REVIEW

CROATICA CHEMICA ACTA

Croat. Chem. Acta **2025**, *98*(2), 79–97
Published online: November 25, 2025
DOI: 10.5562/cca4187

HKD 1926

Small Molecule, Big Potential – Nitroxoline in the Era of Rising Antimicrobial Resistance

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RECEIVED: June 9, 2025 ✶ REVISED: September 11, 2025 ✶ ACCEPTED: October 23, 2025

PROCEEDING OF THE SOLUTIONS IN CHEMISTRY 2024, 11–15 NOVEMBER 2024, SVETI MARTIN NA MURI, CROATIA

Abstract: Nitroxoline (NTX), a synthetic 8-hydroxyquinoline derivative, has re-emerged as a promising antimicrobial agent amid the global rise in antimicrobial resistance (AMR). Despite over 60 years of clinical use, resistance to NTX remains remarkably low. Its unique mode of action, based on metal ion chelation, distinguishes it from conventional antimicrobials and underpins its pleiotropic effects, including anticancer, antiviral, antifungal, antibiofilm, and antiparasitic activities. This review explores NTX's historical development, chemical properties, coordination chemistry, and multifaceted mechanisms of action, as well as its limitations. Special emphasis is placed on its efficacy against multidrug-resistant pathogens, biofilm-associated infections, and its repurposing potential in oncology and parasitology. The paper also highlights critical knowledge gaps, particularly in thermodynamic data for metal complexes, and calls for renewed clinical and pharmacological evaluation. Despite certain limitations, its excellent safety profile, low resistance rates, and broad-spectrum activity makes NTX a valuable, underutilized therapeutic with significant potential for repositioning in modern medicine.

Keywords: antimicrobial resistance, nitroxoline, chelation, biofilm, antibiotics, repurposing, multidrug-resistant bacteria, antimicrobials, antifungals, anticancer, antitumour, amoebae.

1. INTRODUCTION

THE evolution of antimicrobial resistance (AMR) is driven by the never-ending competition for resources among microorganisms. It has evolved long before the introduction of antibiotics as clinical agents, but the overuse of antibiotics and environmental antibiotic pollution in recent times resulted in unprecedented selection pressures, making it a major global public health problem.[1–4] The impact of AMR is evident in higher rates of disease and death, along with considerable financial costs.[5–8] The United Nations Coordinating Group on Antimicrobial Resistance issued in 2019 an alarming report stating that by 2050 multidrug-resistant (MDR) bacteria could cause 10 million deaths each year, exceeding today's cancer deaths, and damage the economy on a scale comparable to financial crises, forcing 24 million people into extreme poverty.[9] The proliferation of AMR is also facilitated by the immense challenges encountered during the new drugs development and their introduction into clinical use.[10,11] In this context, an ancient antibiotic nitroxoline (NTX) has been recognized over the last two decades as a potentially underused antimicrobial drug of substantial promise,[10,12–27] with therapeutic potential extending beyond its traditional antibacterial use.[28–34] Notwithstanding the overall rise in scientific output, Figure 1 shows the evolution of the number of publications referencing "nitroxoline" over the last 100 years, serving as a rough indicator of growing attention to the compound in recent years. It should be noted that the data does not account for variations in terminology across regions and disciplines (e.g., synonyms such as 8-hydroxyquinoline or alternative naming conventions).[35]

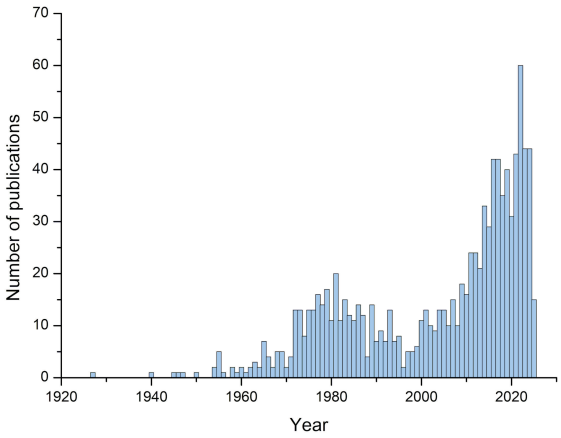

Figure 1. Evolution of the number of publications referencing "nitroxoline" during the last 100 years, based on data obtained from SciFinder.[35]

The reasons for NTX gaining renewed attention appear to be multifaceted; in this review, we outline five key factors that, in our perspective, contribute significantly to its resurgence.

1) Despite being in use for more than 60 years (Chapter 2), which is longer than most modern antibiotics, the resistance rates to NTX remained low, as described in Chapter 6.
2) Its mode of action (MOA) relies predominantly on chelation, which differentiates it from all other classes of antibiotics (Chapter 4). Indeed, NTX does not belong to any of the modern antibiotic classes.[36] This chelation-based MOA provides a basis for its uniquely pleiotropic effects.
3) It is already an approved drug for urinary tract infections (UTIs), with long-standing use in several countries (Chapter 2), and an excellent safety profile (Chapter 8). New generations of drugs within all classes are constantly being investigated, but the rise of AMR has outpaced the slow development pipeline of new antibiotics, leaving a gap in current treatment options.
4) It has shown considerable activity in several domains beyond its primary antibiotic role. Specifically, recent studies have demonstrated its effectiveness as an antibiofilm, antiviral, antifungal, antiparasitic, and anticancer agent (Chapters 5.2 and 7), highlighting its substantial potential for therapeutic repurposing.
5) It is a simple (Chapter 3), synthetic, relatively cheap compound, and could be used as a template for extensive drug development.[18,19]

This review paper presents a comprehensive survey of NTX development and uses through the 20th and 21st century, the molecular basis of its antibacterial/antibiofilm actions and low resistance rates, knowledge gaps regarding its coordination chemistry, challenges met in clinical practice, as well as new revelations regarding its anticancer and antiparasitic effects.

2. BRIEF SURVEY THROUGH HISTORY

The foundation of the NTX structure is quinoline, a compound first isolated in 1834 from coal tar, which still serves as the primary source of industrial quinoline.[37] Its derivative 8-hydroxyquinoline (8-HQ; Chapter 3), NTX's structurally closer predecessor, was used as an antiseptic and disinfectant since 1895, mainly in the form of Chinosol.[38] Its effectiveness as tuberculosis treatment is reported as early as 1910.[39] Chelation as the basis of its MOA was hypothesized in 1923,[40] and proven in 1947 when Albert *et al*.[38] showed that – out of eight HQ isomers – only 8-HQ gave metal ion precipitates. The authors also examined numerous 8-HQ derivatives and found that one of the two derivatives with the best chelating abilities was (then called) 5-nitrooxine, i.e. NTX. It entered clinical use in 1962 for the treatment of UTIs, especially those due to Gram-negative bacteria (*E. coli*).[41] Germany was the forerunner and the most consistent user of NTX, where it remained in clinical use even as its popularity declined elsewhere.[23] It was widely used across Russia and former Soviet states during the Cold War era.[42] It also continued to be prescribed in several Eastern European countries, where resistance rates remained relatively low.[13]

Currently, within Europe, it has marketing authorisation for prophylaxis and treatment of acute and recurrent UTI only in Germany, Croatia, Bosnia-Herzegovina, Montenegro, Poland, Bulgaria and Romania.[16,43] It is not FDA-approved in the USA. In China, it is not approved as a routine antibiotic for UTIs but is approved to enter Phase II clinical trials, for the treatment of non-muscle invasive bladder cancer (Chapter 7.1).[29] In 2024, it was approved (under the investigational name APL-1202) for the treatment of free-living amoebae infections, including rare but deadly brain infections (Chapter 7.3).[44]

3. CHEMICAL STRUCTURE AND DISSOCIATION CONSTANTS

3.1. Quinoline and 8-hydroxyquinoline as Structural Predecessors

NTX is a derivative of quinoline, an important pharmacophore with a broad range of biological effects.[45,46] Quinoline is an aromatic heterocyclic compound, comprising a benzene and a pyridyl ring (Figure 2a). This structure belongs to a group of so-called "privileged structures", as it serves as a scaffold of many biologically active compounds,

including antimalarials, antibacterials, and anticancer agents. Its planar aromatic system allows for π-π stacking and interactions with various biological targets. Additionally, quinoline-based compounds are promising for applications beyond biological, such as the use in polymer photovoltaic and dye-synthesized solar cells.[47]

Quinoline serves as a foundation for a wide class of compounds.[46] The most prominent member of the class is 8-hydroxyquinoline (8-HQ, Figure 2b), due to its frequent use in medicinal chemistry.[45] In nature, 8-HQ is produced by soil *Streptomyces* spp,[48] and it has also been used as a fungicide in agriculture, as a preservative in the textile, wood, and paper industries,[49] and in cosmetic products.[50] Other names for 8-HQ include oxine, oxyquinoline, 8-quinolinol, hydroxybenzopyridine, 8-oxychinolin, etc.

