Rampant proteolysis at the intersection of therapy-induced hypoalbuminemia and acute pancreatitis

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Abstract — Protease inhibition is the intended mechanism of action for drugs across a broad range of diseases: cancer, cardiovascular and stroke, diabetes mellitus, macular degeneration and Alzheimer’s. Treatment for fungal and multiple viral infections, including Sars-Cov-2, also relies upon inhibition of pathogen-specific proteases. This work examines the non-therapeutic proteolytic activity of one such drug, nelfinavir (tradename VIRACEPT™), approved as an inhibitor of HIV protease, the largest, “biotech launch” in history at the time of its introduction. Methods are described in the companion manuscript [Leonard et al. (2022), 4open 5, 11]. These methods are not only suitable for examination of on-target activity but also of off-target activity. Herein, it is demonstrated that nelfinavir is active both as an inhibitor and as a promoter of proteolysis of key blood proteins. Observations are readily connected to known drug induction of acute pancreatitis and attendant hypoalbuminemia. The benefits of expanding molecular-level, early-stage, off-target/off-substrate activity drug candidate evaluation become apparent. Finally, the reality of drug-induced disease places new demands on existing clinical procedures, namely that side effects be approached as symptoms of an induced disease.

Keywords: Proteolysis, Hypoalbuminemia, Acute pancreatitis

Context

Almost every mammalian protein begins and ends its existence as a substrate for proteases. Proteolysis is required to remove small segments of expressed proteins that ensure correct transportation and activation. Many proteins are cleaved at highly specific sites to allow diverse functionality from a single protein. Others undergo proteolysis to allow the extended network formation required for wound healing. All told, the human “degradome” comprises over 550 distinct proteases [1]. Despite their pivotal nature in the whole of human biology [2], proteases and their inhibition remain difficult to assess, especially in a drug discovery context. Detection of the fundamental interaction, namely the interaction of protease and substrate, presents sufficient challenge without the concurrent need to discern and quantify multiple products of whole-protein degradation. Accordingly, the historic and continuing practice is the use of short peptides as substrate surrogates, peptides that, at a minimum, include the suspected scissile amino acid sequence. While such an approach may provide some level of selectivity in the detection of a given protease, it is more than problematic in screening would-be therapeutics. Even if, by some good fortune, the test peptide accurately reflects the activity of its corresponding whole-protein substrate, assessment of off-target and off-substrate activity of the selected drug “hits,” remains virtually impossible. In this report, multiple inadequacies of existing proteolysis methods are reflected in therapy-induced diseases [3], and in off-target/off-substrate promotion of proteolysis. Newly developed methods eliminate most if not all the shortcomings of traditional methods, while concurrently opening opportunities in areas ranging from basic research to drug discovery to disease characterization and diagnosis.

Of proteolysis, proteins and diseases

Serum albumin (SA) in the bloodstream (reference concentration range: 35–50 mg/mL) maintains the fluid volume of the vasculature via colloid osmotic pressure (Fig. 1a) [4]. In doing so, SA levels limit the exchange of material across blood vessel walls and enable the necessary division between the intravascular and interstitial contents. SA also binds a wide range of molecules, including drugs and nutrients like non-esterified fatty acids, and serves as a systemic transporter among other necessary functions. As with all proteins, SA is subject to proteolysis by non-specific,
“digestive” proteases such as those expressed in inactive form in the pancreas. Under normal conditions, inactive digestive enzymes (proteases, amylases and lipases) migrate from the pancreas to the small intestine where activation and digestion of food occurs. It is noteworthy that only trypsinogen is activated to trypsin in the small intestine which then activates all other digestive enzymes. Clinically diagnosable conditions occur (Fig. 1b) when SA levels fall below 35 mg/mL in the serum (hypoalbuminemia) [5] or when elevated digestive enzymes exist in serum and urine (acute pancreatitis, AP) [6]. In research, it is frequently postulated that severe AP is manifest by active, digestive enzymes in the pancreas itself in addition to the cited elevated levels in the serum [7]. Regardless of the site of enzyme activation and regardless of the cause of AP (e.g., gallstones), digestive enzymes exist in locales with few safeguards against digestion of proteins, cells and tissues and their presence in the serum ensures system-wide distribution.

