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Flush with a flash: natural three-component antimicrobial combinations based on S-nitrosothiols, controlled superoxide formation and “domino” reactions leading to peroxynitrite†

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S-Nitrosothiols are 'NO releasing agents renowned for vasodilatory and antioxidant properties. $O_2^{\cdot-}$ promotes their decomposition, forming highly aggressive peroxynitrite ions ($ONOO^-$). Since the production of $O_2^{\cdot-}$ can be controlled by enzymes or by visible light, such otherwise harmless components can be turned into effective antimicrobial and nematicidal combinations with numerous potential applications in medicine.

S-Nitrosothiols (RSNOs), such as S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl-D-penicillamine (SNAP), form a class of biologically interesting agents able to store, transport and release nitric oxide ('NO). These agents are renowned for their vasodilatory and antioxidant properties.^{1–3} In biological systems, the release of 'NO is usually mediated by thiols, such as reduced glutathione (GSH) or, in the case of GSNO, also by enzymes such as redoxins or the gamma-glutamyl transferase.^{4,5} Although the precise mechanism(s) underlying the formation and decomposition of RSNOs *in vivo* are still a matter of some debate and may involve additional redox active species and the nitroxyl anion (NO^-), the products are generally beneficial or at least harmless to the organisms affected.^{6–8}

Besides thiols and a few other “NO releasing agents”, the superoxide radical anion ($O_2^{\cdot-}$) can also promote the decomposition of RSNOs.⁹ This reaction is of particular interest as $O_2^{\cdot-}$ not only liberates 'NO, it subsequently also combines with this radical to form the highly aggressive peroxynitrite anion ($ONOO^-$) (Fig. 1).¹⁰ This radical reaction is fast and efficient. The usually beneficial 'NO is sequestered and the $ONOO^-$ formed is widely (cyto-)toxic and particularly detrimental to smaller organisms.¹⁰

Interestingly, this “cascade” or “domino” reaction sequence of events initiated by $O_2^{\cdot-}$ should be easily controllable *via* the controlled production of $O_2^{\cdot-}$. Intriguingly, $O_2^{\cdot-}$ itself can be produced *in situ* by a variety of means, including biologically relevant processes, such as the illumination of the photosensitizer riboflavin with visible light and as a by-product of the enzymatic conversion of xanthine to uric acid by xanthine oxidase (XO). Therefore, at least in theory, a wider multi-component “domino” reaction sequence based on natural products becomes feasible, which on demand may

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Fig. 1 Schematic illustration of the three-component system generating highly reactive (cyto-)toxic agents from harmless biological ingredients *via* a three-step “domino” reaction *in vivo*, with a comparison to a traditional two-component, binary system.

convert its otherwise harmless components, such as riboflavin, visible light, xanthine, XO and, in particular, RSNOs, into ONOO^- , *i.e.* into an effective antimicrobial, and nematicidal agent. Such a system would require the concerted action of three components to develop its toxicity and therefore, on paper, appears superior to outrightly toxic or even binary (cyto-) toxic agents.^{11,12} Here, we report some of the biological activities associated with two of these three-component systems involving different RSNOs, one based on visible light, the other on the natural enzyme XO.

Overall, the results obtained as part of this study confirm that (a) it is possible to produce ONOO^- “naturally” from two representative RSNOs with the assistance of light/riboflavin or xanthine/XO combinations, (b) the amounts of reactive species generated in both cases are sufficient to reduce the viability and growth of various target organisms, including selected pathogenic bacteria and fungi, (c) the controlled production of ONOO^- involves $\text{O}_2^{\cdot-}$, which itself is also active, albeit to a lesser extent, and (d) such three-component systems could be “switched” between reactive species and hence be of considerable interest in the fields of medicine and also agriculture. These results will now be presented and discussed in more detail.

Initially, four different domino multi-component 'NO release and ONOO^- generating systems were established, based on two widely used RSNOs and two common biological, *i.e.* natural superoxide generating systems. GSNO and SNAP were selected as representative RSNOs, as they are used widely and, in the case of GSNO, also occur naturally in the human body.¹³ Among the various biological $\text{O}_2^{\cdot-}$ producers, the photochemical riboflavin and the enzymatic xanthine/XO systems were of particular interest as both are also associated with human biochemistry, in particular riboflavin, which is an essential vitamin found in milk and dairy products, meat and dark green vegetables.^{14,15} Indeed, generation of $\text{O}_2^{\cdot-}$ and subsequently ONOO^- by a combination of daylight and the natural riboflavin opens up various equally dark green vistas in medicine and also agriculture.

