Emerging therapies for multidrug resistant *Acinetobacter baumannii*

Meritxell García-Quintanilla*, Marina R. Pulido*, Rafael López-Rojas, Jerónimo Pachón and Michael J. McConnell

Unit of Infectious Disease, Microbiology, and Preventive Medicine, Institute of Biomedicine of Sevilla (IBiS), University Hospital Virgen del Rocío/CSIC/University of Sevilla, 41013, Sevilla, Spain.

*MGQ and MRP contributed equally to this work

Corresponding author: Michael J. McConnell (mcconnell.mike75@gmail.com)

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Abstract

The global emergence of multidrug resistant *Acinetobacter baumannii* has reduced the number of clinically-available antibiotics that retain activity against this pathogen. For this reason, the development of novel prevention and treatment strategies for infections caused by *A. baumannii* is necessary. A number of studies have begun to characterize non-antibiotic approaches that utilize novel mechanisms of action to achieve antibacterial activity. Recent advances in phage therapy, iron chelation therapy, antimicrobial peptides, prophylactic vaccination, photodynamic therapy and nitric oxide-based therapies have all been shown to have activity against *A. baumannii*. However, before these approaches can be used clinically there are still limitations and remaining questions that must be addressed.

Acinetobacter baumannii and antibiotic resistance

Acinetobacter baumannii has emerged as an important cause of nosocomial infections, most notably ventilator-associated pneumonia and bacteremia, and less frequently meningitis, skin and soft tissue infections, urinary tract infections, and endocarditis [1]. *A. baumannii* pneumonia and bacteraemia are typically acquired in the hospital setting and are associated with high mortality [2, 3]. Whereas thirty years ago infections caused by *A. baumannii* could be effectively treated with traditionally used antibiotics, the global spread of multidrug resistant strains with resistance to antimicrobials from multiple antibiotic classes has dramatically reduced the number of drugs that retain activity against this pathogen. These trends have necessitated the use of new antibiotic treatment strategies that include the use of tigecycline, the first of a new class of antibiotics termed glycylcyclines, and colistin, an 'old' polymixin antibiotic that was introduced into clinical practice over 50 years ago. Unfortunately, infections caused by strains with resistance to these antibiotics have already been described [4-6], demonstrating the ability of *A. baumannii* to rapidly acquire resistance. Of particular concern are pan-drug resistant strains with resistance to all clinically-used antibiotics [7, 8]. The increased difficulty of clinically managing infections caused *A. baumannii* due to a lack of active antimicrobials has prompted the development of novel strategies for managing these infections. In this review, recent advances in non-antibiotic approaches that are currently being explored for prevention and treatment of *A. baumannii* infections are described.

Phage therapy

Bacteriophages, or phages, are viruses that infect, and in some cases lyse, bacterial cells. The potential use of bacteriophages as antibacterial agents was

66 recognized at almost the same time as their discovery nearly a century ago [9].
67 However, the dawn of the antibiotic era slowed interest in this area in Western counties.
68 In the present context of infections caused by multidrug resistant bacteria for which
69 there are a decreasing number of active antimicrobials, research exploring the use of
70 phage therapy as an alternative treatment has been renewed. In 2010, the first two
71 reports characterizing lytic phages specific for *A. baumannii*, phages AB1 and AB2,
72 were published [10, 11]. Since then, numerous additional phages with lytic activity
73 against *A. baumannii* clinical isolates have been described [12-17], and one of them has
74 been fully sequenced (Phage AB1 accession number HM368260) [18]. In general, the
75 phages were isolated from sewage water, patient sputum, clinical samples or
76 marine/pond samples and all have shown to be highly specific for *A. baumannii* (Table
77 1).

78 The majority of studies characterizing *A. baumannii* phages have consisted of *in*
79 *vitro* studies assessing the ability of the phages to lyse clinical isolates of *A. baumannii*.
80 Phage AB1 was lytic for only one of five clinical multidrug resistant *A. baumannii*
81 isolates tested [11], whereas AB2 was capable of infecting ATCC 17978, ATCC 19606
82 and 25 of 125 (20%) of clinical isolates [10]. Phage Abp53 was able to infect and lyse
83 the ATCC 19606 strain and 7 of 26 (27%) multidrug resistant *A. baumannii* strains [13]
84 while bacteriophage ZZ1 infected only 3 of 23 (13%) multidrug resistant clinical
85 isolates [12]. Phages identified more recently have shown a broader host range, with
86 phages AB7-IBB1, AB7-IBB2 and KM18p lysing 23 of 39 (59%) and 19 of 39 (49%)
87 and 15 of 34 (44%) of clinical isolates tested, respectively [15-17]. In 2012 the *A.*
88 *baumannii* phage AP22 was shown to lyse 89 of 130 clinical *A. baumannii* strains
89 (68%), the broadest host range against *A. baumannii* observed to date [14]. These
90 studies illustrate that a potential limitation of phage therapy is the limited host range of

