Phycobilins Inhibit NADPH Oxidase –
A Personal Perspective on the Discovery

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Bilirubin is a Crucial Physiological Antioxidant

Many important discoveries involve an element of luck. This is certainly true in this instance.

An old friend of mine, science writer David Rorvik, has Gilbert syndrome. This is an innocuous genetic variant associated with chronically elevated plasma levels of unconjugated bilirubin, stemming from the fact that hepatic expression of the enzyme which conjugates bilirubin, glucuronosyltransferase type 1A1, is decreased. People with Gilbert syndrome appear to be no less healthy than those without it – and indeed there is recent epidemiological evidence that people with this syndrome are at greatly reduced risk for coronary disease, hypertension, and carotid atherosclerosis.

Moreover, other epidemiological studies have concluded that, at least in people with healthy hepatic function, relatively high plasma levels of unconjugated bilirubin correlate with reduced risk for vascular disease as well as certain cancers. In numerous rodent and cell culture studies, induction of the enzyme heme oxygenase-1 – which cleaves heme to yield carbon monoxide, ferrous iron, and the bilirubin precursor biliverdin – is associated with potent antioxidant and anti-inflammatory effects, at least some of which can be attributed to the derived bilirubin. And high-expression polymorphisms of this enzyme in humans have been correlated with reduced risk for a wide range of disorders associated with oxidant stress. Indeed, a Japanese study found that these polymorphisms were associated with increased longevity. Bilirubin has radical-scavenging antioxidant activity, and many researchers presumed that this activity was responsible for the apparent health protection associated with elevated plasma free bilirubin and/or high-expression polymorphisms of heme oxygenase-1.

David was well aware of this intriguing research. In conversations with our mutual friend Dr. Shelly Hendler (a formidably brilliant medical scholar whom I introduced to David a number of years ago – they went on to co-author an outstanding series of books on nutrition, culminating in their masterpiece, The Physicians’ Desk Reference for Nutritional Supplements), David commented that bilirubin had remarkable antioxidant activity, and might have great potential as a nutraceutical.

The Antioxidant Potential of Phycobilins

In the course of writing their Physicians’ Desk Reference, Shelly and David had occasion to discuss phycocyanin, a protein found in many cyanobacteria, algae, and green plants that harvests light energy and thus helps to drive photosynthesis. Phycocyanin has radical-scavenging activity, and, in a number of Cuban studies, oral administration of
phycoerythrin to rodents has been shown to exert a wide range of anti-inflammatory and anti-allergic effects. Both the light-harvesting and antioxidant activities of phycoerythrin are largely attributable to covalently bound chromophores known as phycobilins. These phycobilins are derived biosynthetically from biliverdin, and are close chemical cousins of bilirubin.

David’s comments about the health-protective properties of bilirubin led them to recall the Cuban literature pertaining to the intriguing physiological properties of phycoerythrin. Could phycobilins have antioxidant properties comparable to bilirubin – and, if so, did they have important potential as nutraceuticals?

On returning from his visit with David, Shelly told me about David’s Gilbert syndrome, and their joint speculation that phycobilins might have important antioxidant potential. I at first didn’t know what to make of this suggestion, as I had never heard of phycobilins (alas, I hadn’t read their encyclopedic book from cover to cover!) So I began to read the literature on bilirubin and phycoerythrin to alleviate my ignorance.

**Why is Bilirubin so Important?**

It quickly became apparent that hundreds if not thousands of scientists worldwide were highly intrigued by the antioxidant activities of free bilirubin and of heme oxygenase-1. One of the first things I learned was that the antioxidant/anti-inflammatory properties of heme oxygenase-1 were largely contingent on expression of biliverdin reductase, the enzyme which reduces biliverdin to bilirubin. Fortunately, this enzyme appears to be ubiquitously expressed in mammalian cells.

