Antioxidants

DOI: 10.1002/anie.201304548

One Size Does Not Fit All—Bacterial Cell Death by Antibiotics Cannot Be Explained by the Action of Reactive Oxygen Species**

*Nikolai Kuhnert**

antibiotics · antioxidants · oxidative stress · polyphenol · reactive oxygen species

Many scientific theories and concepts are characterized by their simplicity, allowing facile communication to students and the general public. Consequently such simple concepts are frequently adopted in many related and unrelated areas of science. The requirement for simplicity in scientific theories has become known as Ockham's razor, which states that for a scientific theory, all things being equal, the more simple theory should be preferred over the more complex one.[1]

One such simple and highly successful scientific concept in the life sciences is the concept of reactive oxygen species (ROS). ROS are generated in every living organism as a consequence of Fenton chemistry, whereby Fe^{2+} or Cu^{+} ions generate radicals such as $^{\bullet}OH$ from H_2O_2. H_2O_2 appears mainly as a product of flavoenzymes due to an incomplete electron flux. In humans, other factors including smoking or environmental factors such as smog also contribute to excessive ROS generation.[2–4] Highly reactive radicals such $^{\bullet}OH$ react in turn with important biomolecules, with lipids as prime targets, and proteins and DNA as secondary targets, to chemically alter these structures and affect the function of the living cell, ultimately leading to cell death for a single cell or disease for a more complex organism. The generation of ROS in living organisms is inevitable and hence organisms have developed effective defense strategies to quench such reactive radicals. For example, the enzymes superoxide dismutase (SOD) and catalase detoxify H_2O_2, effective radical quenchers such as vitamin C (ascorbic acid) react with ROS in the aqueous phase, and tocopherols (vitamin E) defend lipids in the lipophilic phase.[2] An imbalance of radical generation and detoxification results in oxidative stress, a catchy and suitable term first introduced by Sies et al.[5] The concept of oxidative stress has been successfully employed to rationalize the etiology of a range of degenerative diseases including many types of cancer, cardiovascular disease, and Parkinson's disease.[3]

[*] Prof. Dr. N. Kuhnert
School of Engineering and Science, Chemistry
Jacobs University Bremen
Campusring 8, 28759 Bremen (Germany)
E-mail: n.kuhnert@jacobs-university.de
Homepage: http://www.jacobs-university.de/ses/nkuhnert

[**] Jacobs University is acknowledged for funding.

As a consequence, the theory of antioxidants (a reducing agent able to efficiently quench ROS) evolved, stating that any compound, in particular those taken up with the diet, that are able to detoxify ROS should be beneficial for an organism. Many compounds, in particular naturally occurring polyphenolics, ubiquitous plant metabolites, showed an antioxidant effect in all in vitro and in vivo studies using cell cultures. The antioxidant theory has become so successful that it is accepted by the public as a general truth; "healthy food" is often promoted as "containing antioxidants", the most popular of which are polyphenolics.

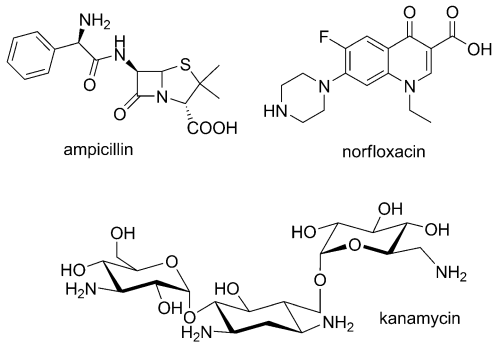
Over the last decade, however, doubts on the validity and usefulness of the antioxidant concept have emerged from various corners. Firstly bioavailability studies showed convincingly that most dietary antioxidants, in particular most polyphenolics, show a low plasma concentration usually in the nM range. Therefore they are unable to vie with ascorbic acid or tocopherols as radical quenchers present in higher μM concentrations (e.g. ascorbic acid 50 μM, tocopherols 30 μM). Secondly it was observed that most hypothetical antioxidants (polyphenolics) undergo extensive, mostly reductive gut floral metabolism, thereby producing metabolites that show an improved bioavailability but are less efficient antioxidants in vitro.[6] Finally 30 years after the emergence of the antioxidant theory gold standard biomarkers giving reliable information on the redox status of an organism emerged that could be easily monitored in human urine, in this case oxidized prostaglandin derivatives otherwise referred to as isoprostanes. Measurement of these isoprostanes clearly showed that in clinical intervention studies even high doses of hypothetical antioxidants given as pure compounds or in a diet rich in fruits and vegetables had no influence at all on the redox status of the organism.[5] As a consequence the antioxidant theory has been put to rest by the large majority of the scientific community previously involved in antioxidant research. The current position is that antioxidant theory plays only a small role, if any, in the undoubtedly health-promoting effects of polyphenolics.[7] A main problem that persists is that in general science education and in the opinion of the general public even a wrong theory has a very long half-life.