These systems were established and investigated for their ability to generate $\text{O}_2^{\cdot-}$ radicals and subsequently ONOO^- . Fig. 2 confirms that both, the photochemical and the enzymatic systems were able to generate ONOO^- . In the case of riboflavin, this generation could be maintained for periods of 2 h to 24 h, whilst in the case of xanthine/XO, the combination produced $\text{O}_2^{\cdot-}$ for up to 1 h before the system was exhausted. Whilst the optical trigger involved a standard lamp (illuminance intensity, 0.6 Lumen; temperature, 310 K), in the case of the enzymatically controlled system, ONOO^- formation could be triggered by adding the “missing” component, *i.e.* the substrate or the enzyme. The kinetics of this reaction could be fine-tuned by varying the concentrations of photosensitizer and light on one side, or substrate and enzyme on the other side. The mean production of $\text{O}_2^{\cdot-}$ radicals amounted to $123.1 \pm 0.2 \mu\text{M}$ in the riboflavin-based system (in the presence of 1000 μM riboflavin) and $173.2 \pm 0.2 \mu\text{M}$ in the xanthine/XO system (400 μM and 50 mU mL^{-1} , respec-

Fig. 2 Production of ONOO^- by both the photochemical (a and b) and the xanthine/xanthine oxidase (c and d) avenue (250 μM , 500 μM and 1000 μM riboflavin and 400 μM xanthine, 50 mU mL^{-1} XO were used; see text for details).

tively) as determined *in vitro* by the standard nitroblue tetrazolium (NBT) assay (200 μM NTB as indicator).¹⁶

It is difficult to establish the exact concentrations of ONOO^- formed in this dynamic system as this reactive species forms and decomposes continuously as long as there is sufficient *S*-nitrosothiol present and $\text{O}_2^{\cdot-}$ is being generated. The qualification and quantification of ONOO^- produced *in vitro* were based on the oxidation of non-fluorescent dihydrorhodamine 123 (DHR-123) into fluorescent rhodamine 123. The fluorescence spectra were recorded at 530 nm after excitation at $\lambda_{\text{ex}} = 485 \text{ nm}$ using a commercial Jasco spectrofluorometer (Jasco, FP-6500). GSNO generated $18.9 \pm 1.6 \mu\text{M}$ and $17.1 \pm 1.7 \mu\text{M}$ of ONOO^- over the time-course in the riboflavin and xanthine/XO systems, respectively. The study was also performed with SNAP which generated similar concentrations of ONOO^- , *i.e.* $20.4 \pm 2.4 \mu\text{M}$ and $15.4 \pm 1.8 \mu\text{M}$ for the riboflavin and xanthine/XO systems, respectively.

As the primary focus of this preliminary study was less on the kinetics or optimization of ONOO^- generation and more on the feasibility of its practical use in a complex biological setting, both ONOO^- generating systems were then tested for their respective biological activities against a selection of organisms, including *Escherichia coli*, *Bacillus cereus*, *Candida tropicalis*, *Saccharomyces cerevisiae*, and *Steinernema feltiae*. These organisms were chosen to reflect possible future applications in medicine. In both cases, extensive controls were employed to account for the various possible combinations of the three individual components each of the two systems were composed of.

The first $\text{O}_2^{\cdot-}$ generating combination tested involved riboflavin (250 μM , 500 μM and 1000 μM) and visible light. Fig. 3 demonstrates that organisms such as the model nematode *S. feltiae* and the Gram-negative bacterium *E. coli* are both insensitive to riboflavin and/or GSNO when kept and cultured in the dark (Fig. 3b and d). This is not surprising, as

Fig. 3 Activities of the three-component system containing GSNO and riboflavin at 250 μM, 500 μM and 1000 μM concentrations against *E. coli* (a and b) and 500 μM and 1000 μM concentrations against *S. felitiae* (c and d), respectively, in the presence (a and c) and absence (b and d) of light. Both, 1000 μM of riboflavin and 1000 μM of GSNO have been employed as controls as indicated by the + sign in the figure. A mixture of growth medium and (micro-)organism was employed as negative control (-ve) whilst a mixture of 10 000 units per mL of penicillin, 10 000 μg mL⁻¹ of streptomycin and 25 μg mL⁻¹ of amphotericin B served as positive control (+ve).

riboflavin is a vitamin widely found in nature and GSNO is also physiologically relevant and usually not particularly toxic. In the presence of light, this system became activated (see above) and the various organisms became affected to different degrees.