Hypoalbuminemia, regardless of its many root causes, leads to a loss of colloid osmotic pressure within the vasculature and fluid flows from the vasculature to the surrounding interstitial space [5] (of those many root causes of hypoalbuminemia, e.g., low expression of SA in the liver, physical insult, poor diet and inflammation, this report deals with those cases of hypoalbuminemia that intersect with acute pancreatitis and their amplification and induction by therapy). Constituents of the blood, molecular and cellular, become more concentrated, upsetting chemical/biochemical and physical/biophysical balances. Flow of blood is impeded and may become blocked, and/or vessels may become increasingly permeable, especially capillaries [8]. Concurrently, excessive amounts of solute-laden fluid enter the interstitial space, again upsetting biochemical and biophysical balance both in the fluid and at cell/extracellular surfaces. It is not surprising, then, that hypoalbuminemia has been generally correlated to “increased short-term mortality, length of hospital stay and complications” for a considerable number of disease states (e.g., COVID-19, HIV/AIDS) [9, 10]. Hypoalbuminemia also predicts disease severity for AP and accompanies a third of diagnosed AP cases [11]. While the cited work establishes a firm clinical connection between hypoalbuminemia and AP, no rationale for the connection is given. However, within the context of this work, it is noted that an increasing number of therapies are known to induce AP including a high percentage of putative protease inhibitors [3]. As will be addressed below in Overlooked Connections, off-target activity of approved protease inhibitors will, in the instances tested, not only connect hypoalbuminemia to AP but can serve to create a proteolysis-driven, degenerative and potentially deadly cycle between hypoalbuminemia and AP. It is also timely to note that one of the approved protease inhibitors known to induce AP is the drug, ritonavir, a component of the therapy (tradename Paxlovid) recently authorized by the US Food and Drug Administration for treatment of COVID-19 [12].

Given that hypoalbuminemia represents a reduction of SA in the blood, it is tempting to envision the “lost” protein reappearing as a corresponding increase of SA in the urine, a condition referred to albuminuria. However, it must be noted that not all albuminuria patients are tested for hypoalbuminemia and vice versa, and no conclusion can be drawn. It is instructive to note that albuminuria, in some cases, manifests as variable amounts of whole-protein SA and SA fragments. For example, 99% of SA in the urine of type 1 diabetes mellitus patients comprise fragments of less than 10 kDa, lending support to the likely in-serum digestion of SA [13]. Albuminuria is generally a phenomenon of impaired glomerular and tubular filtration. Healthy glomeruli don’t pass large proteins, and degradation...
products (peptides) or smaller proteins are recaptured in the tubules. However, excessive protein load will result in urinary leakage [14].

Altering biology to fit methods

Since multiple protease inhibitors are known to induce AP with possibly attendant hypoalbuminemia, two questions arise: (1) How are such molecules “discovered”? and (2) How are the methods discussed in later sections superior to currently employed methods, especially in the selection of new drugs and ready detection of potentially problematic off-target, off-class and off-substrate drug activity? The chapter, “Protease Assays,” [15] contained in the book, Assay Guidance Manual (written by Eli Lilly & Company and managed by the US National Center for Advancing Translational Sciences) comprises, in part, the answer to the first question.

“Protease Assays” focuses on the initial screening of chemical libraries for “hits” that demonstrate the desired effect, in this instance inhibition of the targeted protease. Tens of thousands of molecules may comprise the library, and assay methods are selected to address the need for high throughput. Indeed, recent and ongoing development in the instrument industry targets this demand. While the scale of such an approach and its execution may demand respect, the specifics of the biochemistry can be unsettling, as summarized: given the relatively small sizes of many proteases, their sequence and primary structures are readily assessed, and their preferred scissile point identified. Further, they are available in sufficient quantities for large scale screening. Substrate selection is another matter. Natural substrates tend toward higher molecular weights (viral substrates measure in the hundreds of thousands of Daltons), are not readily available on a massive scale, and proteolytic products are not identifiable by methods suitable to high throughput. Instead, early-stage, library screening substrates are small, typically synthetic peptides labeled at one or more sites with fluorophores. While the preferred scissile site for the test protease is incorporated into the test substrate, little more ensures its biological specificity (e.g., absence of exosite interactions), its relationship to the natural substrate and thus its biological relevance. Given that likely irrelevance, the effectiveness of leads forthcoming from the mammoth exercise is, at best, hit or miss. Even if the commitment were made to utilize, e.g., fragments of the natural substrate, the fluorescent methods employed would likely be unusable, due to the reliance on two or more probes in close proximity. Other high-throughput methods that rely on isolation and separation of products and/or reactants also employ small, synthetic substrates for the reasons cited earlier. Finally, even if some of the leads generated have true potential as therapies, molecular tests are unavailable to confirm that activity or to assess off-target/off-substrate activities that spawn side effects or even create clinically diagnosable conditions. The methods discussed in this work point toward a more general and definitive solution.