But soon I found myself very perplexed. Both bilirubin and biliverdin have been shown to have versatile radical scavenging activity, and most scientists assumed that this was the basis for bilirubin’s protective action. Yet the intracellular concentrations of bilirubin generated by heme oxygenase-1 induction were reported to be in the low nanomolar range – about 50 nM. Most cells contain low millimolar concentrations of the radical scavengers ascorbate and glutathione. And, molecule for molecule, ascorbate is a better scavenger of superoxide than bilirubin is. Why would nanomolar concentrations of bilirubin provide important antioxidant protection in the context of millimolar concentrations of such excellent scavengers as ascorbate and glutathione? And why was bilirubin so much more protective than biliverdin? Something didn’t add up.

I soon enough recognized the solution – bilirubin was protective in pro-inflammatory conditions in which NADPH oxidase was activated. Activated NADPH oxidase is considered to be the chief source of superoxide in most disorders associated with excess oxidant stress. I postulated that bilirubin functioned physiologically as a highly potent and specific inhibitor of NADPH oxidase. On re-reading the relevant literature, the findings appeared to be entirely consistent with this hypothesis. This would also explain why biliverdin needed to be reduced to bilirubin before it could exert important antioxidant activity. This reduction causes bilirubin to twist into a configuration that
makes it highly insoluble; this configuration no doubt is crucial for NADPH oxidase inhibition.

This hypothesis provided a satisfying explanation for the physiology of heme oxygenase-1 induction. This enzyme is induced by oxidant stress – often stemming from overactivity of NADPH oxidase. The induction of this enzyme leads to generation of biliverdin, which is rapidly reduced to yield bilirubin. Bilirubin then provides feedback inhibition of NADPH oxidase, alleviating the oxidant stress that had triggered the induction of heme oxygenase-1. This was a simple and elegant homeostatic mechanism for protecting cells from excess oxidants! (As to the carbon monoxide generated by this enzyme, this can be construed as a “pinch hitter” for the nitric oxide destroyed by ambient oxidant stress: like nitric oxide, carbon monoxide activates guanylyl cyclase, the chief mediator of nitric oxide’s physiological effects.)

I wrote up my hypothesis about bilirubin and NADPH oxidase, and communicated it to Shelly and later David. Several months afterwards, a study by Lanone and colleagues appeared concluding that bilirubin does indeed inhibit NADPH oxidase. Two other such studies have appeared in the last year. The most recent of these demonstrates that bilirubin prevents the migration of p47 to the cell membranes – a crucial step in NADPH oxidase activation.

**Could Phycobilins “Pinch Hit” for Bilirubin?**

Meanwhile, I was still unsure about the antioxidant potential of phycobilins. I found that these were structural homologs, not of bilirubin, but of biliverdin. In other words, unless they could be reduced by biliverdin reductase to insoluble homologs of bilirubin, they weren’t likely to act as NADPH oxidase inhibitors. I did a MedLine search and got only one relevant hit – but that proved to be the only hit I needed!

In 1993, Terry and colleagues had published the seemingly rather abstruse observation that the chief phycobilins – phycocyanobilin (PCB), phytochromobilin, and phycoerythrobilin – are all good substrates for biliverdin reductase. Indeed, biliverdin reductase converts phycobilins to homologs of bilirubin that the authors christened “phycorubins”. The latter compounds don’t exist in nature – at least to any meaningful degree – because algae, cyanobacteria, and green plants don’t express biliverdin reductase. Why Terry conducted this obscure study is still mysterious to me – but it proved to be another stroke of luck that he did!

I was now excited about the prospect that orally administered phycobilins could be converted to phycorubins within cells, and that these compounds might have potential to inhibit NADPH oxidase in a manner analogous to bilirubin. I became even more excited when I devoted careful attention to the Cuban reports about the versatile anti-inflammatory properties of oral phycocyanin in rodents. The effects observed seemed to be consistent with inhibition of NADPH oxidase. Indeed, a report that oral phycocyanin prevents endotoxin-induced circulatory failure in rats was precisely parallel to a recently published study showing that biliverdin administration prevents septic shock in rats. My
interpretation of the Cuban research was that, upon oral administration of phycocyanin, either free phycocyanobilin or a phycocyanobilin oligopeptide was generated in the gastrointestinal tract and partially absorbed; this then was converted to phycocyanorubin (or a phycocyanorubin oligopeptide) in cells, which inhibited NADPH oxidase activity. Everything seemed to be adding up!