A. baumannii phages. However the narrow spectrum of individual phages was overcome in a study by Lin *et al*. in which a cocktail of phages was able to lyse 113 of 127 (89%) *A. baumannii* strains [10]. Only one report has evaluated the use of an *A. baumannii* specific phage for treatment in an animal model of infections [19]. In this study, the phage BS46 was able to protect mice infected intraperitoneally with five times the LD_{50} of a highly virulent *A. baumannii* strain. Clearly, additional studies in animal models are needed in order to characterize the potential therapeutic efficacy of phages specific for *A. baumannii*.

In addition to their potential for therapeutic use, phages could also be used for removing *A. baumannii* from colonized surfaces in the hospital environment. *A. baumannii* can form robust biofilms, which protect the bacteria from environmental insults and allow it to persist on environmental surfaces [20]. It was recently shown that the *A. baumannii* specific phages AB7-IBBI and AB7-ABB2 can inhibit biofilm formation, and also remove approximately 75% of preformed biofilms [15, 16], demonstrating the possible utility of these phages in environmental biocontrol.

Taken together, the studies addressing the use phage therapy for *A. baumannii* infections suggest that the phages isolated to date have lytic potential against clinical isolates of *A. baumannii* and have high host range specificity, an issue of crucial importance as it would be ideal to avoid secondary effects on normal human flora. However, two concerns that have been raised in studies using phages against non-*Acinetobacter* bacteria are the rapid clearance of phages by macrophages and the induction of antiphage antibodies [21-23]. Additional issues such as the appearance of resistant strains and the inflammatory response in humans after administration of phage therapy will also need to be addressed before phage therapy can be considered for clinical use.

Iron chelation and gallium based therapies

Iron is an essential cofactor for many bacterial processes, raising the possibility of using iron chelators and iron competitors as antibacterial agents. While a number of studies have demonstrated the antibacterial activity of various iron chelators [24-31], it is only recently that this approach has been applied to *A. baumannii*. Figure 1 illustrates the structure of the iron chelators that have been used to-date with *A. baumannii*. Deferoxamine is a bacterial siderophore that has been shown to have low antibacterial activity against a number of bacterial pathogens, likely due to the fact that many bacteria have receptors for deferoxamine and thus are able to acquire deferoxamine-bound iron [24]. In agreement with these studies, deferoxamine did not have antibacterial activity against *A. baumannii* grown in a number of different liquid media [24, 32, 33]. Deferiprone is a synthetic bidentate iron chelator that has antimicrobial activity against a number of bacterial species, however showed only modest activity against *A. baumannii* [24]. The same study also evaluated the activity of the iron chelators Apo6619, VK28 dihydrochloride and 2,2- bipyridyl (DIP). The authors observed that if bacteria were grown in Mueller-Hinton broth, DIP showed the highest antimicrobial activity against *A. baumannii* [minimum inhibitory concentration (MIC) = 64 μg/ml] while Apo6619 and VK28 had somewhat less activity (MIC ≥ 128). Interestingly, the antibacterial effect appeared to be dependent on the medium used as bacteria grown in RPMI demonstrated lower MIC values [24].

Gallium (Ga^{+3}) is a transition metal with a similar atomic radius and valence to iron (Fe^{+3}), allowing it to compete with Fe^{+3} for binding to iron-requiring enzymes, proteins and microbial siderophores. However, because gallium cannot undergo oxidation-reduction cycles it acts as an inhibitor to these bacterial components upon binding. Similar to the results seen with iron chelators, the effect of gallium nitrate on

A. baumannii growth depended on the growth medium used [32]. The antimicrobial activity of gallium was increased in serum compared to iron-rich growth media, likely due to the presence of transferrin which sequesters free iron. This study also showed that gallium treatment resulted in lower bacterial loads in the lungs of mice infected intranasally with *A. baumannii*, indicating that gallium maintains activity *in vivo*. Antunes *et al.* recently demonstrated that low concentration of gallium could inhibit growth of *A. baumannii* isolates in human serum and reduce mortality in a *Galleria mellonella* infection model [34]. Importantly, this study also observed a significant *in vitro* synergy between colistin and gallium for both colistin-sensitive and colistin-resistant *A. baumannii* isolates suggesting that the use of gallium in combination with clinically-used antibiotics may be a viable treatment option for multidrug resistant strains. In summary, iron chelation and gallium based therapies show good activity *in vitro*, but additional work is needed to characterize their *in vivo* effiicacy.