Selecting methods to fit biology

One immediate outcome of any proteolytic event is the formation of two protein fragments smaller than the original protein. In terms of molecular dynamics, each fragment is more mobile than the original protein and assessment of that change in mobility can be exploited to track proteolysis. The companion manuscript [16] demonstrates the utility of ESR spectroscopy of spin labelled (ESRSL) proteins to track proteolysis via changes in rotational mobility attendant to proteolysis. Given that ESRSL is sensitive to rotational rates covering six orders of magnitude [17] in its most common implementation and 12 orders of magnitude [18] for all implementations (roughly equivalent to rotational mobilities in molecular weight terms from 200 Daltons to two MegaDalton), it encompasses all likely proteolytic events. The companion manuscript [16] establishes the generality of ESRSL assessment of protease activity and its inhibition by biorelevant modulators. The companion report extends the use of ESRSL into the assessment of therapeutics designed to ameliorate or halt disease via modulation of targeted protease activity. The results of that assessment establish the utility of ESRSL as not only a powerful, new method for lead confirmation of likely drug candidates but also for early-stage determination of likely side effects associated with off-target, off-class and off-substrate activity.

In closing this section, it is noted that the demand for high-throughput screening arises from a common strategy in the pharmaceutical industry, namely the drive to maintain intellectual property for as many would-be-drug molecules as possible. Not only are intellectual property costs high, the costs for creating and maintaining such chemical libraries are also high. The strategy also has the unintended side effect of skewing hit selection toward high throughput methods, regardless of their lack of rigorous biological relevance. Recent advances, hailing back to the early days of target-based discovery, artificial intelligence (AI)-aided design of molecules that bind specifically to sites on targeted proteins is finally producing useful outcomes. Developers in this field acknowledge the need for new physical methods for confirming their hits [19, 20]. AI hit generation coupled to lead confirmation by methods such as those presented here promise effective therapies. Extensive molecular-level screening for off-target, off-class and off-substrate side effects promises truly safe ones.

Off-target effects of the approved HIV aspartyl protease inhibitor, nelfinavir, are summarized in Figure 2 (Data in Fig. 2 represent a very small percentage of the data collected in the companion manuscript [16]. No statistical analysis is applied in either manuscript). Proteolysis of two SL proteins (bovine serum albumin and bovine hemoglobin A) was tracked for three proteases representing three classes of proteases: aspartyl (porcine stomach pepsin), cysteine (papain) and serine (bovine pancreatic trypsin). Manifestation of proteolysis may be inferred from the ESRSL spectrum inset in the chart. As described above, ESRSL spectra reflect the rotational mobility of the moiety to which the label is attached, a property directly related to
The spectrum presented here contains two, clearly identifiable populations as distinguished by widely varying rotational mobility. Small, “mobile” fragments produced by proteolysis are manifest as the three, sharp lines while undigested substrate, more “rigid” in mobility, is manifest by a broader spectrum bounded by the upgoing peak on the far left and the down going peak on the far right. The degree of proteolysis at any point in time is represented by the height of the designated “mobile” peak. Presented are the fractional changes relative to the no – Nelﬁnavir control in the “Mobile” population following a 40-min digestion. The drug inhibits the proteolytic activity of pepsin, an aspartyl protease, in-class with HIV protease. In contrast, Nelﬁnavir promotes proteolysis by cysteiny1 (papain) and serinyl (trypsin) proteases. The promotion effect is most pronounced for the whole-protein substrate, serum albumin.