**A Central Role for NADPH Oxidase in Pathology**

I have long been cognizant of the importance of NADPH oxidase as a source of oxidant stress. Now that I was fairly confident that we could produce a phytonutrient inhibitor of this enzyme complex, I studied the relevant literature intensively. I found that excessive activity of NADPH oxidase had been linked to the pathogenesis of a staggering number of common health disorders – including almost all types of vascular disorders, the complications of diabetes, both osteo- and rheumatoid arthritis, hepatic and pulmonary fibrosis, septic shock, osteoporosis, neurodegenerative disorders such as Alzheimers and Parkinsons disease, UV-mediated skin damage, asthma, allergies – the list goes on and on. Even cancer is involved – NADPH oxidase promotes the proliferative behavior of many cancers, and is also involved in cancer-evoked angiogenesis and in the mutagenesis associated with chronic inflammation. Indeed, I have been led to the conclusion that, in perhaps the majority of non-infectious disorders, NADPH oxidase becomes activated in the affected tissues, and the resultant oxidant stress often either mediates or exacerbates the pathology. Thus, an agent that could produce partial inhibition of NADPH oxidase, and that is reasonably safe within specified dose levels, may have staggeringly versatile potential for prevention and treatment of disease. Perhaps it wouldn’t be “the cure” for any given disorder, but it could play a worthwhile role in the amelioration or prevention of a great many pathologies – perhaps as close to a “panacea” as we are ever likely to find!

Of course, we mustn’t lose sight of the fact that NADPH oxidase plays an important physiological role – particularly in regard to destruction of phagocytosed microorganisms. Excessively potent inhibition of NADPH oxidase activity would likely have immunosuppressive effects that could be harmful. Moreover, relatively low levels of NADPH oxidase activity have a physiological signal modulating function in many tissues; complete suppression of these signals presumably would be inappropriate. But experience with Gilbert syndrome teaches us that moderate down-regulation of NADPH oxidase activity can be achieved without any overtly negative consequences. And, if serious infection develops, the use of NADPH oxidase inhibitors could be temporarily discontinued. So it is reasonable to anticipate that pharmacological modulation of NADPH oxidase activity has important clinical potential. Indeed, some of the beneficial effects of statins and ACE inhibitors are thought to reflect partial inhibition of NADPH oxidase activity in certain tissues.

**Proving the Theory**

But I was only conducting “thought experiments” – direct proof that phycobilins could inhibit NADPH oxidase was evidently needed. And first we needed some phycobilins –
these compounds are so obscure that you can’t buy them from chemical catalogs (albeit phyocyanin, the holoprotein that contains phycobilins, is readily available commercially, and indeed has been used both as a chromophore in biological research and as an approved food dye – it, like PCB, is intensely blue.) So I asked my friend Dr. Jan Zielinski, an outstanding organic chemist with whom both Shelly and I have worked in the past, if he would join our effort. He obligingly set to work with some freeze-dried spirulina that Shelly had obtained from Cyanotech. Spirulina is exceptionally rich in phycocyanobilin (PCB – not to be confused with polychlorinated biphenyls!); this is what puts the “blue” in “blue-green algae”. Using techniques that had previously been described in the literature, he was eventually able to generate about 12 mg of highly pure PCB.

