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The history of the tetracyclines

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The history of the tetracyclines involves the collective contributions of thousands of dedicated researchers, scientists, clinicians, and business executives over the course of more than 60 years. Discovered as natural products from actinomycetes soil bacteria, the tetracyclines were first reported in the scientific literature in 1948. They were noted for their broad spectrum antibacterial activity and were commercialized with clinical success beginning in the late 1940s to the early 1950s. The second-generation semisynthetic analogs and more recent third-generation compounds show the continued evolution of the tetracycline scaffold toward derivatives with increased potency as well as efficacy against tetracycline-resistant bacteria, with improved pharmacokinetic and chemical properties. Their biologic activity against a wide spectrum of microbial pathogens and their uses in mammalian models of inflammation, neurodegeneration, and other biological systems indicate that the tetracyclines will continue to be successful therapeutics in infectious diseases and as potential therapeutics against inflammation-based mammalian cell diseases.

Keywords: tetracyclines; history; bioactivity; mechanism; resistance; uses

The antibiotic era

In early 1948, five-year-old Toby Hockett (Fig. 1) was rushed by his parents to Johns Hopkins Children's Hospital in Washington, DC, with severe abdominal pain, and diagnosed with a ruptured appendix. Although emergency surgery was successful, a serious infection and complications set in, and the few antibiotics clinically useful at the time proved ineffective, leaving him facing imminent death.[1]

However, a new experimental antibiotic had recently arrived on campus for clinical use, a compound that had hardly been used in humans and was still being evaluated as a new chemotherapeutic agent. In this post–WWII era, antibiotics were considered novel therapeutics, with penicillin being heralded as a "wonder drug" having saved countless lives on the battlefields. It was also a period of chemical discovery, where the scientific methods of microbiology and organic chemistry were merging, and the promises of infectious disease chemotherapy became a major drive of medical research in academia and the chemical industry.

Since the antibiotics of the day failed Toby Hockett, his parents in desperation consented for him to be treated with the yellow-colored compound recently sent by the Lederle Laboratories Division of American Cyanamid, under the name Aureomycin™. It was a risk without choice; "I remember being put on the operating table, screaming and crying, and seeing the gas mask coming down on my face and being in that hospital for a very long time after that," he recalled. Within months he fully recovered and was one of the first of many people whose lives were saved by Aureomycin.[1]

Aureomycin had been discovered almost five years earlier in the early 1940s by Lederle, whose mission to generate new compounds and drugs, particularly antibiotics, had begun even earlier in the late 1930s.[2] Industrial chemical producers in this era were aware of the discovery and commercial value of penicillin, and it changed the course of their business. Normally they were resigned to producing consumer products, but now they began hiring scientists from many medical disciplines and started screening chemicals, biologics, immune-sera, and

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Figure 1. Five-year-old Tobey Hockett, one of the first patients treated with Aureomycin.

other promising and potential molecules against a host of diseases in a spirit of optimism, growth, and medical discovery that was unprecedented in the history of the emerging pharmaceutical industry.

In 1938, Cyanamid president William B. Bell unveiled to his executives a new mission statement, "You may come up with nothing, but you may discover a single drug that may conquer even one major disease, then the public will be well served and our company will prosper,"[2] thus formalizing the company's entry into the area of antibiotic discovery.

The discovery of the tetracyclines

In the early 1940s antibiotic discovery was progressing rapidly, best exemplified through the work and methods of microbiologist René Dubos[3] and the chemical diversity derived from the soil actinomycetes shown by Selman Waksman and colleagues.[4] It was evident that the microbial world produced a wealth of natural products and antibiotic compounds capable of fighting microbial diseases. But it was their medical and financial potential that drove the expansion of many pharmaceutical companies within the United States, with American Cyanamid as one of the first to commit to antibiotic research and development.