Each protease/substrate combination was tested in the presence and absence of nelﬁnavir and the degree of change in digestion noted from the height of the designated “mobile” population peak. The fractional change in proteolysis induced by nelﬁnavir was assessed after a 40-min incubation and is represented in the columnar bars. Within the strictest interpretation of “target-based” discovery, no effect of nelﬁnavir treatment should be seen for any combination of these off-target proteases and off-substrate substrates. Quite to the contrary, nelﬁnavir induced both inhibition and, surprisingly, promotion of proteolysis, and the nature and degree of that induced change is both protease and whole-protein substrate dependent.

For both substrates, SA and hemoglobin, nelﬁnavir inhibits proteolysis by 20–25% for the aspartyl protease pepsin, in-class with the HIV protease. A surprise occurs for the cysteiny1 protease, papain in that nelﬁnavir promotes proteolysis by 10–20% for both whole-protein substrates. The large, and troubling, effect is the dramatic promotion of digestion of SA by the common serinyl protease, trypsin, with nelﬁnavir nearly doubling the extent of proteolysis. Given that the promotion of proteolysis of hemoglobin by trypsin is much smaller than for SA, it likely that nelﬁnavir binds to SA, making it labile to proteolysis.

While most of the off-target/off-substrate data may be tolerable within a treatment regimen, the enormous increase in proteolysis of SA by a common digestive protease cannot be ignored. Indeed, as is addressed in the next section this molecular-level side effect may well induce and/or accelerate a condition known to coincide with indication of poor outcomes for treated patients: “The only risk factor associated with hypoalbuminemia was current antiretroviral therapy . . .” [10].

Figure 2. The effect of the drug, Nelﬁnavir, an approved HIV protease inhibitor, is shown for three different classes of proteases and two, whole-protein substrates. The inset ESR31, spectrum reflects both digested and undigested protein, with the sharp lines reflective of low molecular weight or “Mobile” fragments released by digestion of the whole protein. Presented are the fractional changes relative to the no – Nelﬁnavir control in the “Mobile” population following a 40-min digestion. The drug inhibits the proteolytic activity of pepsin, an aspartyl protease, in-class with HIV protease. In contrast, Nelﬁnavir promotes proteolysis by cysteiny1 (papain) and serinyl (trypsin) proteases. The promotion effect is most pronounced for the whole-protein substrate, serum albumin. 
Overlooked connections and rampant proteolysis

As was noted in Figure 1, a third of all AP cases are accompanied by hypoalbuminemia [11]. Figure 3 diagrams the rampant degradation cycle that develops when the conditions co-exist. Digestion of serum albumin in the intravascular space leads to reduction of intravascular volume and corresponding increase in interstitial volume. In this model blood vessels are also damaged by proteolysis, enabling increasingly large molecules, such as trypsin, a known activator of pancreatic enzymes, to cross into the interstitial space. In-pancreatic, digestive enzymes are activated within the pancreas, wreaking havoc on pancreatic tissues and blood vessel walls from the interstitial space. Vessels become even more leaky and activated, digestive enzymes – elastase, indiscriminate proteases, lipases and amylases – enter the intravascular space, attacking not only serum proteins but also blood cells. Local tissues are damaged, likely permanently, digestive enzymes are circulated into the systemic vasculature and the organism is deprived of nutrition. The low probability of survival under these conditions is well appreciated.

The degradation cycle can, hypothetically, originate in either the intravascular or interstitial spaces. The presence, for example, of bacterial, trypsin-like proteases [21] in the vasculature may initiate serum albumin proteolysis leading to nascent hypoalbuminemia and attendant leakage of protease activators into the pancreatic, interstitial space. Leaked protease activators (nominally trypsin) proceed to activate pancreatic digestive enzymes with concomitant introduction of digestive enzymes into the interstitial space, completing the cycle. Conversely, independent development of advanced AP implies the presence of activated digestive enzymes in the interstitial space with attendant destruction of blood vessel walls and leakage of digestive enzymes into the vasculature, kicking off the initiation of hypoalbuminemia, again completing the cycle. Regardless, it is feasible that both hypoalbuminemia and AP may arise not from an existing disease state, but from a treatment that is administered (Fig. 4) for a condition wholly unrelated to either hypoalbuminemia or AP.