Antimicrobial peptides

Antimicrobial peptides are key elements of the immune system of higher organisms, making them potential candidates for use as antibacterial agents [35]. Although numerous distinct antimicrobial peptides have been developed, most of them share a cationic character and fold into amphipathic conformations that allow them to act as bacterial membrane disruptors, although some peptides have also been shown to exert intracellular mechanisms of action [35, 36].

Several antibacterial peptides have been studied *in vitro* and *in vivo* against *A. baumannii*. The cecropin A-melittin hybrid peptides demonstrated *in vitro* bactericidal activity against multidrug resistant and colistin-resistant *A. baumannii* strains [37, 38], and showed local efficacy in an experimental model of peritoneal sepsis caused by a

166 pan-drug resistant strain [39]. However, the peptide exhibited a short half-life after
167 administration, limiting its systemic efficacy. Several peptides derived from frog and
168 toad skin and their analogs have also been studied. The brevinin-2-related peptide [40]
169 and six cationic a-helical frog skin-derived peptides [41] showed good activity (MIC 4 –
170 128 μg/ml) against multidrug resistant strains of *A. baumannii* and low hemolytic
171 activity. One of the frog skin peptides, Alyteserin-2a, presented relatively weak
172 antimicrobial activity and produced hemolysis. Interestingly, however, its analog
173 containing D-lysines at positions 7 and 11 showed higher potency against multidrug
174 resistant *A. baumannii* and lower hemolytic activity and cytotoxicity [42]. A3-APO is a
175 proline-rich antibacterial peptide that, despite high MIC values (32 - >64 μg/ml),
176 showed improved *in vivo* efficacy compared with imipenem in mouse models of
177 carbapenem-resistant *A. baumannii* infection, when used either intravenously or
178 intramuscularly [43]. Moreover, in an *A. baumannii* blast injury model, A3-APO
179 improved survival and reduced bacterial loads in the blood and in wounds, and
180 improved wound appearance significantly more than treatment with imipenem or
181 colistin [44].

182 Although antimicrobial peptides have shown promising results in *in vitro* and in
183 animal models, a number of issues still remain to be addressed. A major technical
184 challenge that must be resolved before antimicrobial peptides can be used systemically
185 will be the design of peptides that maintain antibacterial activity but are resistant to
186 proteolytic degradation in serum. This limitation, however, is not problematic when the
187 peptides are administered topically, as demonstrated by a recent clinical trial [45]. In
188 addition, the hemolytic activity that is seen with some peptides will need to be reduced
189 in order to prevent toxicity upon systemic administration. Studies aimed at developing

peptide analogs through the introduction of structural changes that modify the peptide's stability and toxicity profiles are addressing these limitations.

Prophylactic vaccines

The increased prevalence of infections caused by *A. baumannii*, as well as the identification of factors associated with higher risk for acquiring these infections, has prompted research aimed at developing a prophylactic vaccine. Populations that would potentially benefit from prophylactic vaccination for *A. baumannii* could include patients at high risk for requiring mechanical ventilation, patients with extensive burn injury, and members of the military at high risk for sustaining war-related trauma. The ideal vaccine would target bacterial components that are highly conserved among strains within the *A. baumannii* species. In addition, the targeted antigen(s) should be highly expressed during infection and would be present on the bacterial surface to allow for interaction between the antigen and the antibodies produced by vaccination.

The first reports describing the development of vaccines for *A. baumannii* were based on formulations that incorporated multiple bacterial antigens into the vaccine. An inactivated whole cell vaccine was highly immunogenic and elicited antibodies against multiple bacterial antigens [46]. Tissue bacterial loads were approximately 5 $\log_{10}$ lower in vaccinated mice compared to control mice 12 hours post-infection, and serum pro-inflammatory cytokine levels were lower in vaccinated mice. Importantly, the inactivated whole cell vaccine was able to protect mice from infection with multiple *A. baumannii* strains, including a pan-resistant clinical isolate. Similar results were obtained in independent studies with vaccines based on *A. baumannii* outer membrane vesicles and outer membrane fractions [47, 48]. Although these vaccines were highly effective in mouse models, their use in humans may be complicated by the high levels

of lipopolysaccharide present in the vaccine preparations and the difficulty in standardizing the composition of the vaccine between production lots due to the high number of antigens present.