Particularly noteworthy is the activity against *E. coli*, whereby growth after 24 h was reduced to just 20% of the control in the light (Fig. 3a), whilst virtually no toxicity was observed when an identical incubation was performed in the dark (Fig. 3b), confirming the importance of light in the development of toxicity. This toxicity was clearly concentration-dependent and in virtually all organisms studied increased once the concentrations of GSNO and SNAP were raised from 250 μM to 1000 μM. Interestingly, the generation of O₂⁻ on its own, *i.e.* in the absence of GSNO, also impacted on the growth of *E. coli* and other microorganisms (see below), albeit to a lesser extent, *i.e.* by reducing growth to around 60% of the control in the case of *E. coli*, *B. cereus* and *S. cerevisiae* (Fig. 3 “no GSNO” and Fig. 4a–d). This is not particularly surprising, as O₂⁻ is known to be toxic, at least at higher concentrations, although its toxicity is considerably lower than the one usually associated with ONOO⁻.¹⁷

The riboflavin-based system was also evaluated against *B. cereus* and *S. cerevisiae*, and its activity was slightly less pronounced against these microorganisms (data not shown), pointing towards a more selective action against specific targets. Such a selectivity is not entirely unexpected, as such a three-component system depends critically of the availability of all three components to be present and to act in concert at the place and time of action. The penetration of the individual components into different organisms may differ, resulting in different degrees of action. Interestingly, the nat-

Fig. 4 Activities of the three-component system containing GSNO (a and c) or SNAP (b and d) at 250 μM, 500 μM and 1000 μM concentrations and xanthine (400 μM)/xanthine oxidase (50 mU mL⁻¹) against *B. cereus* (a and b) and *S. cerevisiae* (c and d), respectively. A mixture of growth medium and microorganism was employed as negative control (-ve) whilst a mixture of 10 000 units per mL of penicillin, 10 000 μg mL⁻¹ of streptomycin and 25 μg mL⁻¹ of amphotericin B served as positive control (+ve).

ural presence of one or even two components within such a target organism *a priori* may actually turn this limitation into an advantage thanks to improved selectivity.

Here, the enzymatic system turned out to be highly toxic against *B. cereus* and *S. cerevisiae*, reducing growth to just 6% and 9%, respectively, primarily due to presence of xanthine whilst *C. tropicalis* seemed to be slightly less sensitive at least under the experimental conditions employed (see ESI†), Importantly, these activities are not limited to GSNO, as SNAP also provided a comparable activity (Fig. 4a–d).

Overall, the riboflavin and the enzymatically controlled ONOO⁻ release systems both have proven to be highly effective and, at the same time, controllable. Still, there are notable differences between the two which will now be discussed in some detail.

Two of the major advantages of the riboflavin-based system are its simplicity on the one side and the use of light, rather than chemicals, as the third component, on the other, as light is easy to control and amenable to virtually all kinds of organisms. Indeed, in practice, the enzymatic trigger based on xanthine and XO turned out to be more complicated, as some of the organisms appear to be sensitive towards the xanthine substrate (see below).

Still, the xanthine/XO system has some attraction. In the case of *S. feltiae*, the viability was affected strongly by both systems, once all three components were applied together and ONOO⁻ was generated, with a reduction of viability to less than 10% observed in the xanthine/XO system. In contrast, lack of any one of the three components rendered the system inactive, with the notable exception of the xanthine/XO (only) combination, which generates O₂⁻, but not ONOO⁻. As before, this combination showed some – albeit a clearly reduced – activity when compared to the complete three-component system, with a reduction of viability to around 50%. The activity of the ONOO⁻ and of the O₂⁻ generating systems in both cases, *i.e.* for the riboflavin and enzymatic system, was concentration-dependent.

Similar, sometimes even quite impressive results were obtained for most of the other organisms under investigation, with the exception of *E. coli*, which unfortunately was sensitive to xanthine at the concentrations required to produce sufficient amounts of O₂⁻.

These findings raise the question if such ONOO⁻ generating systems eventually may also be toxic to humans, and hence of limited practical use. Indeed, ONOO⁻ is not entirely harmless, still concentrations required to cause damage to relevant human cells, such as intestinal epithelial cells, are in the order of 50 μM or above, as demonstrated for T84 cells.¹⁸ These concentrations are more than twice the concentrations generated in our systems, and probably reflect the pronounced ability of human cells to “neutralize” ONOO⁻ with the assistance of its various cellular antioxidant defence mechanisms. Furthermore, the systems reported here, at this stage, are not designed for systemic applications and hence a wider exposure of and hence toxicity for human organs is unlikely.

Taken together, the results obtained as part of this preliminary study into the three-component “domino” systems are therefore encouraging. In all cases, it has been possible to combine three virtually inactive or even biologically beneficial components to initiate a sequence of reactions resulting in the formation of a highly active toxic reactive nitrogen oxygen species (RNOS). Whilst this kind of chemistry may appear obvious and straight-forward, it also has its particular charm (Fig. 1).