While in most cases ROS generation is detrimental for an organism, it can as well be highly useful in defense against a variety of enemies. This concept has become known as the

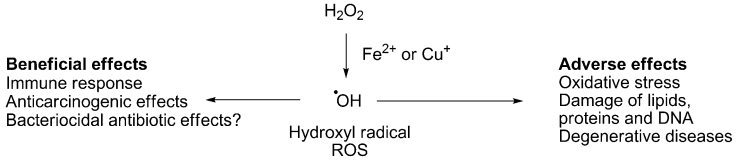
prooxidative effect. In particular all chemical species able to increase the generation of Fenton metal ions switch on oxidative stress and therefore allow indirectly the generation of ROS that attack the enemy. This concept has been successfully employed in explaining the cancer protective activity of many dietary polyphenolics[8] and also to explain the mechanism by which T-lymphocytes kill foreign invaders during the human immune response (Scheme 1).

Most recently this concept has been extended by Kohanski et al. to explain the bacteriocidal activity of many antibiotics, thereby providing a unified theory of antibiotic activity.[9] Kohanski et al. showed in a series of elegant and convincing experiments that selected antibiotics from several classes, such as ampicillin [a β-lactam (cell-wall biosynthesis inhibitor)], norfloxacin [a quinolone (DNA gyrase inhibitor)], and kanamycin [an aminoglycoside (bacterial protein biosynthesis inhibitor)] (Scheme 2), which all share bacteriocidal activity rather than bacteriostatic activity, are able to switch on ROS production in *Escherichia coli* with lethal effects. As a consequence it was concluded that the target binding of the antibiotics does not play the crucial role in killing the microorganisms. The generation of ROS was established by using an ·OH-specific dye (hydroxyphenyl fluorescein, HPF) monitored after incubation with H_2O_2. Dye oxidation was switched off by a typical ·OH quencher such as thiourea. The authors suggested that ROS production occurred after damage to Fe-containing enzymes following by release of Fe^{2+} into the cellular fluid, leading to generation of ·OH by Fenton chemistry followed by induction of DNA and protein damage. Addition of ferrous ion chelators inhibited the cellular death. In a subsequent differential gene expression analysis including data on knock-out strains, the authors showed that catabolic NADH depletion following a metabolic response involving the citric acid cycle triggers the leaking of ferrous ions, ultimately stimulating Fenton-mediated formation of hydroxyl radicals. A simple concept was adapted to an important area of science, giving a consistent picture for the mechanism of action of antibiotics. Hope was raised that this new theory could provide a new impetus for the development of novel antibiotics aimed at resistant strains. The only feature of the experiments that could be criticized was the assumption that a dye would react selectively with the highly reactive ·OH radical. Also an unrealistically high concentration of H_2O_2 was employed, which at 1 mM is 6000 times higher than physiological values of around 0.15 μM experimentally determined in *E. coli*.[10] This last point is also a mistake that was frequently made in nutritional antioxidant research leading to misleading outcomes.