Meanwhile, we needed to find an NADPH oxidase expert to test the impact of phycocyanobilin in cell cultures. Our initial choice, Shelly’s old friend and free radical expert Dr. Sampath Parthasarathy, became unavailable after Hurricane Katrina destroyed his lab at Tulane University! Fortunately, I was able to establish contact with Dr. Toyoshi Inoguchi of Kyushu University, a noted authority on the role of NADPH oxidase in pathology, particularly as it pertains to diabetic complications. After I explained my ideas to Toyoshi, he was quite enthusiastic, and agreed to test our phycocyanobilin in the cell culture assays he had developed in his lab. Within several months, Toyoshi was able to send us data indicating that this agent does indeed inhibit NADPH oxidase in human cell cultures, including those derived from vascular endothelium, vascular smooth muscle, and renal mesangium. He further showed that biliverdin had comparable activity in these systems, and that its activity was highly parallel to that of phycocyanobilin. The effect of these agents was dose-dependent, becoming significant at about 1 micromolar, and maximal at about 20 micromolar (albeit PCB was not quite as potent as biliverdin in the mesangial cells). We presume that direct inhibition of NADPH oxidase is actually mediated by bilirubin and phycocyanorubin evolved by intracellular biliverdin reductase. Toyoshi continues to work with PCB, hoping to define the mechanism whereby it (and bilirubin) inhibit NADPH oxidase.

These outstanding new data enabled us to file a use patent application covering use of isolated phycobilins as NADPH oxidase inhibitors, and as components of nutraceuticals and cosmetics. Our next step is to begin to generate quantities of partially purified PCB sufficient for use in rodent studies and ultimately clinical studies – and to raise investment capital that will help us to expedite the further development of this promising phytoneutrient. (Remarkably, everything accomplished so far has been done with no funding – all of the scientists involved have donated their efforts, working in their spare time. Even the patent application was drawn up without charge by a friend with expertise in biomedical patents.)

Prospects for Commercial Production of Phycobilins

The reason why phycocyanobilin may have important practical potential is that Spirulina can contain close to 1% PCB by dry weight! (By comparison, commercial bilirubin is derived from ox bile, and biliverdin has to be chemically synthesized by a complex
process.) Up to two-thirds of this PCB can be extracted in free form from Spirulina using hot methanolysis, which slowly cleaves the thioether linkages between PCB and the phycocyanin apoprotein. A good way to proceed is to extract broken Spirulina cells with cold methanol – thereby removing most of the carotenoid and chlorophyll content. The PCB remains behind in the insoluble protein fraction. This fraction can then be subjected to hot methanolysis, which can be expected to extract much of the PCB. The resulting extract can then be dried, to yield a Spirulina extract highly enriched in free PCB that may be suitable for use as an orally administrable nutraceutical.

However, even though Spirulina is an exceptionally rich source of PCB, it has the disadvantage that it is comparatively expensive to grow, owing to the fact that it requires sunlight for growth and thus can only grow to a low density. Effective clinical doses of PCB derived from Spirulina are unlikely to ever be as inexpensive as we would like (especially given the likelihood that a high proportion of the world’s population may want to use this nutrient some day!) An exciting alternative possibility is that it may be feasible to develop bacteria that generate large amounts of PCB. Indeed, a Japanese group has recently developed a bioengineered E. coli that produces PCB! Since bacterial cultures have been used to produce a range of vitamins and amino acids at low cost, it may some day be possible to produce PCB efficiently this way.

Pending the commercial availability of PCB supplements, ingestion of whole spirulina represents a feasible alternative. A heaping tablespoon of spirulina – approximately 15 g – contains about 100 mg PCB; extrapolation from rodent studies with dietary spirulina suggests that 1-2 heaping tablespoons daily could provide clinically significant antioxidant activity. Spirulina can be rendered palatable by inclusion in “smoothies” featuring ingredients such as soy milk, fruit juices, and whole fruits. My favorite recipe: blend 1 cup vanilla soy milk, a sliced banana, and a heaping tablespoon of spirulina.

The appended figures are from our patent application. Figures 1-3 summarizes Toyoshi’s findings regarding the inhibitory impacts of PCB and of biliverdin (BVD) on NADPH oxidase activity in human cell lines derived from arterial endothelium, arterial smooth muscle, and renal mesangium. Figure 4 depicts the structures of biliverdin, the chief phycobilins, and the various “rubins” produced from these by biliverdin reductase activity.