Cyanamid built new laboratories in Pearl River, NJ under the direction of general manager Wilbur Malcolm and their head of research, Yellapragada Subbarow. They then began a search for an antibiotic they felt should rival Waksman's streptomycin, enlisting as consultant 71-year-old Benjamin Minge Duggar (Fig. 2), a retired professor of plant physiology and economic botany from the University of Wisconsin, to head their soil screening department.

Duggar was world renowned for his extensive knowledge and study of soil fungi; he collected soil samples from all over the world sent to him by friends from sites he instructed would yield actinomycetes soil bacteria, or "ultra-molds" as he called them, those with ground coverings left undisturbed and natural. The samples were subjected to culture and broth dilution assays performed by his technicians, in which the microorganisms were plated, and the colonies assayed for antibiotic activity against a panel of Gram-positive and Gram-negative bacteria.[2] Although many soil organisms were known to produce antibiotics, most were toxic or had undesirable properties, and the team encountered many false leads.

One sample, however, drew their attention early on. It was marked A-377 and sent by William Albrecht, dug from Plot 23 on Sanborn field, a dormant timothy hayfield on the University of Missouri campus, outside Columbia, Missouri. It yielded an unusual yellow-colored colony that inhibited the growth of all their strains in an initial panel of bacteria, and produced remarkably large zones of growth inhibition in agar. This was an unheard-of property at this point, as compared to the few antibiotics available for comparison. They further found that even crude extracts of the colony retained remarkable antibacterial activity against lethal scrub typhus and the rickettsias, such as Rocky Mountain spotted fever, an infection for which there was no

Figure 2. (Left) Septuagenarian Benjamin Minge Duggar, who discovered the "ultra-mold" *Streptomyces aureofaciens*, a soil bacterium producing Aureomycin. (Right) Technicians screening soil samples in the Lederle laboratories.

cure.[2] Soon enthusiasm about its broad range of activity and potency against lethal pathogens led to the labeling of the unknown substance as a "broad spectrum" antibiotic, becoming one of the first in medical history to attain this title. Duggar named the compound *aureomycin* in reference to its yellow color and the gold-colored *Streptomyces* strain from which it was extracted. He continued the study of the ultra-mold and its medical microbiology, taxonomy, and physiology, naming it *Streptomyces aureofaciens and* first publishing his results in 1948 in the *Annals of the New York Academy of Sciences*.[5] This established Aureomycin as a new and potent broad-spectrum antibacterial agent that was safe and effective, although its exact chemical structure had yet to be determined.

The efforts at Cyanamid were expanded, bringing in R.D. McCormick to produce the compound using advanced fermentation methods. Soon the company was producing Aureomycin in commercial quantities. By December 1, 1948, the drug was approved by the FDA for clinical use and was an immediate success in the clinic, saving countless lives against a broad spectrum of infectious diseases, and generating notoriety and profits for the company. It appeared that the mission statement of Cyanamid by William Bell had come to fruition.[2]

Within a short time, other chemical companies were announcing their own discoveries of new bioprospected antibiotics, and by 1950, Alexander Finlay and colleagues at Charles Pfizer Co., Inc., Groton, CT, had gathered thousands of soil samples from around the world, and isolated the soil bacterium *Streptomyces rimosus*.[6] Their organism produced a compound with similarity in color to Aureomycin, but it was slightly more water soluble and had better bioactivity, giving it a medical and competitive edge over Aureomycin in the treatment of infectious diseases. The compound was named Terramycin in reference to *terra*, Latin for *earth*, and perhaps its origin, Terre Haute, Indiana. It was approved by the FDA in 1950, competing directly with Aureomycin while gaining success in the treatment of a broad spectrum of infectious diseases.[7]