More recent studies have used individual bacterial components for the development of a vaccine. Vaccination with the *A. baumannii* biofilm-associated protein Bap elicited high levels of antigen-specific titers, reduced post-infection tissue bacterial loads and was able to protect mice in an intraperitoneal infection model [49]. Although these results are promising, it is unclear if a vaccine based on Bap will have activity against strains that do not produce biofilm. The bacterial porin OmpA is highly conserved and expressed at high levels on the bacterial outer membrane making it an attractive candidate for vaccine development. A vaccine based OmpA was highly immunogenic in mice and reduced post-infection bacterial loads in multiple tissues [50]. Importantly, survival studies using a diabetic mouse model of disseminated sepsis showed that the vaccine based on OmpA provided partial protection from infection. Antibodies against the bacterial outer membrane transporter Ata and the membrane polysaccharide poly-*N*-acetyl-b-(1-6)-glucosamine (PNAG) were each shown to promote the opsonophagocytolysis of *A. baumannii in vitro* and to reduce tissue bacterial loads after passive immunization [51, 52]. It remains to be seen if active immunization with these antigens is able to induce protective immunity in survival studies.

In short, prophylactic vaccination represents an approach that could be highly effective for preventing and treating the most common and serious infections caused by *A. baumannii*. However, issues that remain to be defined, in addition to the technical challenges of appropriate antigen selection and design, are determining which patient populations should receive prophylactic vaccination and when they should be

vaccinated. Epidemiological studies that have identified and characterized risk factors for acquiring infections by *A. baumannii* will help to address these questions.

Photodynamic therapy

Photodynamic therapy was initially developed over 100 years ago, and while today it is most commonly used for cancer and ophthalmological treatments, the emergence of multidrug resistant pathogens has prompted its application as a novel antimicrobial approach. Photodynamic therapy involves the generation of reactive oxygen species through the use of a combination of oxygen, visible or near infrared light and a photosensitizer, a non-toxic dye that is photoreactive. Photosensitizers are typically aromatic molecules that, when excited by a photon, reach a high-energy, excited state. The excited photosensitizer can then relax to its ground state through interaction with molecular oxygen producing reactive oxygen species formed by either type I (electron transfer) or a type II (energy transfer) reactions (Figure 2). These reactive oxygen species then react locally with the target cells (e.g. DNA and membrane components) to produce damage which results in target cell death.

The activity of two different classes of photosensitizers against *A. baumannii* has been evaluated: tetrapyrroles (such as porphyrins) and phenothiazinium salts. The tetrapyrroles deuteroporphyrin, Cd-texaphyrin, tetra-methylpyridyl porphine and propyl gallate have all demonstrated activity against multidrug resistant *A. baumannii in vitro* [53, 54]. More recently, the photobactericidal activity of porphyrin-cellulose nanocrystals against *A. baumannii* was also demonstrated [55]. This study also showed that the reactive oxygen species produced by photodynamic therapy can diffuse up to 1.5 mm in solution, supporting the use of this approach as local therapy. Photosensitizers can also be combined with other molecules in order to increase their

bactericidal activity, as was shown in a recent study in which the polycationic biopolymer chitosan increased the effect of hematoporphyrin and the phenothiazinium toluidine blue on *A. baumannii* planktonic cells [56]. There have been few *in vivo* studies addressing the effectiveness of photodynamic therapy in the treatment of *A. baumannii* infections in animal models. In a study using a mouse model of thermal injury it was shown that the tetrapyrrole cholin(e6) reduced bacterial counts 1000-fold in infected tissue after topical application and exposure to red light [57]. Similar results using the same animal model were obtained with the phenothiazinium new methylene blue [58].

Although photodynamic therapy has been used clinically for the treatment of skin infections caused by a variety of pathogens, this approach has not been evaluated in humans for the treatment of *A. baumannii* infections. The advantages of photodynamic therapy are that it has been shown to destroy lipopolysaccharide [59], and that it does not result in the selection of resistant strains after repeated cycles of treatment [60] However, potential limitations of photodynamic therapy are that it can only be used topically, and that the reactive oxygen species produced by this technique could potentially damage host as well as target cells, issues that must be taken into account when considering this treatment approach.