Besides the fact that all of the components employed, with the possible exception of xanthine, are generally harmless, such systems can be triggered by one of three components, which renders them superior to out-rightly toxic substances or even the kind of enzymatic two-component binary systems investigated by us and others in the past.^{11,12} Indeed, when

compared to the alliin/alliinase or glucosinolate/myrosinase systems, which both generate (cyto)toxic species upon addition of either substrate or enzyme, the three-component systems presented here are considerably more sophisticated, facet-rich, controllable and flexible.^{11,12} It is possible, for instance, to generate O₂⁻ instead of ONOO⁻ with a two-component approach and then switch to ONOO⁻ production by including the third component, as shown in Fig. 1. Intriguingly, if left “inactivated”, such a system would eventually generate NO rather than O₂⁻ and ONOO⁻, adding a third biologically active molecule with its own spectrum of biological actions to the system.

Compared to the previous two-component systems based on plant enzymes, the two three-component systems rely on (harmless or beneficial) components found widely in mammalian organisms. This, in turn, implies not only better applicability of the components, it also raises the possibility that some of these components may be present naturally, for instance in the target organisms, and hence may not even have to be added at all, also raising the spectre of selectivity for specific target organisms (see above). It may be possible, for instance, to apply riboflavin in the dark and then to sit back and wait for the sunrise to trigger ONOO⁻ production in organisms rich in GSNO, whereas O₂⁻ production dominates in organisms low in RSNOS.

A direct comparison of the two – enzymatic and photochemical – systems studied is difficult. At first sight, the photochemical system has its particular attractions since it is less complicated and its components, taken individually, are more common, more readily obtainable, less toxic and mostly even beneficial. Then again, such an approach requires light, and as any kind of photodynamic therapy is limited to specific applications, in this particular case probably primarily but not exclusively to the fields of agriculture.

Conclusions

When considered from the perspective of the initial hypothesis, the investigations presented here support the idea of three-component antimicrobial systems, which are based on harmless, even beneficial biological components and turn toxic once two, and especially three of them are applied together. Such systems rely on “domino” reactions *in vivo* and can be initiated enzymatically or photochemically. Future studies will need to consider the underlying mode(s) of action and optimization of these systems for potential biological applications. In parallel, other multi-component antimicrobial systems based on natural products may be considered, with three or even more components, and based on *S*-nitrosothiols, O₂⁻, enzymes, light or indeed any other suitable ingredients. As such binary and especially “domino” multi-component systems compare highly favourably with toxic substances and binary agents, such investigations are particularly enticing, from a basic scientific and also from a more applied practical perspective.

Experimental

The xanthine/XO three-component system was composed of xanthine (400 μM), XO (50 mU mL^{-1}) and three concentrations (250 μM , 500 μM and 1000 μM) of each RSNO, *i.e.* GSNO and SNAP. For the riboflavin + RSNO system, riboflavin (250 μM , 500 μM and 1000 μM) and the same three concentrations of RSNO were prepared and used. Plates were incubated at 37 °C for 24 h using a direct day light source (LED – Tageslichtleuchte, Briloner Leuchten GmbH, Brilon; illuminance value, 0.6 Lumen), or under dark conditions. The concentrations of ONOO^- and O_2^- produced by the RSNOs were determined by spectrofluorometric quantification in the dihydro-rhodamine 123 and the NBT assay, respectively.^{19,20}

Each of the three-component systems was then evaluated for activity against representative organisms. The antimicrobial assays were performed using Gram-negative (*E. coli*) and Gram-positive (*B. cereus*) bacteria and the fungi *S. cerevisiae* and *C. tropicalis*. The antimicrobial activities were evaluated employing the microbial growth assay based on optical density measured using a micro plate reader (E800, BioTek, Winooski, VT) at 570 nm.^{21,22} Optical density values were determined at 0 h and then following a 24 h incubation at 37 °C. Nematicidal assays were carried out using *S. feltiae* purchased from Sautter & Stepper GmbH (Ammerbuch, Germany) and according to protocols established in our laboratory.^{12,23} For the antimicrobial assays, the medium with a culture of the respective microorganism was employed as negative control, whilst a mixture of 10 000 units per mL of penicillin, 10 000 $\mu\text{g mL}^{-1}$ of streptomycin and 25 $\mu\text{g mL}^{-1}$ of amphotericin B was employed as positive control. The solvent controls necessarily differed between the two systems studied. For the xanthine/XO system, six different controls were used, namely: xanthine, XO, xanthine/XO, xanthine + RSNO, XO + RSNO, and RSNO. Regarding the riboflavin system, the negative and positive controls were as described above. Additionally, a riboflavin control with three different dilutions and an RSNO control with three different dilutions were employed. All experiments were carried out in triplicate and at three different occasions ($n = 9$). The mean values for the replicates were calculated and differences between means were checked for statistical significance by one-way ANOVA performed using GraphPad Prism, Version 5.03 (GraphPad Software, San Diego, CA). A significance threshold of $p < 0.05$ was adopted for all analyses.

Conflicts of interest

There are no real or potential conflicts to declare.

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