The chemical structures of both Aureomycin and Terramycin, however, were difficult to solve and remained elusive for both companies, although they shared their respective compounds with each other in order to determine their common structural features and substructures and settle their disparate molecular identities. In this era of chemical characterization of natural products, instrumental analysis was limited to ultraviolet-visible spectroscopy (UV-Vis) and infrared spectroscopy, and structural proofs routinely relied on chemical modifications and degradation studies that few laboratories in the world were equipped to perform. Scientists at Pfizer, led by Karl Brunings (Fig. 3), and in collaboration with the legendary Harvard University chemist Robert Woodward, raced to prove the chemical structures of both compounds. By 1952 the preliminary chemical structures of both Aureomycin

Figure 3. Pfizer group members from the structure determination and tetracycline team (left to right): Frederick Pilgrim; Lloyd Conover, inventor of tetracycline; Karl Brunings, director of chemical research; Phil Gordon; and Charles Stephens, inventor of doxycycline.

and Terramycin (Fig. 4) were solved by the Pfizer-Woodward team, postulating that both compounds possessed a DCBA naphthacene core with similar functional groups with only minor differences in structure.[8] The core scaffold for this new family of antibiotics became descriptively known as the tetracyclines; however, Terramycin possessed an additional C5 position hydroxyl group and was devoid of a C7 chlorine atom, compared to Aureomycin.

The major structural features of the molecules were published by the Pfizer-Woodward group in a landmark paper in 1954 titled "The Structure of

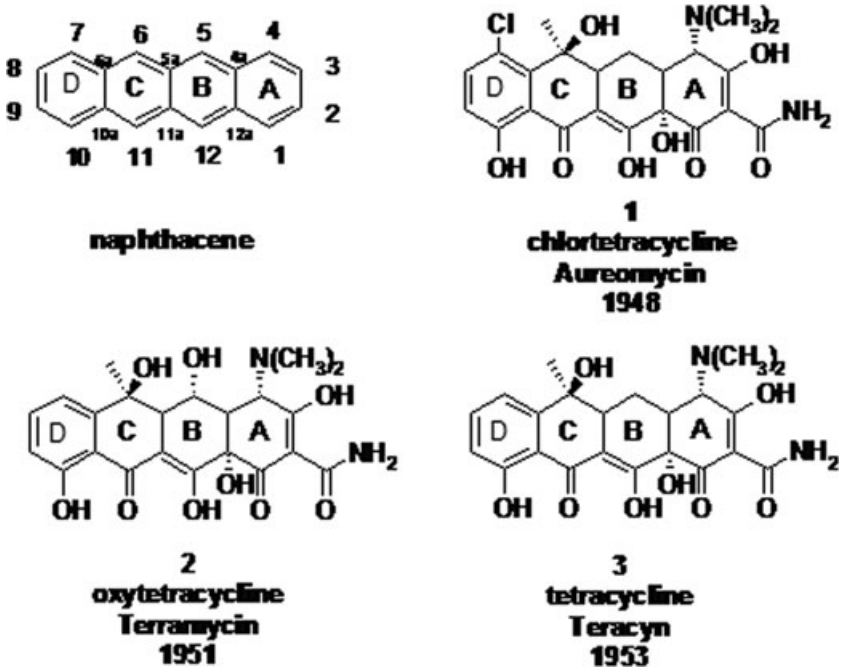

Figure 4. The naphthacene ring system and its structural locants of the first-generation antibiotics: chlortetracycline (1), oxytetracycline (2), and tetracycline (3), followed by the year approved by the FDA.

Aureomycin," thus setting the stage for the first semisynthetic derivative of this new family of antibiotics to emerge.[9]

In this scientific era, it was believed that chemical modifications of antibiotics decreased their activity. But Pfizer's Lloyd Conover took the opposite view, that the C7 chlorine in Aureomycin was not responsible for activity, and that it could be removed or modified to produce a more active analog with improved pharmacological and antibiotic characteristics. By catalytic hydrogenation of Aureomycin, using palladium metal and hydrogen, the C7 deschloro derivative was synthesized, producing a compound of higher potency, a better solubility profile, and favorable pharmacological activity; it was subsequently named *tetracycline*.[10] This compound was approved by the FDA for clinical use in 1954, as the first novel tetracycline by modification of a natural product, and it was one of the first commercially successful semisynthetic antibiotics used in medicine.