The addition of hydroxyl group at C8 enables 8-HQ to act as a strong metal ion chelator. In acidic solutions, the pyridyl nitrogen is protonated, and this cationic species can be viewed as a diprotic acid.[51,52] The first and the second dissociation steps entail deprotonation of the pyridyl nitrogen and the hydroxyl group, respectively.[52,53] Note that throughout the research history, there was much confusion regarding the two steps and the constants describing them: the differentiation was sometimes omitted, a particular step disregarded, or inconsistent terminology was used (e.g. pK_a2 intermixed with basicity constant).[50,54] Also, in modern databases, the reported values of dissociation constants originate from studies dating back to the middle of the 20th century.[52,54,55] In aqueous solutions, the values of pK_a1 and pK_a2 range from 4.91 to 5.82 and from 9.55 to 10.00, respectively, depending on the temperature and ionic strength.[35,52,54,55]

The addition of different chemical moieties onto the 8-HQ molecule has been recognized for a long time as a means of improving and widening its effects.[22,52,56,57] As an example, the presence of chlorine, bromine, or iodine atoms in the C5 or C7 positions improved its antifungal activities, which was not observed with the fluorinated compounds. Clioquinol, an antiseptic drug effective against multidrug-resistant Candida, is a dihalogenated 8-HQ. The 7-chloroquinoline moiety was extensively studied due to its antimalarial properties,[46] etc.[12,49,56,58–61]

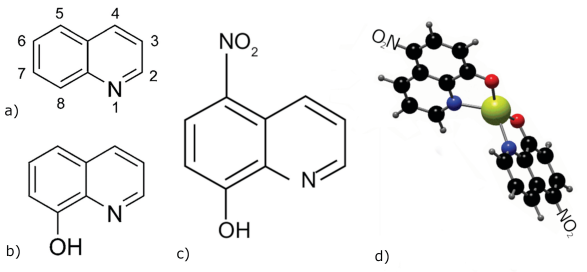

Figure 2. Structures of a) quinoline; b) 8-hydroxyquinoline; c) nitroxoline; and d) metal-nitroxoline chelate. Figure 2d adapted from Ref. [51]

3.2. Nitroxoline, 5-nitro-8-hydroxyquinoline

The addition of the nitro-group to a para-position of the 8-HQ phenyl ring yields NTX, 5-nitro-8-hydroxyquinoline (also known as 8-quinolinol, 5-nitroquinolin-8-ol, 8-hydroxy-5-nitroquinoline, 5-Nitrox, 5-nitrooxine, etc.) (Figure 2c). Electron-withdrawing properties of the nitro group cause an increase of the hydroxyl group acidity,[52,53] which results in NTX's enhanced chelating power and consequently improves its pharmacological characteristics. pK_a1, describing the deprotonation of pyridyl nitrogen, decreased from ~5.3 (an average value for 8-HQ, section 3.1.) to 1.95 – 2.64; pK_a2, describing the deprotonation of the hydroxyl group, decreased from ~9.7 (an average value for 8-HQ) to 6.04 – 6.40.[52,53,55,62,63] The data are valid for aqueous solution. In dioxane–water solutions, the pK_a1 and pK_a2 values are 1.61 and 7.19, respectively; in ethanol–water solutions, the pK_a1 and pK_a2 values are 2.80 and 7.10, respectively.[52] Empirical evidence showed that complete dissociation of NTX is achieved at a pH above 8.4.[64]

It seems astonishing that until 2018 it was not certain whether the NTX molecule is planar or not. Tikhonov *et al*. [65] established that – in a gas phase – the nitro group is twisted by the angle of 8 ± 3° with respect to the 8-hydroxyquinoline plane, due to the interatomic repulsion of the nitro-group oxygen from the closest hydrogen, similar to its structural analogue 1-nitronaphthalene. The twist distorts the planar structure formed by the π-π conjugation of the nitro group and the 8-HQ aromatic system. However, despite distortion, the conjugation is retained. In the condensed phase, the molecule is planar. This is due to the surrounding molecules that compensate for the interatomic repulsion in a single molecule.

Also, the protonation state of the NTX molecule in its crystalline form — whether neutral or zwitterionic due to intramolecular proton transfer from the hydroxyl group to the pyridyl ring nitrogen — was not determined until 2001.[66] The DFT (density functional theory) calculations showed that the zwitterionic form is less stable (the difference is 31 kJ/mol), but not sufficiently to exclude it from consideration. Spectrophotometric and X-ray results showed that NTX crystallizes in its neutral state. For the zwitterionic state, the UV-VIS spectra would have contained absorption bands throughout the visible region, while in reality, the NTX crystals are light yellow. In a crystal, neutral molecules form dimers *via* two hydrogen bonds, with an interplanar distance of app. 0.35 nm between stacked dimers.

4. CHELATION AND STABILITY CONSTANTS OF METAL COMPLEXES

The chelation of metal cations essential for microbial survival as the main mechanism in the quinolines' MOA was recognised almost a century ago (Chapter 2). The chelates consist of a metal cation that effectively replaced the hydrogen from the hydroxyl group and coordinated to the nitrogen of the pyridyl ring, thus forming a stable five-membered ring (Figure 2d). 8-HQ thus acts as a bidentate ligand, and one metal cation is linked to two 8-HQ anions (the chelates obey 2:1 stoichiometry). As the metal reacts directly with the anion, the chelating abilities can be affected through manipulation of the hydroxyl group acidity. Also, Zborowski *et al*.[51] studied chelatoaromaticity of 8-HQ complexes; the study showed that aromatic properties of the pyridyl and benzene rings within 8-HQ differ and that during complexation the aromaticity of the ligand increases, causing the stabilisation of the studied metal complexes by chelatoaromatic effect. The stability constants of 8-HQ complexes with chosen metal cations are given in Table 1.

Out of numerous 8-HQ derivatives, it was shown that the positioning of nitro-group at C5 (thus obtaining NTX) yields the highest improvement of 8-HQ chelating abilities.[38] As aforementioned, the electron-withdrawing abilities of the nitro group facilitate deprotonation of the hydroxyl group (lowering pK_a2 from ~9.7 to ~6.2, section 3.2), which results in enhanced chelating power. Indeed, for 8-HQ, a linear relationship was established between the chelates' stability constants and the hydroxyl group dissociation constant.[64] Since the chelating power depends on the hydroxyl group dissociation, the pH of the medium plays a key role: higher pH enhances the dissociation and the chelation abilities. However, there have been reports that NTX antibacterial activity is more pronounced in acidic than in alkaline urine.[24] Also, Ca^{2+} is not chelated at physiological pH.[64]

Regarding stability constants of NTX-metal chelates, there is a surprising lack of quantitative data, which is particularly astonishing given that transition metal chelation is central to NTX's biological activity. A comprehensive search of the NIST SRD 46 database[55] and major chemical literature repositories revealed only a single, non-critically evaluated entry, which was for an NTX-Zn^{2+} complex, and that was from the study that dates back to 1968.[67] To the best of our knowledge, no values are reported even for NTX-iron chelates, as the most prominent ones in the context of NTX's antibacterial activities (Chapter 5.1). The lack of thermodynamic data represents a significant gap in NTX's coordination chemistry and highlights the need for renewed experimental efforts to characterise its metal-binding properties quantitatively.

In an attempt to estimate the stability constants of the NTX-iron complexes, 8-HQ can serve as a useful reference. As given in Table 1, reported log *K* values for 8-HQ complexes with Fe^{3+} in the range of 13.4 – 13.7 (there is no relevant data regarding Fe^{2+}).[55] In NTX, the nitro group at C5 facilitates hydroxyl group deprotonation, which should enhance the complex stability. On the other hand, the nitro group may slightly reduce the donor strength of the pyridyl nitrogen, so the overall effect might turn out to be somewhat, but not markedly, favourable, depending on pH and ionic strength.