Nitric oxide-based therapies

Nitric oxide (NO) is a short-lived, hydrophobic free radical with a small atomic radius of 3-4 Å. These properties allow NO to diffuse across biological membranes and exert effects on both intra- and extracellular components [61] (Figure 3). NO can be oxidized to dinitrogen trioxide (N_2O_3), which causes nitrosative stress on biological membranes [62], and it can react with thiol groups (S-nitrosation) of both intra- and

290 extracellular proteins [63]. In addition, nitryl radicals (NO_2) can provoke lipid
291 peroxidation and subsequent membrane disorganization [64] and NO can induce DNA
292 damage via an N-nitrosating intermediate or by oxidative cleavage [63]. For these
293 reasons, the use of NO as a topical treatment for *A. baumannii* wound infections has
294 begun to be explored. NO-containing nanoparticles were shown to decrease the bacterial
295 load at wound sites and reduce healing time in a mouse model of *A. baumannii* wound
296 infection. NO nanoparticle-treated mice also experienced a reduction in inflammation
297 at the wound site and decreased collagen degradation compared to control mice. Local
298 cytokine profiles were altered in mice treated with NO nanoparticles, supporting the
299 idea that the inflammatory response had been altered [65].
300 The major limitation of NO-based therapy is that it can only be used topically,
301 preventing its application to the most common infections caused by *A. baumannii*,
302 pneumonia and bacteremia. However, its ease of application together with its multiple
303 mechanisms of bactericidal activity make it an attractive approach for use in wounds
304 and burn injuries. In addition to wound treatment, NO may also find a role in
305 environmental biocontrol as evidenced by a recent study showing that it has inhibitory
306 effects on the formation of *A. baumannii* biofilms [61], which warrants further study.
307
308 **Concluding remarks**
309 The global emergence of multidrug resistant *A. baumannii* requires that novel
310 approaches for prevention and treatment are developed. The studies reviewed here
311 highlight recent advances that aim to address this issue using approaches that employ
312 novel mechanisms of action for achieving antimicrobial activity against *A. baumannii*.
313 However, for each approach there are still questions that must be answered and
314 limitations that must be addressed (Box 1) before these potential therapies can be

315 applied in clinical practice. In addition, it remains to be determined how these
316 approaches affect bacterial physiology and if *A. baumannii* can develop resistance to
317 these therapies as it has done in response to many antibiotics.

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319

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499
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Table 1. Characterized lytic bacteriophages that infect *A. baumannii* clinical isolates

Phage	Genome	Genome size (kb)	Tail	Lysis of clinical isolates	Reference
AB1	dsDNA	45-47	Long, non-contractile	20% (1 of 5)	11
AB2	dsDNA	40	Short	20% (25 of 125)	10
Abp53	dsDNA	95	Contractile	27% (7 of 26)	13
ZZ1	dsDNA	166	Contractile	13% (3 of 23)	12
AB7-IBB1	DNA	75	Long, non-contractile	59% (23 of 39)	16
AB7-IBB2	ND	170	Short	49% (19 of 39)	15
Km18p	dsDNA	45	No tail	44% (15 of 34)	17
AP22	dsDNA	46	Contractile	68% (89 of 130)	14

Abbreviations: dsDNA, double-stranded DNA; ND: not determined

Box 1. Outstanding questions

- Bacteriophages with lytic activity against clinical isolates of *A. baumannii* have shown limited host range. Are there phages with broader host ranges that are yet to be discovered? Can the use of 'cocktails' of phages overcome this limitation?

- What is the nature of immune response produced upon the administration of bacteriophages and what side effects could be produced by these reactions?

- Can toxicity and stability issues that have been observed with antimicrobial peptides be overcome using peptide derivatives (e.g. by substituting D-amino acids)?

- Which populations would benefit from prophylactic vaccination for *A. baumannii* and when should they be vaccinated?

- Can *A. baumannii* develop resistance to photodynamic therapy and NO-based treatment approaches?

519 **Figure Legends**

520 **Figure 1. Chemical structure of iron chelators.** Chemical structures of the iron
521 chelators that have been tested for activity against *A. baumannii*.

522

523 **Figure 2. Production of reactive oxygen species with photodynamic therapy.** The
524 schematic represents the process by which photoactivation (*f*) of photosensitizers (PS)
525 results in an excited state that reacts with oxygen either through type I or type II
526 reactions in order to produce reactive oxygen species (ROS) which induce cellular
527 damage.