Therapy-induced disease

A growing body of work confirms that AP may be induced by hundreds of current treatments [3]. The World Health Organization lists 525 such drugs [22]. Included are not only nelfinavir but protease inhibitors and antivirals generally. The incidence of AP in HIV/AIDS patients is, e.g., 40% versus 2% in the general population and there’s growing concern that AP is induced by routine treatment [23]. As noted above, AP can initiate the development of hypoalbuminemia (and vice versa). The data in Figure 2, admittedly for a single drug, demonstrate that treatment known to induce AP can also amplify degradation of serum albumin, assuming the presence of indiscriminate proteases, e.g., of bacterial origin, in the serum.

An alarm has been sounded [22, 23]: a large and growing number of treatments can induce serious, even deadly diseases. As of this writing, funding to interrogate treatment-induced disease is all but non-existent, nor has any

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**Figure 3.** When hypoalbuminemia and acute pancreatitis coexist, a degradation cycle comes into play. Intravascular proteases digest serum albumin and induce blood vessel leakage. Serum-origin proteases migrate into the interstitial space. Trypsin, in particular, serves as activator of pancreas-origin proteases, lipases and amylases. In-pancreatic activation of digestive enzymes damages pancreatic tissues and attacks vasculature from the interstitial space, allowing passage of even more destructive proteases into the serum. Systemic impacts include distribution of digestive enzymes throughout the vascular system and denial of nutrition.
regulatory body acknowledged what must be perceived as a growing crisis. Figure 5 presents the depth to which attitudes and practices must be adapted to this new reality. Consider that a patient presents with a recognizable set of symptoms. Based on those symptoms, tests are administered, diagnosis is made, and treatment prescribed. Predictably, side effects to the prescribed treatment will occur which may or may not warrant immediate concern. Within the context presented here, however, side effects must be considered symptoms of a disease induced by the treatment. Symptoms of side-effects, just as with symptoms generally, must be interrogated by testing and diagnosis made. Side-effect-based diagnosis dictates possible treatments which may or may not include discontinuance of the original treatment regimen. Potential conundrums arise, weighing, e.g., the relative negative impacts of an untreated primary disease to those of treatment-induced disease. On a more positive note, the potential for development of a new class of biomarkers – those which translate clinical observables into drug discovery tools or vice versa – is an untapped and potentially enormous opportunity.

**Connecting the dots**

Figure 6 attempts to consolidate the wide variety of disciplines and observations in this document. Existing drug screening for putative protease inhibitors tests the native, or on-target, protease ability to cleave a peptide serving...
Figure 6. The shortcomings of existing drug discovery for protease inhibitors give rise to products such as nelfinavir, a putative HIV protease inhibitor. Nelfinavir is now known to induce acute pancreatitis and this work demonstrates that it promotes digestion of serum proteins, especially serum albumin, by digestive proteases. A diagnostic for acute pancreatitis demonstrates the presence of digestive enzymes in the serum. Taken together, it is postulated that the unspecified “catabolism” driver for hypoalbuminemia is proteolysis. The relative benefits of ESR$_{SL}$ for drug screening and potential use in the clinic are shown.

What now?

Consider, for example, that a single institution with diverse capabilities chooses to aggressively embrace the many challenges, and opportunities, implied by treatment-induced disease. New classes of biomarkers must be developed alongside of more robust methods for assessment of the existence and degree of induced-disease severity. Logically, new clinical-research lab biomarkers combined with more robust, bio-relevant methods places that institution in the driver’s seat of early-stage drug discovery. It is not a stretch of the imagination to anticipate a true revolution in much of disease treatment and management brought about by the foresight and commitment of a single institution.
Abbreviations
AIDS Acquired immunodeficiency syndrome
AP Acute pancreatitis
ESR Electron spin resonance spectroscopy
ESRsl ESR of SL materials
HIV Human immunodeficiency virus
SA Serum albumin
SL Spin labeling, spin labeled

Conflict of interest

All authors own stock in New Liberty Proteomics Corporation. Ray Perkins is a senior member of the editorial board for Life Sciences and Medicine at 4open.

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