Chelation of metal ions provides a basis for a whole group of medical treatments often termed chelation therapy.[68] Besides the disruption of the specific biological functions that require a certain metal ion (iron, zinc, manganese, magnesium, cobalt, and molybdenum are

Table 1. Stability constants of 8-hydroxyquinoline complexes with chosen metal cations in aqueous solutions, at respective temperatures and ionic strengths.[55]

metal ion	log *K*	temperature *t* / °C	ionic strength *I* / (mol dm^{-3})
Fe^{3+}	13.7; 13.5; 13.4	25	0.1; 0.5; 1.0
Zn^{2+}	8.52; 8.56	25; 20	0.1; 0.0
Ni^{2+}	9.27	25	0
Co^{2+}	8.65	25	0
Mn^{2+}	6.24	25	0.1
Ag^{+}	5.20	20	0.1
Cu^{2+}	12.0; 12.56	25; 20	0.1
Cd^{2+}	7.34; 7.78	25; 20	0.1; 0.0
Pb^{2+}	9.02	25	0.0
Ga^{3+}	14.5	20	0.1
In^{3+}	12.0	25	0.1
alkaline earths (without Be^{2+})	1.26 – 4.74	20	0.0 – 1.0

essential for the enzymatic function of more than 36 % of proteins in every enzyme class[69]), chelation also disturbs the regulation of intracellular metal pools, which can result in a production of reactive oxygen species *via* Fenton reactions.[68,70] In bacteria, metal ions are crucial in a plethora of functions, which provides a platform for NTX's pleiotropic effects, as described in the following Chapter.

5. ANTIBIOTIC ACTION

5.1. Bacteriostatic and Bactericidal Action

During infections, bacteria acquire metal ions from the extra- and intracellular environments of host organisms. Transition metals are essential for bacterial survival due to their role in a wide range of biochemical and physiological processes, which can be broadly categorised into redox catalysis, non-redox catalysis, or non-catalytic functions.[69,71] At the same time, they can be toxic due to these frequent catalytic functions, thus requiring careful maintenance of the optimum concentrations. Iron cations are found in cytochromes and iron-sulphur clusters, which are involved in electron transport and redox reactions, oxygen transport, gene regulation, DNA synthesis, etc.[68,72] Other redox enzymes, like cytochrome c oxidase, require copper ions. Zn^{2+} is a cofactor in DNA/RNA polymerases and other enzymes, while Mn^{2+} acts as an antioxidant cofactor in superoxide dismutase. Chelating agents like NTX thus interfere with bacterial metal acquisition and bioavailability, disrupting their nutrition and growth, normal functioning of metal-dependent proteins, and virulence.[68]

For the virulence of most microorganisms, iron cations are of special importance (Figure 3).[3,70,72,73] It is worth noting that one of the rare truly innovative recently developed drugs is the cephalosporin derivative cefiderocol, which incorporates an iron-chelating moiety to facilitate bacteria cell entry.[11,36]

For bacteria, acquiring iron from their surroundings is a difficult and energy-consuming task, all the while the level of free iron in the human body remains far below their physiological needs. Specifically, the concentration of Fe^{3+} in human serum is 10^{-24} M, which is around 20 orders of magnitude lower compared to Fe^{3+} concentrations in bacteria (such low levels in the serum are maintained by iron transport protein transferrin, with its extraordinarily high constants for Fe^{3+} binding, namely, log K_1 = 22.7 and log K_2 = 22.1).[74–76] To overcome this limitation, bacteria (and fungi, and plants) have evolved several sophisticated mechanisms for Fe acquisition,[69,71] the most important of which is based on the secretion of small, high-affinity iron-chelating molecules known as siderophores. Their stability constants for iron binding range from 10^{10} to as high as 10^{49}, with siderophore enterobactin having the highest known affinity.[74,77] The reason for the production of a plethora of other lower-binding-affinity siderophores lies in a fascinating fight between bacteria and the human immune system (Figure 3): for example, to neutralize the enterobactin from *Salmonella*, the immune system produces a protein lipocalin-2 (neutrophil gelatinase-associated lipocalin[71]) that binds and sequesters it, to which bacteria respond by secreting salmochelins, to which lipocalin-2 cannot bind. Also, by varying siderophore chemical structures, bacteria optimise iron extraction from different sources and conditions. Newer studies demonstrate that the role of siderophores extends beyond iron acquisition: they can also act as zincophores, signalling molecules, interact with manganese (due to the ability to accommodate the Jahn-Teller distortion of the high-spin d^4 Mn^{3+} centre), and – in the case of softer donor atoms – with copper.[74,78,79] Siderophores produced by *Pseudomonas* spp. (pyochelin and pyoverdine) can form complexes with over 15 different metal cations, of transitional as well as main group metals, which is believed to be the reason behind *Pseudomonas* prevalence in soil and marine environments, together with its pathogenicity in humans.[80]

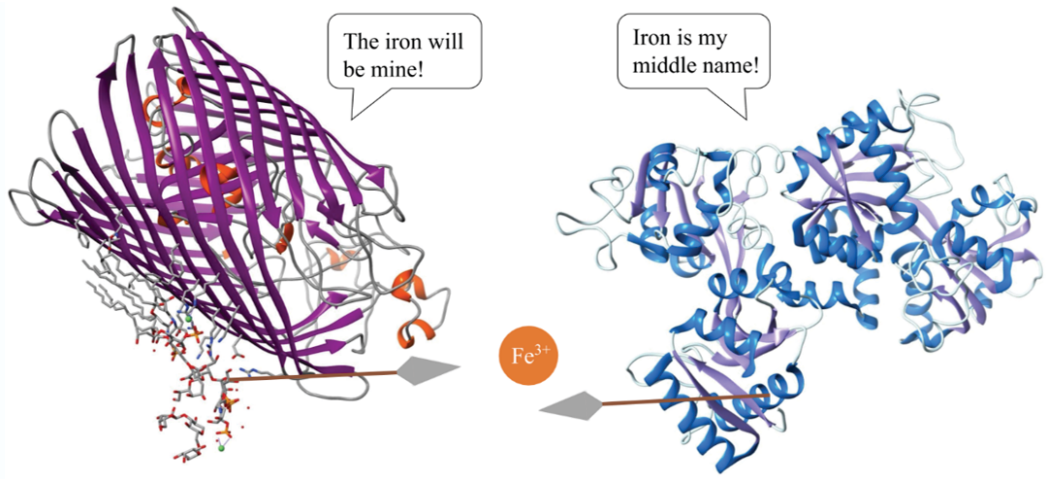

Figure 3. Cartoon depicting the "iron wars" between human and bacterial iron-sequestering proteins, *E. faecalis* siderophore (right) and human protein lactoferrin (left). Image adapted from Ref. [81]

Zinc is among the most abundant transition metals in bacteria, often second only to iron in biological importance and abundance, due to its role as a cofactor in numerous enzymes (non-redox catalyses) and structural proteins.[82,83] Indeed, one of the means of fighting bacterial infections in humans is the secretion of zinc-sequestering proteins, which is – in general terms, not just for Zn – known as nutritional immunity.[69]

From this broad scope of roles that transition metals assume in bacterial life, growth, and virulence, and sophisticated bacterial response to the disturbance of metal ion homeostasis, it appears obvious that transition-metal-chelating agents like NTX must have a complex pleiotropic antibacterial effect. Nevertheless, the majority of studies limit their explanations of NTX's MOA to "chelation of divalent cations, inhibiting RNA polymerase, biofilm formation, and adhesion",[14,17,24,84] based on the studies of Pelletier *et al.*,[64,85] thus leaving a comprehensive understanding of NTX action unclear. Note that the inhibition of RNA polymerase was actually established for 8-HQ, not NTX.[86] In the 1990s, it was assumed that NTX chelates cations that stabilise bacterial outer membrane, and this was taken as the basis of its antibacterial activity. Only very recently the studies analysing NTX's activity in more depth started to emerge, as described in the following.

In 2025, Cacace *et al.*[10] investigated a broader view of NTX's direct and indirect cellular effects, whether they are dependent or independent of its perturbation of metal homeostasis, and its effects on metal concentrations. Having *E. coli* as a reference, as the main pathogen responsible for UTIs and the only bacterial species for which NTX clinical breakpoint was determined up to now,[43] it was established that NTX has a broad activity spectrum against a range of Gram-negative bacteria. This includes wildtype strains of *A. baumannii*, a pathogen for which multidrug-resistant variants pose significant treatment challenges.[11] Besides the expected bacteriostatic effect, the first evidence of NTX bactericidal activity against *A. baumannii* wildtype was given. This is concordant with the results of Repac-Antić *et al.*,[87] which showed NTX bactericidal activity against other common uropathogen *E. faecalis*. Both studies also established that cell death is caused by damage to the cell wall, resulting in the leakage of cellular material. It was deemed plausible that NTX affects the bacterial outer membrane integrity through complexation with Mn^{2+} and Mg^{2+} and through the genetic regulation of the levels of membrane proteins. Specifically, the downregulation of expression of proteins likely connected to membrane porins was observed only for the NTX-treated mutant. Relying on the study of Dreschner *et al.*,[21] the porins in question might be OmpC and OmpF, and membrane-bound maltoporin LamB.