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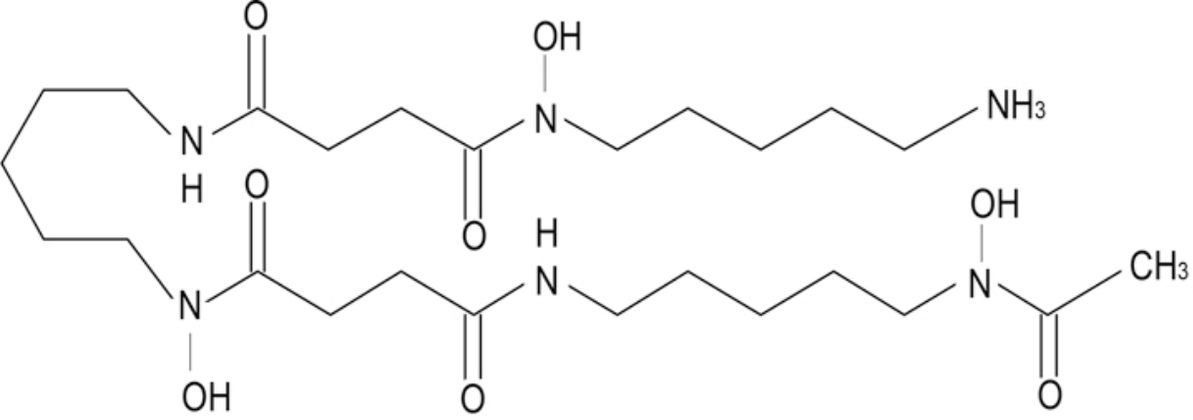
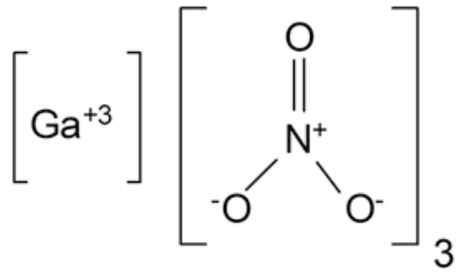
529 **Figure 3. Cell damage produced by nitric oxide-based therapies.** The schematic
530 represents the mechanisms by which nitric oxide (NO) induces damage to cellular
531 membranes, proteins and DNA.

532

533



Deferoxamine



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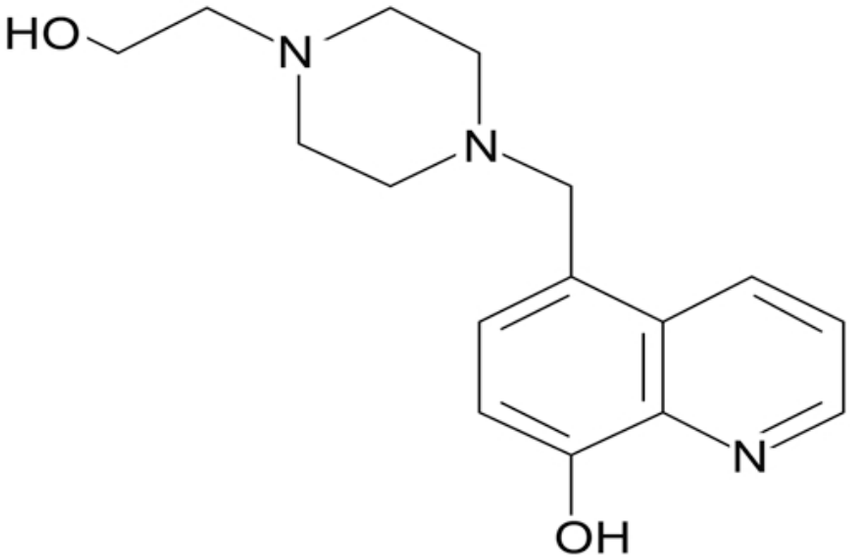

