While photocaged amino acids have been applied to the optical control of a range of different proteins, reversible control through photoswitchable amino acids is underdeveloped to date (Bose et al., 2006; Hoppmann et al., 2015; Luo et al., 2018). One reason for this is placement of the UAA: caged amino acids can be directly positioned into the active size of an enzyme, allowing for prediction of optical activation, since photolysis generates the native protein. In contrast, photoswitchable amino acids and their unnatural chromophores remain attached to the enzyme after optical triggering as they only undergo a change in configuration. These UAAs in particular will benefit from allosteric control over catalytic function, since irradiation never generates the native protein, limiting their placement into active sites. The allosteric control approach reported by the Sterner lab should pave the way for further development of light-activated and light-switchable proteins based on UAA mutagenesis and may allow for optical regulation of previously "uncagable" targets that do not contain suitable lysine or tyrosine residues in their active sites.

ACKNOWLEDGMENTS

A.D. acknowledges support from the National Institutes of Health (R01GM132565). T.M.C. was supported by a National Science Foundation Graduate Research Fellowship.

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Dawn of a New Era of Targeted Antioxidant Therapies

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https://doi.org/10.1016/j.chembiol.2019.11.003

In this issue of *Cell Chemical Biology*, Shah et al. (2019) report an *in vitro*, high-throughput assay that predicts the ability of compounds to suppress peroxidation of phospholipids. This approach provides a way to design and optimize targeted antioxidants that suppress specific oxidative event in cells, potentially overcoming previous failures with generic antioxidants.

In 1838, the Dutch chemist Gerardus Johannes Mulder proposed that certain albumin-like substances from plants and animals be called proteins, a term suggested by the chemist Jöns Jacob Berzelius from the Greek for "standing in front," to emphasize the priority of proteins in biology (Hartley, 1951). The relative uniformity of the composition of protein substances from different sources led Mulder to suppose that all protein molecules were relatively similar. We now know that there are hundreds of thousands of distinct protein types within each cell and that the specific composition of each protein provides it with a unique structure and function. Thus, to have a single generic assay for all proteins would be nonsensical, given our current understanding of the massive diversity in the composition and functions of proteins.

The field of redox biology, and specifically the study of antioxidants, is undergoing a similar transformation, analogous to how our understanding of proteins evolved from their first description 180 years ago. Antioxidants were originally developed to slow oxidation of rubber for industrial applications and then adapted to food science uses, to prevent spoilage of food when exposed to oxygen for extended periods of time (Mattill, 1947). Indeed, antioxidants are routinely used and highly effective in industrial applications. In contrast, they have not been beneficial for treating or preventing diseases (Halliwell, 1989), despite intense clinical and pre-clinical

study of this possibility (Commoner et al., 1954; Sies et al., 2017). It has been unclear why there is a disconnect in the utility of antioxidants for these very different applications that both involve oxidative chemistries.

One of the key distinctions between the industrial and biological applications of antioxidants is in the diversity of targets of antioxidants in biological systems compared with industrial uses. There is a limited number of relevant molecular targets in the autooxidation of hydrocarbon fuels, compared to the substantial diversity in the identity, reactivity, and localization of reactive oxygen species (ROS) in biological systems. While researchers have succeeded in identifying and characterizing the functional importance of many biologically relevant ROS in the past several decades (Sies et al., 2017), methods for defining the reactivity of antioxidants toward each of these species have not kept pace. Thus, many researchers continue to use a monolithic and simplistic but convenient assay format, such as the color change produced upon reduction by an antioxidant of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. However, this assay, originally developed almost a century ago in 1922, despite its convenience and relevance to industrial applications, doesn't address the relevant activity of a potential antioxidant toward the specific molecular species that occur in biological systems (Li and Pratt, 2015).