Regarding genetic regulation, it was determined that in *E. coli* NTX causes a decrease in the stability of the Ferric uptake regulator (Fur).[10] Fur is a key transcriptional repressor in bacteria, which is metal-dependent and controls the expression of genes involved in iron homeostasis. In an environment abundant with iron, Fur binds Fe^{2+} and attaches to specific DNA sequences (called Fur boxes) to repress transcription of iron uptake genes.[82,88] When iron is scarce, Fur is inactive, and those genes are derepressed, allowing the cell to increase iron acquisition. By chelating Fe^{2+}, NTX inactivates Fur, which in turn causes the upregulation of several iron acquisition systems, including the proteins responsible for siderophore enterobactin biosynthesis. Fur destabilisation also caused the increased abundance of the manganese importer MNTH. At the same time, NTX increased the stability of IscR ("Isc" for "Iron-sulphur cluster"), a metalloprotein that acts as a transcriptional regulator controlling genes involved in Fe-S cluster biogenesis in bacteria. There are two forms of IscR: one that represses the *isc* operon (operates under normal conditions), and the other that activates the *suf* operon (induced under stress conditions such as oxidative stress or iron limitation). Through interaction with IscR, NTX promoted activation of the *suf* operon and repression of the *isc* operon. The *Suf* system is less efficient than the *Isc* system under normal conditions, but it is more robust under stress: it better protects Fe-S cluster intermediates from oxidative damage and functions effectively when iron is scarce. However, the mechanism behind the NTX stabilisation of IscR was not further investigated. The study also showed that NTX increased intracellular levels of copper, zinc and manganese, causing metal intoxication of the bacterial cells, and indicating that NTX antibacterial properties include its ability to chelate zinc and copper. Counterintuitive at first, these results imply that NTX binds Zn and Cu ions, chelates are able to enter the cell, metals ions are then released within the cell, metal homeostasis is disrupted, and the concentration of ions is elevated to toxic levels. As a response, bacteria upregulate the production of copper and zinc exporters, siderophores (metal stress causes damage to Fe-S clusters), and phenols and polyamines (antioxidants needed upon metal intoxication). This mechanism might explain the results of proteome analysis of NTX treated *E. faecalis* mutants that exhibited the upregulation of numerous proteins upon NTX treatment,[87] which would imply that the proposed mechanism is probable for bacterial species other than *E. coli*.

5.2. Antiadhesion and Antibiofilm Action

To persist in hostile environments and enhance resistance to antibiotics and host immune responses, bacteria develop multifaceted, structured, surface-associated communities called biofilms (Figure 4).[89–91] 40 % – 80 % of bacteria on

Earth can form biofilms.[92] The efficacy of the biofilms as a main survival strategy is clearly demonstrated by the notion that antibiotic concentrations needed to eradicate bacteria in a biofilm are 100 – 1000 times higher than those needed to kill bacteria in a planktonic state.[91,93] This characteristic biofilm property is called "recalcitrance".[94] Biofilms exhibit spatial organization, metabolic heterogeneity, coordinated gene expression, and enable bacteria to adhere to biotic (e.g., extracellular matrix components, cell and/or tissue) and every probable abiotic surface.[68,95] Biofilms formed on the surfaces of medical devices (vascular grafts, heart valves, intrauterine devices, pacemakers, prosthetic joints, catheters, sutures, and contact lenses) are the leading cause of nosocomial (hospital-acquired) infections, posing a major public health concern and financial burden on the healthcare system.[94–96] It is therefore all the more surprising that their role as a primary stage in the pathogenesis of up to 80 % of bacterial infections was recognized only relatively recently.[91,97] Notably, not all biofilms are harmful; many are beneficial (such as those comprising the human microbiome) and collectively cover 300 m^2 – 400 m^2 of tissue in the human body.[98] With a change of conditions, like upregulation of virulence factors, virulent bacteria can disperse from the original "healthy" biofilm into normally sterile regions as the middle ear, lungs, brain, and blood.[97]

Among medical devices, urinary catheters are particularly susceptible to biofilm formation, causing notorious catheter-associated urinary tract infections (CAUTIs), the most common nosocomial infection.[15,101,102] UTIs in general are one of the most common reasons for medical consultation and prescription of antibiotics.[14] For every day of using an indwelling catheter, the risk of infection increases by 7 % – 10 %, and bacteriuria will inevitably develop in almost 100 % of patients on long-term catheterisation (>30 days).[103,104] Bacteria ascend the catheter, then colonize the bladder, and form multispecies biofilms, further complicating the treatment. Additionally, CAUTIs are frequently caused by multidrug-resistant bacteria.[15,17,104] Therefore, as the NTX is used in the treatment and prevention of UTIs, its antiadhesion and antibiofilm actions constitute an integral part of its antibiotic effect. Indeed, the importance of these NTX's abilities is additionally underscored by the notion that the advances in effective antibiofilm therapies present one of the most pressing challenges in antimicrobial drug development.[91] Antiadhesion and antibiofilm actions, of course, depend on the physicochemical processes behind bacterial adhesion[105–107] and the chemistry of the biofilm.[89,92,95,108]

Biofilms are composed of microbial cells and polysaccharides, proteins, and extracellular DNA, a complex mixture known as extracellular polymeric substances (EPS). Polysaccharides provide mechanical stability, help in cell adhesion, and retain water and nutrients. Proteins obtain various roles: enzymatic, structural, and as adhesins. Extracellular DNA also stabilizes biofilm structure and enables horizontal gene transfer, antibiotic resistance, and immune evasion. Biofilm development and stability depend on metal cations – namely Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+} and Zn^{2+}. The role of iron metabolism and transport in normal biofilm development is well established.[70,82,109–111] Hancock *et al*. found that *E. coli* produced significantly less biofilm in the presence of an iron chelator.[112] Iron and other metal cations contribute to multiple stages of biofilm formation (typically subdivided into four stages, including initial adhesion and maturation, before which the bacteria are said to be in a planktonic – freely suspended – state), but also to matrix stabilization and quorum sensing. Adhesion, as a first step in colonization, invasion, and biofilm formation, represents the Achilles heel of crucial virulence functions, the

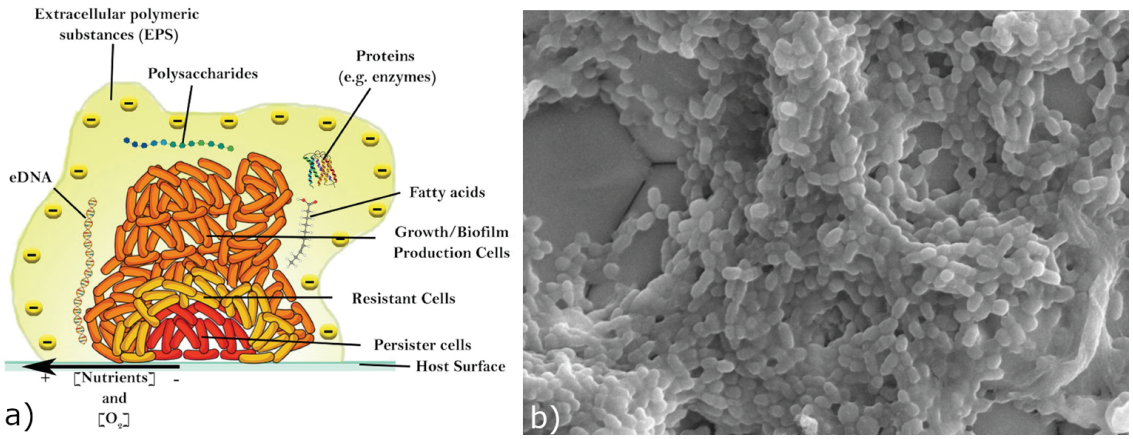

Figure 4. a) Schematic representation of a bacterial biofilm; b) SEM micrograph of a mixed biofilm on hydroxyapatite. Images a) and b) adapted from Ref. [99] and Ref. [100], respectively.