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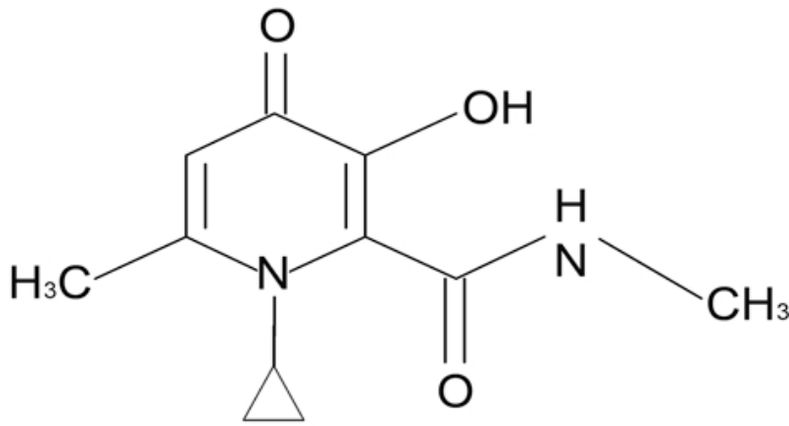
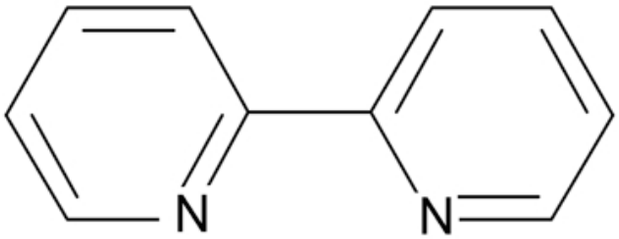
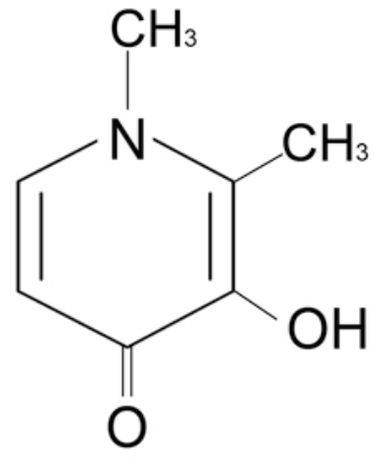