By the mid-1950s there were now three tetracyclines used clinically, their complex chemical structures had been solved, and their chemical names became in order of their discovery, chlortetracycline (Aureomycin), oxytetracycline (Terramycin), and tetracycline (Fig. 4). More importantly, these compounds saved tens of thousands of lives and generated much revenue for both companies yearly; recognition was given to the Pfizer-Woodward group for their structural elucidation and to Lloyd Conover for the invention of the most described molecule within its family, tetracycline.

Second-generation semisynthetic tetracyclines

Structural features within the tetracycline nucleus and the application of organic synthetic reactions separately by Lederle and Pfizer led to further modifications of their proprietary tetracycline scaffolds, with the goals of generating more potent and active tetracycline antibiotics. Pfizer chemists, led by Robert Blackwood, chose a disjunctive approach, modifying the C-ring of oxytetracycline to afford chemical stability, where halogenation and C6-dehydration yielded the antibiotic methacycline (Fig. 5).[11] While this new class had desirable pharmacological properties compared to its progenitor, it was never subjected to FDA approval within the United States. However, it was used as a starting material by Charlie Stephens (Fig. 3) to produce an analog with remarkable activity, stability, and pharmacological efficacy: doxycycline was approved for use by the FDA in 1967.[12] Doxycycline is still widely used today as an antibiotic with activity against a broad spectrum of community-acquired bacterial infections, and a diverse range of microbes, from the causative agent of anthrax infections, *Bacillus anthracis*, to malaria caused by the intracellular schizont of *Plasmodium falciparum*.[13] More recently doxycycline has been shown to inhibit the growth of bacteria in the genus *Wolbachia*,[14] symbionts that bear close taxonomic relationships with the α-proteobacteria, the rickettsias, the apicoplast of malarial schizonts,[13] and animal mitochondria.

Both companies also studied other antibiotic-producing soil bacteria within the Actinomycetales order and created biochemical mutants of their *Streptomyces* strains in an effort to induce higher yields of products as well as to discover other novel tetracyclines. One bioengineered strain by Lederle scientists produced a new tetracycline they named demeclocycline (Figs. 5 and 6)[15] a tetracycline that possessed unique C6 and C7 functional groups. While hardly bioactive, demeclocycline was chemically modified to yield an intermediate that retained activity while harboring the most minimal structure needed for antibacterial activity. This new intermediate became known as sancycline. Further conjunctive modifications of the aromatic D-ring by Robert Church produced novel C7 and C9 derivatives of sancycline.[16] One analog possessing a C7-dimethylamino group was found to exhibit far greater antibacterial and pharmacological activity against a larger range of bacteria compared to the first-generation compounds and doxycycline. The compound was named minocycline and was approved for clinical use in 1971. It became one of the most widely used of the tetracyclines to the present day, although it would be the last of new tetracyclines to enter the clinic for the next 35 years.

Tetracycline structure versus activity

Many of the chemical modifications of both the first- and second-generation tetracyclines produced variably active or inactive compounds, which led to a general description of the structure–activity relationships for tetracycline antibacterial activity. An active tetracycline with its minimum pharmacophore must possess a linearly arranged DCBA