inhibition of which is recognized as an attractive alternative approach to fighting infections.[104,107,113–115] When the approach is based on targeting adhesion, the problem of the inability of antibacterial agents to penetrate the biofilm – which presents a major contributing factor in biofilm resistance – is circumvented.[91]

The antiadhesion effect of NTX has been demonstrated a long time ago by several studies.[87] Bourlioux *et al*. conducted a series of research (responsible for a local maximum at Figure 1) showing NTX's major inhibitory effect on bacterial adhesion on epithelial cells from human bladder mucosa and on the urinary catheter surface, with inhibition levels as high as 90 % (while norfloxacin has no effect).[116–119] The authors did not provide a molecular mechanism for those findings. Latrache *et al*. found that the NTX treatment makes the bacterial surface of *E. coli* more hydrophobic, and in this way decreases the adherence to the catheter surface.[120] Note that bacterial surface hydrophobicity is one of the most important physicochemical parameters controlling early bacterial adhesion.[107]

Antibiofilm action of NTX was shown for *P. aeruginosa* biofilm by Sobke *et al*.[18] Potent antibiofilm activity, demonstrated by 50 % lower biofilm thickness and markedly reduced surface coverage compared to control biofilms, was attributed to the chelation of iron and zinc ions. NTX activity was found comparable to EDTA, and superior to pure iron chelators like 2,29-dipyridyl, deferoxamine mesylate, or diethylenetriaminepentaacetic acid. Unlike those chelators, NTX is orally applicable, less toxic, and efficient in concentrations achieved *in vivo* during oral treatment. Regarding EDTA as a reference, it is worth noting that EDTA treatment of *P. aeruginosa* biofilms resulted in 1,000-fold greater killing compared to antibiotic gentamicin.[109] Another study showed a broader antimicrobial spectrum compared to nitrofurantoin (a first-line treatment drug, but problematic due to lung toxicity and insufficient coverage of Enterobacteriaceae other than *E. coli*), which included MDR bacterial isolates and rare resistance rates.[17] Abouelhassan *et al*. reported broad-spectrum biofilm-eradicating activities of NTX against multiple human pathogens, including MDR *A. baumannii*, *E. coli*, MRSA (Methicillin-resistant *Staphylococcus aureus*), MRSE (Methicillin-Resistant *Staphylococcus epidermidis*) and VRE (Vancomycin-Resistant Enterococci).[19] This was deemed extremely rare as there were reports of only one other non-membrane-active broad-spectrum biofilm-eradicating agent of similar scope of action (mitomycin C). The authors emphasize the potential of NTX-based therapies as powerful new means of fighting persistent biofilm-associated bacterial infections (i.e. device-related biofilm infections, wound infections). However, again, the molecular mechanisms for these activities were not provided. Dobrindt *et al*. studied the resistance mechanisms against UTI drugs (described in the next chapter) and concluded that NTX seems to be the preferred choice for the treatment of biofilms in the urinary tract.[15]

6. ANTIBIOTIC RESISTANCE MECHANISMS TO NITROXOLINE

One of the most prominent advantages of NTX in comparison to other antimicrobials is the fact that resistance rates have remained low even though it has been in use for more than half a century. For *E. coli*, the most frequent uropathogen, no increase in resistance has been observed.[14] A more recent study showed that among 600 *E. coli* urine-isolated strains susceptible to penicillins, third-generation cephalosporins, and quinolones, only three were resistant to NTX.[15] Based on phenotypic resistance data from a large number of urine isolates, structural characterisation of an MDR plasmid and publicly available genomic data of resistant enterobacteria, the study showed that NTX could be used instead of cotrimoxazole against MDR uropathogens. However, a molecular or genetic explanation of resistance mechanisms specific to NTX, nor the mutational pathways or resistance gene profiles associated with NTX's resistance, were not provided.

Indeed, the mechanisms of bacterial resistance to NTX have begun to unravel only during the last two years. Deschner *et al*.[21] found that the low-level resistance of *E. coli* to NTX is associated with mutations in efflux-related genes. Efflux pumps actively expel NTX from the bacterial cell, reducing its intracellular concentration and effectiveness. Resistance-related mutations were also established in pleiotropic regulators and sensor histidine kinases such as EnvZ, which affect global gene expression and membrane permeability. The analysis was performed on the low-level mutants that authors succeeded in generating using a long-term exposure setup; astonishingly, those mutants exhibited reduced motility, altered metabolism, and diminished virulence *in vivo*, in zebrafish infection models. The results thus showed that developing even a low-level resistance carries a high cost of fitness loss, somewhat slower growth rates, and diminished virulence. Puértolas-Balint *et al*. also studied an increase in resistance of *E. coli* to NTX.[84] In the work that preceded the aforementioned prominent study of Dreschner *et al*.[21] by four years, they identified the upregulation of the efflux pumps (that expel NTX from the bacterial cell) as a key factor in NTX resistance in *E.coli*. They were also the first to establish that building resistance carries fitness costs.

Cacace *et al*.[10] found that NTX targets metal-dependent enzymes and DNA topoisomerases, but its binding sites differ from those of fluoroquinolones. This is

relevant because it makes cross-resistance less likely. Cross-resistance is the phenomenon where a microorganism that has developed resistance to one antimicrobial agent also exhibits resistance to one or more other agents, to which it has never been exposed. The phenomenon occurs due to a shared or overlapping MOA or resistance pathway. In accordance with the results of Deschner *et al*.[21], the results showed that the most recurring resistance mechanism is the upregulation of Resistance-Nodulation-Division (RND) efflux pumps. Mutations in genes that regulate membrane permeability were also observed. A typical resistance mode – stabilisation of a specific protein target – was absent, which strongly implies the NTX's pleiotropic effect and explains low resistance rates.

In contrast to the aforementioned study by Cacace *et al*.[10], which investigated NTX activity against wildtype *A. baumannii*, Fuchs *et al*.[27] conducted a focused analysis on carbapenem-resistant *A. baumannii* (CRAb) isolates from urinary tract sources. Some of the 34 molecularly characterized CRAb strains exhibited co-resistance to ciprofloxacin and trimethoprim/sulfamethoxazole. Despite this, NTX demonstrated excellent *in vitro* activity across all isolates. However, for biofilm-associated CRAb, higher concentrations were required for complete eradication, as described in Chapter 9. These findings highlight NTX's promise as an oral treatment option for lower UTIs caused by WHO-priority drug-resistant *A. baumannii*.

The results of Repac-Antić *et al*. on *E. faecalis* showed the upregulation of a protein specific to *van*B-positive isolates, but only upon the combined treatment with NTX and gentamicin (GENT), as opposed to NTX or GENT monotreatments.[87] "*van*B"-positive isolates designates the strain that carries the *van*B gene, which confers resistance to vancomycin through the production of enzymes responsible for altering the bacterial cell wall target of vancomycin, making the antibiotic ineffective. The results imply synergistic NTX/GENT effect, proven very effective, but also carrying a higher risk of resistance development in comparison to monotreatments.

Dobrint *et al*.[15] showed a low prevalence of NTX's resistance among enterobacterial uropathogens, even among MDR strains. On the other hand, the results show that current guidelines for UTIs bear the risk of promoting MDR uropathogens. NTX stands out compared to standard treatments like cotrimoxazole due to high *in vitro* activity against ESBL (Extended-Spectrum Beta-Lactamase)-producing, carbapenem-resistant, and fluoroquinolone-resistant *E. coli* and *Klebsiella pneumoniae* strains, while cotrimoxazole and other guideline-recommended drugs often show reduced efficacy due to widespread resistance. The distinct metal-chelating and redox-active properties of NTX MOA reduce the likelihood of cross-resistance with other antibiotic classes. For now, NTX is not widely included in current UTI treatment guidelines, likely due to limited recent clinical data, historical underuse, and lack of global regulatory approval. Authors thus call for greater consideration of NTX in the treatment of UTIs, and point out its potential for prophylactic use, especially in patients prone to recurrent UTIs caused by MDR bacteria. It is worth noting that the aforementioned ESBL-producing bacteria have become a global scourge in the past few decades, expanding from nosocomial problems to community-acquired infections, and amounting to the presence of 55 % – 65 % in China and 67 % – 79 % in India.[3]

A pivotal study by Fuchs and Hamprecht[26] provided the first comprehensive analysis of nitroxoline activity against carbapenemase-producing Enterobacterales (CPE), including strains harboring rare carbapenemases. Out of 105 well-characterized CPE isolates, 95.2 % were susceptible to NTX based on the EUCAST breakpoint[43] for *E. coli* (≤ 16 mg/L). Notably, all *E. coli* isolates were susceptible, with MICs ranging from 2 – 8 mg/L. The study demonstrated that NTX retained activity even against pan-drug-resistant strains and showed no correlation between NTX MICs and carbapenem MICs, suggesting a distinct resistance profile. These findings underscore NTX's potential as an oral treatment option for uncomplicated UTIs caused by multidrug-resistant Enterobacterales, including those with carbapenemase production.