Ap6619



2,2 Dipyridyl



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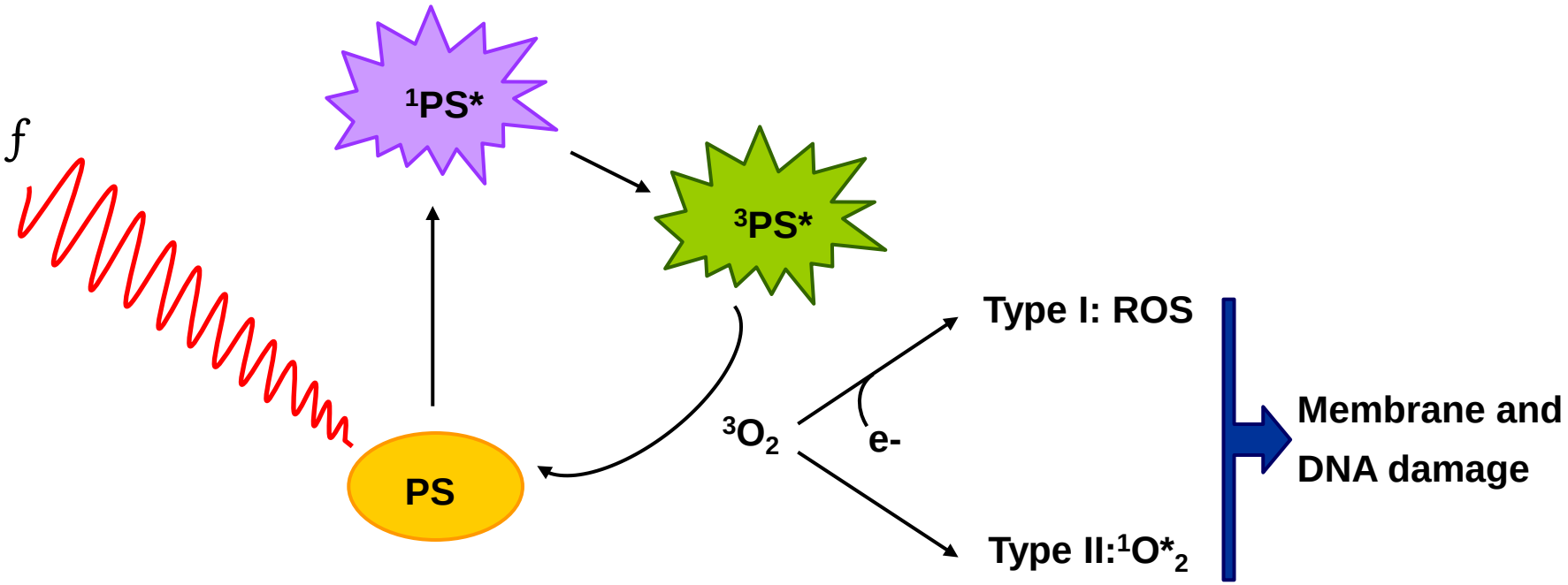
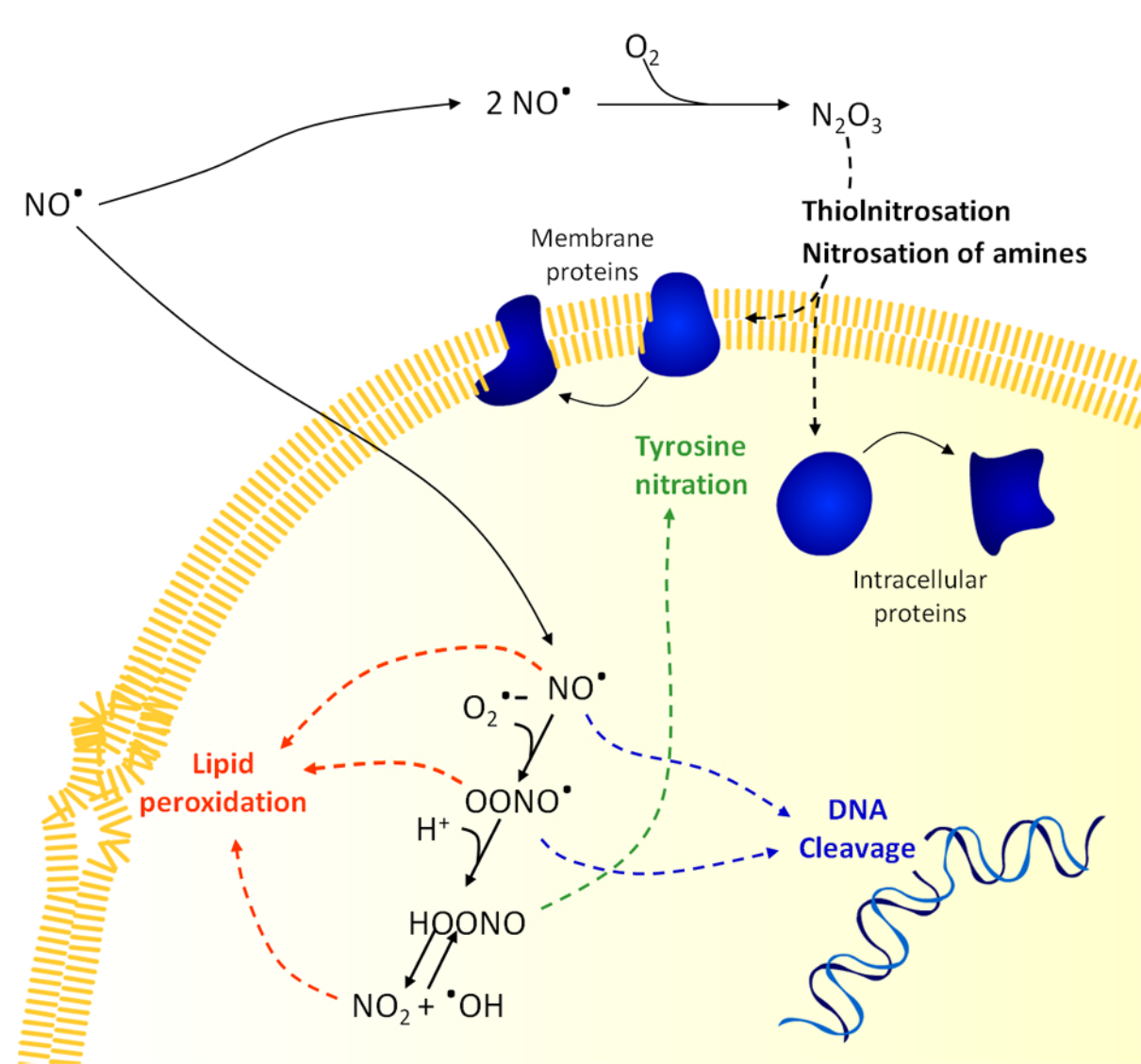

f
1PS*
3PS*
PS
3O2
e-
Type I: ROS
Type II:1O*2
Membrane and
DNA damage


O_2
2 $NO^{\bullet}$
N_2O_3
$NO^{\bullet}$
ThiolNitrosation
Nitrosation of amines
Membrane proteins
Tyrosine nitration
Intracellular proteins
$NO^{\bullet}$
$O_2^{\bullet-}$
Lipid peroxidation
$OONO^{\bullet}$
H^+
DNA Cleavage
HOONO
NO_2 + $^{\bullet}OH$