This year two back-to-back papers have been published by the groups of Imlay[11] and Lewis[12] in *Science*, both independently showing in a series of well-planned experiments that the concept of cell death from antibiotics depending on reactive oxygen species cannot be supported. The two groups have used the same antibiotics as those Kohanski's work: ampicillin, kanamycin, and norfloxacin, although at different concentrations. *E. coli* served as the model organism and the Lewis group provided additional evidence in *Pseudomonas aeruginosa*. Both groups could show that incubating bacteria under anaerobic conditions in the presence of all three antibiotics did not result in a reduced killing rate. Thus the relevance of oxygen as the precursor for H_2O_2 production and ultimately cell death was excluded. Imlay et al. measured both intracellular ferrous ion concentrations by EPR spectroscopy and H_2O_2 concentrations in the growth medium following antibiotic treatment and observed that neither compound levels increased following antibiotic treatment. Similarly mutants devoid of catalase and SOD, which should possess a higher intracellular H_2O_2 concentration, showed no increased sensitivity towards any of the antibiotics. Quantitative real-time polymerase chain reactions of the *oxyR* regulon expressed at H_2O_2 concentrations above 1 μM were neither increased nor could an increase of hypersensitivity of DNA repair mutants be observed. Increased cell survival rates after thiourea treatment could be reproduced; however, alternative ·OH radical quenchers such as ethanol did not show any effect on survival rates. The observation by Kohanski of HPF dye oxidation as a marker for ·OH radical activity was investigated in detail by Lewis's group. They reported that after separation of fluorescent and nonfluorescent cells using flow cytometry, no difference of survival rates were observed in sorted colonies. For dye oxidation, Lewis et al. suggested that dying cells generate products other than the ·OH radical able to cause dye oxidation, whereas the group of Imlay suggested from in vitro experiments that



Scheme 2. Chemical structures of antibiotics employed in studies of Kohanski et al., Imlay and Liu, and Lewis et al.[9,11,12]



Scheme 1. Generation and biological consequences of reactive oxygen species for human health.

FeO^{2+}, a typical Fenton intermediate, can cause dye oxidation.

In conclusion, the evidence provided by the Imlay and Lewis groups is convincing and shows that ROS are not involved in antibiotic-mediated cell death. The concept of ROS undoubtedly has its place in many fields of biological research; however, it does not fit all areas involving cellular damage or cell death. Like the antioxidant theory, the concept of ROS, although simple and elegant, does not hold in antimicrobial research. A better understanding of both areas—the biological effects of dietary "antioxidants" and the mechanism of bacterial cell death—must start from the beginning.

Biological systems are inherently complex and although well-designed experiments seem to provide consistent and superficially convincing evidence for novel concepts, as many control experiments as possible are required to soundly substantiate a novel concept. On the use of Ockham's razor in biological systems one finds another reason to agree with Francis Crick who once said: "*[w]hile Occam's razor is a useful tool in physics, it can be a very dangerous implement in biology. It is thus very rash to use simplicity and elegance as a guide in biological research*".[13]

Received: May 27, 2013
Published online: September 3, 2013

[1] S. Roowi, A. Stalmach, W. Mullen, M. E. J. Lean, C. A. Edwards, A. Crozier, *J. Agric. Food Chem.* **2010**, *58*, 1296 – 1304.
[2] B. Halliwell, J. M. C. Gutteridge, *Arch. Biochem. Biophys.* **1986**, *246*, 501 – 514.
[3] B. Halliwell, J. M. C. Gutteridge, C. E. Cross, *J. Lab. Clin. Med.* **1992**, *119*, 598 – 620.
[4] B. Halliwell, M. V. Clement, L. H. Long, *FEBS Lett.* **2000**, *486*, 10 – 13.
[5] P. C. H. Hollman, A. Cassidy, B. Comte, M. Heinonen, M. Richelle, E. Richling, M. Serafini, A. Scalbert, H. Sies, S. Vidry, *J. Nutr.* **2011**, 141.
[6] A. Crozier, I. B. Jaganath, M. N. Clifford, *Nat. Prod. Rep.* **2009**, *26*, 1001 – 1043.
[7] P. C. H. Hollman, *Ann. Nutr. Metab.* **2013**, *62*, 149 – 150.
[8] H. Y. Khan, H. Zubair, M. F. Ullah, A. Ahmad, S. M. Hadi, *Curr. Drug Targets* **2012**, *13*, 1738 – 1749.
[9] M. A. Kohanski, D. J. Dwyer, B. Hayete, C. A. Lawrence, J. J. Collins, *Cell* **2007**, *130*, 797 – 810.
[10] B. Gonzalezflecha, B. Demple, *J. Biol. Chem.* **1995**, *270*, 13681 – 13687.
[11] Y. Liu, J. A. Imlay, *Science* **2013**, *339*, 1210 – 1213.
[12] I. Keren, Y. Wu, J. Inocencio, L. R. Mulcahy, K. Lewis, *Science* **2013**, *339*, 1213 – 1216.
[13] F. Crick, *What Mad Pursuit: A Personal View of Scientific Discovery*, **1988**, pp. 138 – 146.