A research group of Sobke *et al*. reported the emergence of NTX-resistant isolates under NTX treatment of lower UTIs in geriatric patients, but the mechanism of resistance was not discussed.[17,121] In an *in vitro* study, a low level of resistance was established for all examined species, including ESBL-producing isolates of *E. coli* and *Klebsiella pneumoniae*, MRSA, and VRE, but excluding *P. aeruginosa*. An important observation of the study, done in 2018, is that minimum inhibitory concentrations (MICs) resemble those reported in a study from 1978, indicating no major emergence of NTX-resistant clones within the last 40 years.[17]

Following the study of Hoffmann *et al*.[122], which demonstrated promising *in vitro* activity of NTX against drug-resistant *Mycobacterium abscessus*, Xu *et al*.[123] elucidated the underlying resistance mechanism. Their findings identified mutations in the *marR* transcriptional repressor (*MAB_2648c*) as a key factor in modulating NTX activity. These mutations significantly affect the regulation of efflux pumps (concretely, the MmpS5–MmpL5 efflux pump system), leading to reduced NTX susceptibility. Although the efflux-related mechanism was clarified, the specific molecular target of NTX remained unknown. The authors emphasized the need for future studies that would lead to precise understanding of NTX mode of action and, thus, of the resistance development.

7. REPURPOSING OF NITROXOLINE

"It takes too long and costs too much to bring new drugs to market.", as Chong and Sullivan summed up the main reason for efforts put in repurposing existing drugs.[124] Due to NTX being an approved drug, with an excellent safety profile, well-characterized pharmacokinetics, and the MOA that enables its activity against different microorganisms, in the last few years there were numerous studies indicating its repurposing potential as an antiviral,[32,125,126] antifungal,[31,56,127] antiparasitic[30], antituberculosis[28], neuroprotective[128] and – most prominently – anticancer agent.[29,33,129–136]

7.1. Antitumour Actions

NTX repurposing in oncology is based on its efficacy across various tumour models, including glioblastoma, prostate cancer, multiple myeloma, cholangiocarcinoma, pancreatic cancer, and small-cell lung cancer.[131] The molecular bases for NTX's antitumour actions are multifaceted and include a broad spectrum of molecular mechanisms targeting key hallmarks of cancer, including proliferation, angiogenesis, invasion, and immune evasion.

Shim *et al.*[132] first reported on NTX anticancer activity. The study showed that NTX affects the growth of human bladder cancer by dual inhibitory action: first, it inhibits MetAP2 (Type 2 methionine aminopeptidase), the enzyme involved in endothelial cell proliferation; second, it inhibits SIRT1 (sirtuin 1), which is a NAD^+-dependent deacetylase that regulates angiogenesis and cell survival (Figure 5a). Besides suppressed angiogenesis (the formation of new blood vessels), the inhibition resulted in the induction of senescence – a state in which cells permanently stop dividing – and reduced tumour growth, both *in vitro* and *in vivo* models. It is noteworthy that NTX was chosen for the study because it was identified from a high-throughput screen of 175 000 compounds for MetAP2 inhibitors and from a parallel screen to identify currently used clinical drugs that can also inhibit human umbilical vein endothelial cell proliferation. The NTX's chelating abilities seemed irrelevant as part of its antitumour mechanism: even though MetAP2 is a metal-dependent enzyme, the inhibitory effect is not due to metal chelation but attributed to a direct enzymatic inhibition. The description of chemical interactions between NTX and the enzyme was not provided in the study.

However, the description of chemical interactions between NTX and another tumour-related enzyme was provided by Mirković *et al.*[129] The study focused on cathepsin B (catB), a cysteine protease with both endo-peptidase and exopeptidase activity, which contributes to extracellular matrix breakdown and tumour cell invasion. NTX is identified as a potent, reversible, and selective inhibitor of catB, which enables its effect on cancer progression. NTX binds to the S2' subsite of catB's active site *via* its nitro group that forms symmetric interactions with two histidine residues in the occluding loom. This occluding loop is a structural feature unique to catB, thus enabling selective inhibition (Figure 5b). Pyridyl ring nitrogen and the hydroxyl group at position 8, crucial for NTX chelating abilities (see Figure 1d), are not involved in direct interactions with the protein. The authors also demonstrated antitumour actions of ruthenium-based NTX derivatives[133]

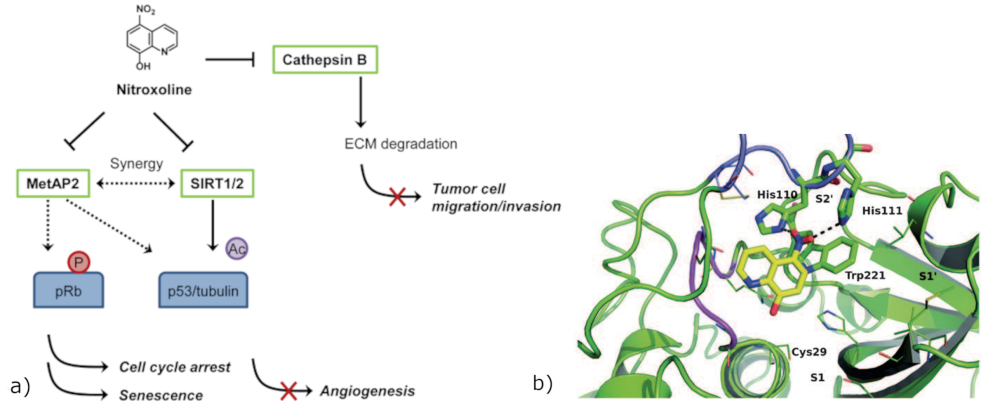

Figure 5. a) Anticancer mechanism of action of nitroxoline (NTX): NTX inhibits MetAP2 (Methionine Aminopeptidase 2) and sirtuins (SIRT1 and SIRT 2), the enzymes in human endothelial cells. MetAP2 inhibition helps preserve pRb tumour suppressor activity. SIRT1/2 inhibition increases acetylation of p53 and tubulin, respectively, leading to p53 activation and microtubule destabilization, making cancer cells more vulnerable. b) Binding of NTX (yellow) to the cathepsin B (catB) active site cleft. CatB is a cysteine protease of high pharmacological relevance, with a role in tumour progression. NTX is identified as a potent, reversible, and selective inhibitor of catB. The binding occurs via the NTX nitro group that forms symmetric interactions with two histidine residues. Quinoline ring nitrogen and the hydroxyl group at position 8, crucial for NTX chelating abilities, are not involved in direct interactions with the protein. The images reproduced from Refs. [129,130] (Figure 5b is reproduced under license from John Wiley and Sons, license number 6093530293304).

Veschi *et al*. analysed the effect of NTX on pancreatic cancer (PC) cell lines, as a single agent and in combination with erlotinib, a drug approved for PC treatment.[135] The study showed that NTX caused decreased viability, induced apoptosis through activation of caspases (enzymes that execute programmed cell death), affected the expression of relevant cell cycle proteins, and drastically impaired the self-renewal capacity of the cells in the three PC cell lines. The action of NTX was comparable or in some cases more pronounced than erlotinib. The chelating abilities of NTX do not seem to be a relevant part of the antitumour mechanism.

Zhang *et al*.[29] performed a preclinical pharmacodynamics evaluation of NTX's anticancer activities, and found that NTX dose-dependent inhibition of various cancer cell lines, with urological cancer cells being especially sensitive. Non-cancerous cells remained largely unaffected, demonstrating NTX selectivity and safety. Remarkably, oral doses equivalent to those used for UTI treatment in humans significantly inhibited tumour growth (up to 85 %), while higher doses enhanced efficacy without increasing toxicity. This anticancer activity is accomplished *via* anti-angiogenesis actions, in accordance with Shim *et al*.[132] results described above. In this study also, the anticancer effects are attributed to direct cellular and molecular actions, and not to NTX chelating abilities.

