mechanics of cell growth or apoptosis, and so may provide valuable adjuncts to other therapies.

A more exciting application of ECM-modulating therapeutics is in Regenerative Medicine. Regenerative Medicine seeks to repair disease- or age-related damage through one of two broad strategies: stimulating endogenous repair mechanisms that have been damaged or down-regulated, or providing exogenous cells or tissues. It is now well-known that cells grown in tissue culture mimic *in vivo* cell behaviour better if the cells are grown in a 3D matrix mimicking the ECM, rather than on flat glass or plastic surfaces, and that, far from being inert, many components of the ECM have powerful signalling effects on cells[2]. Even cell growth that is dependent on growth factors such as TGF-β is in fact also dependent on the mechanical properties of the ECM *in vivo*, because TGF-β is stored in the ECM as bound complexes, and released by cell-specific proteases or mechanical tension[30].

It is now understood that the quality of the ECM *in vivo* can also be decisive in determining how well cells function to repair tissue after damage. Several studies have suggested that the failure of older tissue to repair itself as well as young tissue

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is due at least as much to the 'old' ECM as to the cells themselves being old. For example, the replicative and differentiation potential of mesenchymal stem cells from old mice could be 'rescued' by growing them on ECM generated by young stem cells[31]. Kang and Lightman tracked regenerating peripheral nerves in the mouse and showed that the poorer regeneration in old mice over young ones was due to the lack of ability of the growing axon tips to clear debris from damaged nerves from their path. In the absence of debris, they grew and innervated muscle as well as nerves from young animals[32]. Loss of functional fibroblasts in the dermis of the skin, and hence loss of skin strength, elasticity and thickness with 'normal' ageing is primarily driven by degradation of the collagen matrix to which the cells attach, including glycation[33]. Thus ECM repair is likely to be a key component of either of the regenerative medicine strategies[34].

The structural proteins of the body, especially collagen, have traditionally been viewed as non-starters as drug discovery targets, because they are not obviously active as catalysts or signal transduction molecules. The recent work summarised above shows that this is not necessarily a good argument for ignoring the ECM. As an alternative route to identifying the role of every gene in every cell in a complex disease such as chronic inflammation or in cancer, targeting damage to the ECM may prove a valuable approach to new treatments for a range of diseases and disabilities of old age. DDW

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