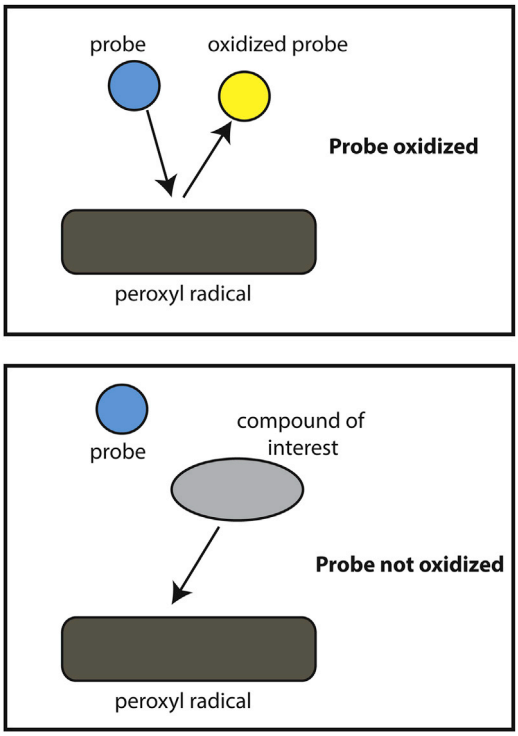

Figure 1. A Probe that Detects the Reactivity of Antioxidants toward Peroxyl Radicals

The probe normally reacts with peroxyl radicals and becomes oxidized, resulting in a change in fluorescence (top). In the presence of an antioxidant compound with greater reactivity than the probe toward peroxyl radicals, the probe remains unoxidized, and the difference in fluorescence can be readily measured (bottom).

Specifically, the DPPH assay does not predict the ability of antioxidants to suppress the accumulation of phospholipid hydroperoxides, which have been implicated in numerous disease processes and drive the execution of a form of cell death known as ferroptosis (Dixon and Stockwell, 2019). Ferroptosis represents death by lipid peroxidation, in which a failure to repair or eliminate phospholipid peroxides results in their accumulation to lethal levels (Hirschhorn and Stockwell, 2019). Some radical-trapping antioxidants (RTAs) can prevent the accumulation of these lethal phospholipid peroxide species and thus prevent cell death by ferroptosis; however, the mechanism of action of these protective compounds, and an assay to identify them reliably, has been lacking (Gaschler et al., 2018). While cellular assays can define the potency with which various compounds suppress ferroptotic cell death, they do not provide information on the mechanism by which this cytoprotection occurs. On the other hand, activity in the DPPH assay has almost no correlation (R^2 = 0.01) with the potency of compounds as inhibitors of ferroptosis.

In this issue of *Cell Chemical Biology*, Pratt and colleagues tackled this problem by developing a microplate-based high-throughput assay for detecting the ability of compounds to suppress phospholipid peroxidation in a well-defined *in vitro* phospholipid bilayer system (Shah et al., 2019). This assay unraveled the mechanism underlying the superior cellular potency of aromatic amines as inhibitors of ferroptosis compared to classic phenolic antioxidants, such as α-tocopherol. Furthermore, it provided a platform for rapid testing of new inhibitors of phospholipid peroxidation, as well as a guide to designing similar amine-based compounds with higher potency.

Shah et al. (2019) reasoned that a predictive *in vitro* assay should be based on the ability of RTAs to compete kinetically with phospholipids for hydrogen atom transfer to a peroxyl radical, the molecular species that is responsible for propagating the lipid peroxidation chain reaction. They developed a colored probe that competes with candidate RTAs for reaction with peroxyl radicals and thus were able to monitor the relevant activity of antioxidant RTA compounds (Figure 1). The authors thus had a key insight—that the crucial feature of effective antioxidants for suppressing ferroptosis is the ability of such compounds to compete with lipids for reacting with already-formed peroxyl radicals in lipid bilayer environments. Their assay reports on precisely this feature of the test compounds, in a rapid and convenient microplate format.