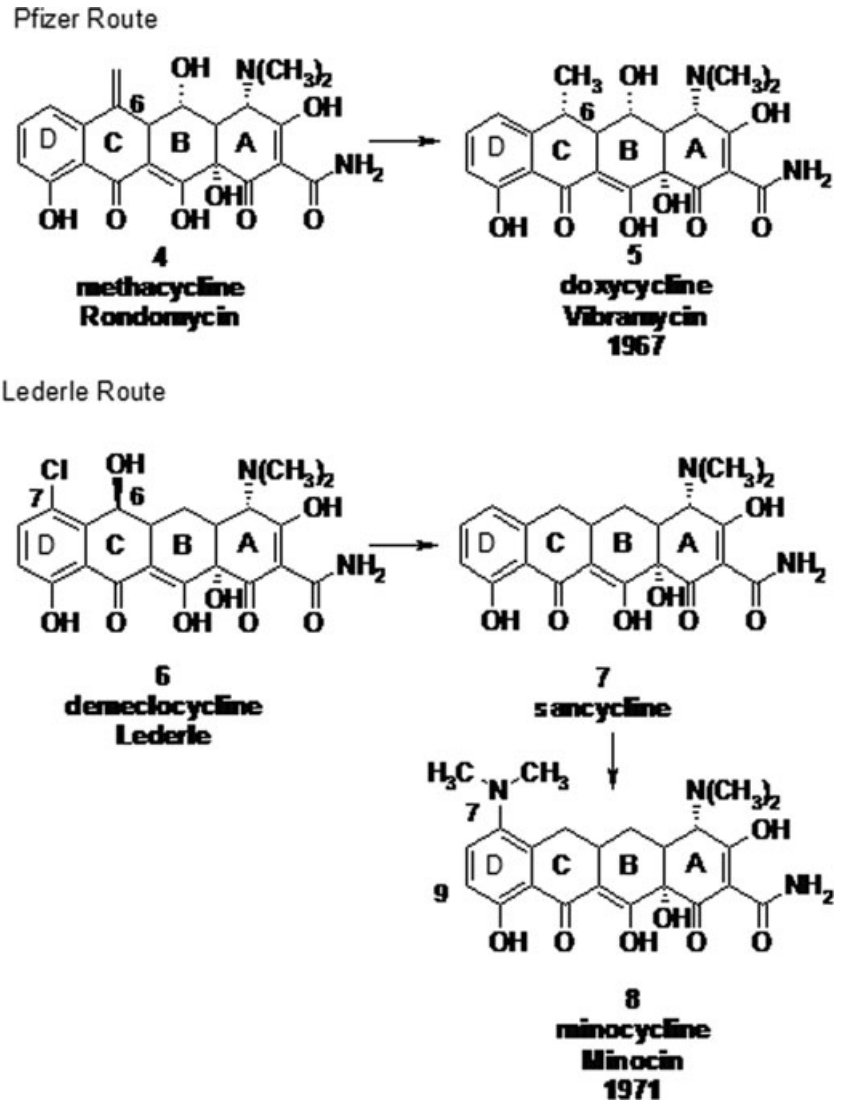

Figure 5. Semisynthesis routes for the second-generation tetracyclines chosen by the Chas. Pfizer Co. for doxycycline (5), and Lederle Laboratories for minocycline (8). The chemical name is followed by the year approved by the FDA.

naphthacene ring system with an A-ring C1-C3 diketo substructure and an exocyclic C2 carbonyl or amide group. It also requires a C10-phenol and a C11-C12 keto-enol substructure in conjunction with a 12a-OH group (Fig. 6) outlining a lower peripheral region, where chemical modification abolishes bioactivity. Furthermore, a C4-dimethylamino group with its natural 4S isomer is required for optimal antibacterial activity, while epimerization to its 4R isomer decreases Gram-negative activity, as first reported by Albert Doerschuk at Lederle.[17] By contrast, positions C5 to C9 can be chemically modified to affect their bioactivity as antibiotics and are designated the upper peripheral region, generating derivatives with varying antibacterial activity.