Other studies on oncological repurposing of NTX show a similar pattern: NTX exhibits potent antitumour activity, combined with negligible toxicity and good selectivity, and a potential to improve its potency and selectivity by serving as a scaffold for new derivatives. The mechanism of antitumour action is completely unrelated to its antimicrobial MOA, which rests on metal chelation. For a more comprehensive survey on NTX antitumour application, a reader is referred to review papers of Mitrović *et al*.[131] and Shim *et al*.[130]

7.2. Antiviral Action

Even though NTX is primarily considered an antibiotic drug, numerous studies have recently shown its antiviral and antifungal activities. The study of Bojkova *et al*.[126] demonstrated its potency against mpox (previously known as monkeypox) and emphasized that, as an antibiotic, NTX can simultaneously target sexually transmitted bacteria that were found to be co-transmitted with mpox virus during the 2022 outbreak. Until recently, the mpox virus caused only zoonotic outbreaks in Africa, but in 2022 it spread for the first time by human-to-human transmission outside of Africa. By 2023, it reached 111 countries, with a considerable death toll. The drugs currently used for mpox treatment (cidofovir and brincidofovir) are burdened with therapy-limiting side effects and increasing resistance, while tecovirimat stocks were insufficient to cover the ongoing outbreak. The study showed that NTX inhibited mpox viruses, including a tecovirimat-resistant strain, at therapeutically achievable concentrations. Additionally, it can be used in combination with the two approved drugs and increase their activity, and (unlike tecovirimat) it did not induce resistance in the mpox virus even after multiple passages. Remarkably, the addition of divalent cations (Fe^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}), that impair NTX's antibacterial actions,[36] did not affect its antiviral activity. This indicated that antiviral MOA is not based (exclusively) on metal chelation. It was established that NTX reduces phosphorylation and interferes with two key host signalling pathways critical for viral propagation. However, a direct molecular binding of NTX to a specific host protein was not defined.

Japanese encephalitis virus (JEV) is a major cause of Japanese encephalitis (inflammation of the brain), with a mortality rate of about 20 % – 30 %. It is an epidemic in the Asia Pacific region, putting at risk more than 3 billion people, yet with no available effective drugs. Zhang *et al*. performed a high throughput screening of 1443 FDA-approved drugs.[32] NTX was one of the five newly discovered inhibitors of JEV.

7.3. Antifungal Action

Fuchs *et al*.[31] studied NTX antifungal action against the yeast *Candida auris*, and established excellent *in vitro* activity, with MIC values 0.125 mg/L – 1 mg/L, which are an order of magnitude lower than those for *E. coli*.[43] NTX's convenient pharmacokinetic properties, namely, high urinary concentrations after oral administration (Chapter 8), are of special importance for the treatment of *Candida*. The exact antifungal mechanism remained unclear, but it was hypothesized that iron chelation interferes with metal-dependent enzymes, plus a possible inhibition of RNA synthesis, which was observed in another yeast species (*Schizosaccharomyces pombe*) after the treatment with NTX analogue 8-HQ (Section 3.1.).[137]

Another study of Fuchs *et al*.[127] demonstrated potent *in vitro* antifungal activity of NTX against a diverse collection of *Candida* species isolated from the urinary tract, including strains resistant to commonly used antifungals such as amphotericin, anidulafungin, fluconazole, voriconazole, and posaconazole. Despite its frequent occurrence in catheterized patients and as a consequence of antibiotic therapy, candiduria treatment is still controversial.[138] These findings position NTX as a promising alternative for managing candiduria and it may be approved or used in clinical practice for this indication.

7.4. Other Applications

Amoebae are unicellular eukaryotes, much larger and more complex than bacteria. Yet, several studies reported NTX's

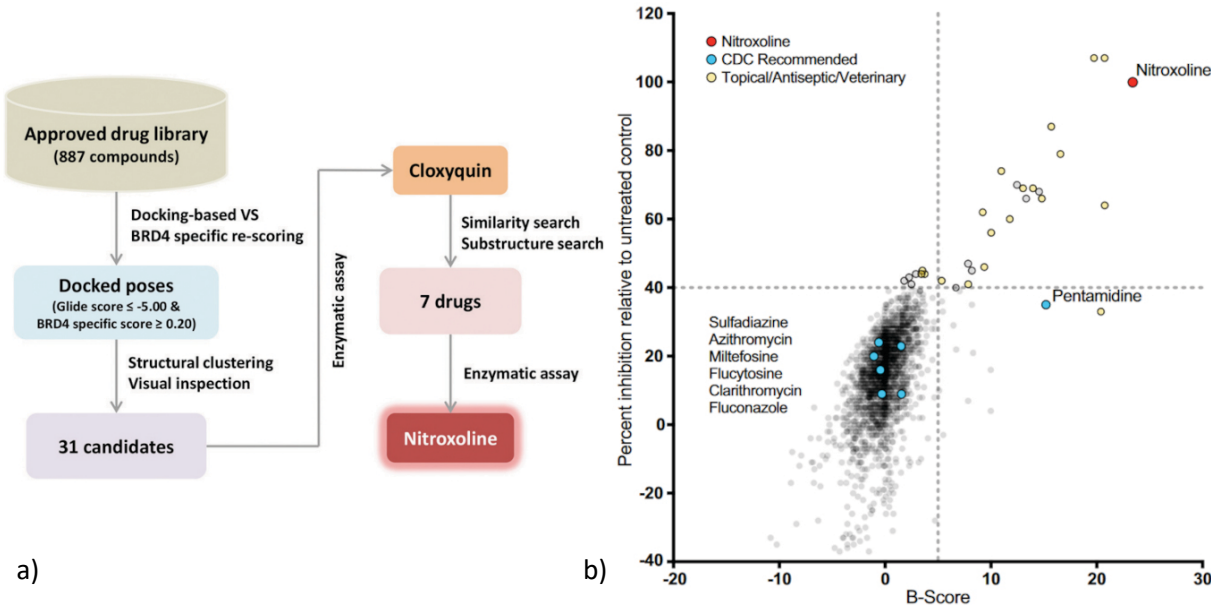

Figure 6. a) Schematic overview of the identification of nitroxoline as an inhibitor of BRD4 (bromodomain-containing protein 4, a key promoter of tumour progression) *via* virtual screening. b) The results of the screening of 2177 clinically approved compounds identified nitroxoline as the only compound with high selectivity for inhibition of *B. mandrillaris* viability. *B. mandrillaris* is an amoeba causing rare but almost always fatal infection of the central nervous system. Adapted from Refs. [140] and [30] respectively. (Figure 6a is reproduced under license from Royal Society of Chemistry, license number 1643033-2).

impressive amoebicidal actions. Laurie *et al.*[30] screened 2177 clinically approved compounds for *in vitro* activity against *B. mandrillaris*, a rare but almost always fatal infection of the central nervous system (mortality rate > 90 %). The screen selected NTX as the only compound with high selectivity for inhibition of *B. mandrillaris* viability (Figure 6b). Its effect is directly associated with its chelating abilities: NTX analogues lacking hydroxyl group at position 8 (see Figure 2d) were inactive. On the other hand, 1,10-phenanthroline, not an NTX analogue but can act as a bidentate ligand, demonstrated some activity. The authors pointed out the intriguing possibility that NTX may have activity against similar other free-living amoebae such as *Acanthamoeba* spp. and *Naegleria fowleri*.

5 years later, a patient with granulomatous amoebic encephalitis, caused by the aforementioned amoeba *B. mandrillaris*, was hospitalized in California.[139] After unsuccessful treatments with approved drugs for 109 days, the FDA authorized the treatment with NTX through an emergency use authorization (NTX is not an FDA-approved drug in the USA, Chapter 2). After 1 week of NTX treatment, MRI showed decreased size of the cerebral abscesses and no new lesions, resulting in a discharge of the patient from the hospital, but under NTX medication for 1 year.

Another screening, described by Jiang *et al.*,[140] included 877 compounds of the approved drug library, to find the inhibitors of the BET (Bromodomain and Extra-Terminal) family of BRD proteins (bromodomain-containing proteins). Seven compounds were filtered for BRD4 binding evaluation, and among them, NTX exhibited the most potent inhibition with very low IC_{50} (Figure 6a). Overactivation of BET proteins, especially BRD4, is linked to cancer (leukaemia), inflammation, and cardiovascular diseases. NTX binds to the acetyl-lysine binding pocket, disrupting the interaction with the enzyme's normal target (acetylated histones). The binding occurs through hydrogen bonds with Asn residue and *via* water bridge to Tyr, hydrophobic interactions within the bromodomain pocket, and water-mediated interactions involving the NTX nitro group. The prevention of enzyme binding to acetylated histones leads to reduced transcription of key oncogenes, and this presents the base of NTX anti-leukemic and anti-inflammatory activity. NTX chelating abilities are not involved in the mechanism.