After optimizing this assay, Shah et al. (2019) used it to analyze the properties of antioxidant RTA compounds that result in the most potent ability to suppress lipid peroxidation. They discovered that hydrogen bonding between the RTA and the phospholipid head group had a significant inhibitory effect on the ability of RTAs to transfer a hydrogen atom to a lipid peroxyl radical. In other words, hydrogen bonding to the phospholipid head group competes with hydrogen atom transfer needed to suppress the peroxidation reaction. Avoiding such head group interactions now allows for the design of more effective RTAs that can more potently suppress ferroptosis.

In summary, Pratt and colleagues (Shah et al., 2019) developed a powerful new *in vitro* assay for assessing the ability of compounds to suppress lipid peroxidation in a relevant context; this assay system predicts the potency of such RTA ferroptosis inhibitors in cellular contexts, which has been virtually impossible to do until now. In addition, these authors more generally paved the way for a new

era of targeted antioxidants, in which *in vitro* assay systems are developed to report on the selectivity and potency of antioxidants toward particular ROS in a context that models their cellular microenvironment (Figure 2). Such assays can reliably predict the cellular activity of compounds toward ROS and significantly advance the field by moving away from generic targeting of all ROS with poorly predictive assays to a more rational and targeted future.

Just as we now understand the vast diversity in the function, structure, and relevant environment of proteins, Shah et al. (2019) have prodded us to appreciate the diversity in the function, localization, and reactivity of oxidative species in a living system.

ACKNOWLEDGMENTS

B.R.S.'s research is funded by grants from the National Cancer Institute (R35CA209896 and P01CA087497) and the National Institute of Neurological Disorders and Stroke (1R61NS109407).

DECLARATION OF INTERESTS

B.R.S. is an inventor on patents and patent applications related to ferroptosis and holds equity in and serves as a consultant to Inzen Therapeutics.

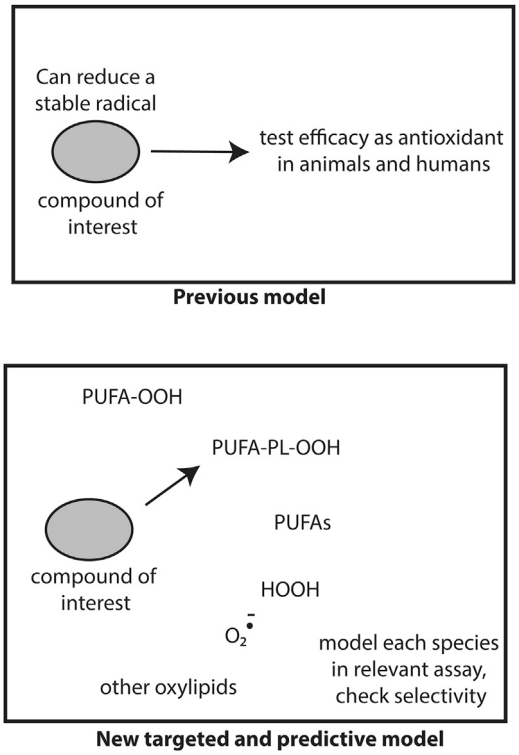

Figure 2. A New Model for Measuring the Reactivity of Antioxidants toward Specific Species in a Relevant Context

In the previous paradigm, a compound that could reduce a stable radical would be evaluated in animal models and clinical studies as a generic antioxidant (top). In the new paradigm, a candidate compound is evaluated for its ability to target a particular species (PUFAs, PUFA-OOH, PUFA-PL-OOH, HOOH, superoxide, or other oxylipids) in a relevant context (bottom). PUFA, polyunsaturated fatty acid; PUFA-OOH, polyunsaturated fatty acid hydroperoxide; PUFA-PL-OOH, phospholipid with hydroperoxide PUFA tail; HOOH, hydrogen peroxide; $O2^{-\bullet}$, superoxide.

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