Following the aforementioned work of Laurie *et al.*,[30] Chao-Pellicer *et al.*[141] examined NTX action against another amoeba, *Naegleria fowleri*, a parasite against whom there is no standardized treatment, causing death shortly after the onset of infection, with a fatality rate of > 97 %. The results showed that NTX treatment increases plasma membrane permeability and a loss of mitochondrial membrane potential, leading to an 85 % reduction in ATP

production, indicating mitochondrial collapse. Even though the authors did not attribute this directly to NTX's ability to chelate ions responsible for membrane structural stability (but suggested that hydroxyl and nitro groups on NTX are critical for its amoebicidal activity), it seems obvious that the results are in accordance with findings of other studies described in Section 5.1. However, more pharmacokinetics research is needed to establish whether NTX may achieve relevant concentrations in the human central nervous system to be effective amoebic meningoencephalitis treatment.

8. CLINICAL (UNDER)USE

NTX is an orally administered antimicrobial agent approved in several European countries for the treatment and prophylaxis of acute or recurrent uncomplicated UTIs, including those caused by multi-resistant uropathogens. Note that UTI is one of the most common reasons for medical treatments and for prescription of antibiotics.[14] No severe adverse effects of NTX have been reported so far [17,24,132], and it has no qualitative or quantitative effect on the faecal flora,[24] since it is excreted only *via* urine (and not parallelly *via* intestine, like, e.g., cotrimoxazole).[15] Alongside its low resistance rates (Chapter 6), its specific pharmacokinetic properties are also one of its advantages for most applications. It is known that it is rapidly absorbed into the plasma, within half an hour after oral administration, and excreted into urine. In rats, oral administration at a dose of 5 mg/kg resulted in the elimination of approximately 77 % of the NTX from plasma within 6 h, while free NTX concentrations exceeding 1 µmol/L persisted in plasma for over 10 h. Remarkably, urinary retention is even more prolonged, which is a prerequisite for successful therapy of uropathogens.[15,41] In human subjects, a single oral dose of 400 mg (within the standard therapeutic range of 400 mg/day – 750 mg/day) yielded urinary concentrations as high as 10 µmol/L after 24 hours.[142] However, despite its potential, NTX was not incorporated into treatment guidelines (in Germany) until 2016, primarily due to insufficient data regarding resistance rates, MIC distributions, and the absence of EUCAST-validated (European Committee on Antimicrobial Susceptibility Testing) clinical breakpoints.[17] It is surprising that for a drug in use for more than half a century, clinical breakpoints were not available until 2016, and then, only for *E. coli*.[43] MIC distributions are available from EUCAST only for eight bacterial species and seven genera.[10]

This point actually exemplifies part of the reasons why other drugs for UTIs, like nitrofurantoin, were prevalent over NTX in clinical practice; other reasons include the fact that nitrofurantoin is an FDA-approved drug, well-established in many countries, as opposed to local application of NTX only in several European countries (see Chapter 2); nitrofurantoin endures longer-standing inclusion in guidelines, better characterized pharmacokinetic profile, more clinical data and broader familiarity among clinicians.[13] NTX clinical trials are outdated, which limits its perceived reliability in clinical decision-making. However, nitrofurantoin (and other common UTI therapeutics like trimethoprim, cotrimoxazole, and fluoroquinolones) resistance rates are high in many countries worldwide, and appear to increase with patient age; hence, NTX – with the addition of its fewer side-effects and low resistance rate – encounters increasing interest as a candidate for the first-line treatment drug for UTIs.[14,17,24] As a repurposed drug, as aforementioned in Chapter 2, it entered Phase II clinical trials for bladder-cancer treatment in China,[29] where it was also approved for the treatment of free-living amoebae infections.

9. LIMITATIONS OF NITROXOLINE USE

Despite its broad-spectrum activity and renewed interest in nitroxoline (NTX) as a therapeutic agent, several limitations constrain its clinical application and should be acknowledged.

NTX achieves high urinary concentrations following oral administration, but systemic bioavailability is low, which restricts its use primarily to intraluminal infections of the urinary tract. According to EUCAST, concentrations in urine can exceed 200 mg/L, while systemic levels remain significantly lower, limiting its efficacy in treating infections outside the urinary tract.[43] This pharmacokinetic profile explains why NTX is currently approved only for uncomplicated UTIs in select European countries (Chapter 2).

Besides in pharmacokinetic distribution, there are also limitations regarding susceptibility of certain pathogens. While NTX shows excellent *in vitro* activity against *E. coli* and some multidrug-resistant uropathogens, intrinsic resistance has been observed in several clinically relevant species, namely, *Aerococcus sanguinicola* and *Pseudomonas* spp. Ahmadzada *et al*.[25] demonstrated that all tested *A. sanguinicola* isolates had high MICs ($MIC_{50}/_{90}$ = 64/128 mg/L), suggesting intrinsic resistance. In contrast, *A. urinae* isolates were largely susceptible. Regarding *Pseudomonas* spp., EUCAST explicitly states that *Pseudomonas aeruginosa* and related species are resistant to NTX, and no clinical breakpoints have been established for these species.[43] These findings underscore the importance of species-level identification and susceptibility testing before initiating NTX therapy.

There are also some conflicting data on biofilm eradication. Although NTX has demonstrated antibiofilm activity (section 5.2), its ability to eradicate established

biofilms remains controversial. In a study on carbapenem-resistant *A. baumannii* (CRAb), NTX showed promising MIC and MBIC values (≤ 4 mg/L), but minimum biofilm eradication concentrations (MBECs) were substantially higher (16 – 128 mg/L), often exceeding achievable urinary concentrations.[27] This suggests that NTX may inhibit biofilm formation but is less effective at eradicating mature biofilms, particularly *in vivo*.

Limitations are also encountered in clinical data and manifested as variable outcomes. Despite decades of clinical use, robust pharmacodynamics and clinical outcome data are lacking. For example, a prospective observational study in geriatric patients reported microbiological failure despite NTX treatment, raising concerns about its reliability in certain populations.[121] PK/PD breakpoints have not been established, and Monte Carlo simulations are unavailable, limiting the ability to predict clinical success based on MIC values alone.[43]

10. FUTURE PERSPECTIVES AND RESEARCH NEEDS

While NTX demonstrates promising *in vitro* activity and a favourable safety profile, its broader clinical application remains constrained by limited systemic bioavailability, intrinsic resistance in certain pathogens, and incomplete understanding of its pharmacodynamics. To overcome these barriers and address its perceived underusage, future research should prioritize well-designed clinical trials that evaluate NTX's efficacy beyond UTIs, particularly in biofilm-associated and systemic infections. Additionally, studies exploring NTX's pharmacokinetic optimization – such as formulation improvements or combination therapies – could enhance its therapeutic reach. A deeper molecular understanding of NTX's pleiotropic mechanisms, including its interactions with metal-dependent enzymes and regulatory pathways, is essential to predict resistance development and guide rational repurposing. Rather than solely emphasizing its *in vitro* potency, future investigations should aim to bridge the gap between laboratory findings and clinical applicability, thereby clarifying NTX's role in modern antimicrobial therapy.

11. CONCLUSION

This review paper presents overwhelming evidence of NTX's potential not just as an underused ancient antibiotic resurfacing in the era of rising antimicrobial resistance, but also as the repurposed drug for a wide range of microorganisms and various pathological states. Its well-known metal-chelating abilities enable its pleiotropic effect in the antimicrobial and antibiofilm realm, while newly discovered inhibitory actions, unrelated to chelation, open completely novel pathways for its use. Combined with the notion that it is an approved drug in use for more than 60 years, of simple structure easy to synthesize, with low resistance rates, NTX's rising importance on a global scale seems undisputable.

However, while NTX remains a valuable option for oral treatment of UTIs, especially in the context of rising antimicrobial resistance, its limited systemic distribution, intrinsic resistance in certain pathogens, and uncertain efficacy against biofilms highlight the need for cautious interpretation of *in vitro* data and further clinical studies. These limitations should be considered when evaluating NTX for broader therapeutic use or repurposing in non-urinary infections.

Acknowledgments. The graphical abstract incorporates modified icons and illustrations downloaded from Freepik (www.freepik.com), used under Freepik's free license. We acknowledge the original creators as per Freepik's attribution guidelines. This work was funded by University of Rijeka, grant number uniri-iskusni-prirod-23-92.

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