The clinical utility of the tetracyclines has demonstrated that they are active against a wide array of infectious disease agents, from Gram-positive and Gram-negative pathogens, to mycoplasmas, intracellular chlamydiae, rickettsias, and protozoan parasites. The many primary and secondary indications for the tetracyclines are represented in Table 1. Their

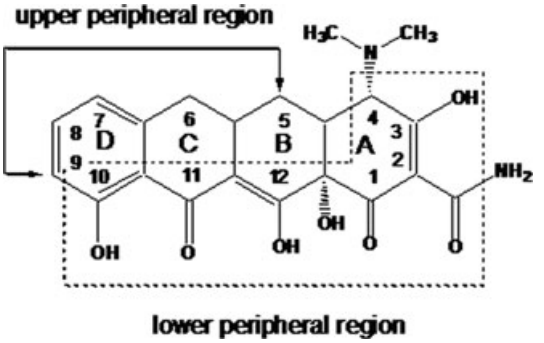

Figure 6. The designated upper and lower peripheral regions of the tetracycline molecule.

clinical efficacy and biology have been the subject of numerous comprehensive reviews,[18,19] books,[20,21] and chapters in medical microbiology and pharmacology texts,[22] readily detailing their usefulness in chemotherapy.

Table 1. Uses of the tetracyclines against bacterial and microbial infections[a]

Primary indications	Secondary indications[b]
Rickettsiae	Streptococcus species[c]
Mycoplasma pneumoniae	Amebiasis[d]
Psittacosis and Ornithosis	Severe acne[e]
Borrelia recurrentis and other species	*E. coli*[c]
	Enterobacter aerogenes[c]
Hemophilis ducreyi	*Shigella* species[c]
Yersinia pestis	*Klebsiella* species[c]
Bacteroides species	
Vibrio cholera and other species	
Neisseria species	
Treponema species	
Clostridium species	
Bacillus anthracis	
Chlamydia species	
Malaria prophylaxis	

[a]This current list is considerably affected by the high frequency of tetracycline resistance today, limiting the use of tetracyclines against common disease agents.
[b]When other antibiotics, such as penicillin, are contraindicated.
[c]When bacteriological testing indicates susceptibility.
[d]Adjunct to amebicides.
[e]Adjunct to standard therapy.

Tetracycline biology

The early biology of the tetracyclines pointed to a specific mechanism of action that was first described in 1953, when they were shown to block protein synthesis in *S. aureus* cells and inhibit cell growth in a bacteriostatic manner.[23] Later studies showed that they bound to a single site within the bacterial 70S ribosome,[24] with a dissociation constant determined to be between 1 and 30 $\times$ 10^{-6} M, although weaker binding sites were present.[25] Cell-free ribosome experiments concluded that the ribosome was indeed the primary site of action of the tetracyclines,[26] while later photoaffinity labeling experiments demonstrated photo-incorporation of [^{3}H]tetracycline into the 30S subunit,[27] labeling minor proteins S18 and S4 along with others. Cooperative binding dynamics between the ribosomal proteins and tetracycline were demonstrated, where experiments with 70S ribosomes revealed a higher affinity than with 30S or 50S particles alone.[28]

Not all tetracyclines acted similarly against bacterial ribosomes. Tested in a cell-free ribosome preparation,[29] a series of tetracyclines differed in ribosomal inhibitory activity; minocycline proved to be the most potent inhibitor of protein synthesis while tetracycline was the least.

Structural and mechanistic details of the drug–ribosome interaction soon followed, and many reports were conflicting, but it was soon demonstrated that the A-site region was involved[30] and that tRNA binding to this site was affected.[31] It was Gerardo Suarez, however, who first hypothesized the binding of tetracyclines and aminoacyl-tRNA to the 30S ribosome at therapeutic concentrations.[32] More detailed models of the binding of tetracyclines to RNA were subsequently described,[33] and molecular details of the drug–RNA interaction were proposed by Harry Noller, narrowing the site of binding to specific bases within the 16S ribosomal particle, the site of action on the ribosome that is accepted today.[34] More recent X-ray studies led by Venki Ramakrishnan, using ribosomes from *Thermus thermophilus,* confirm this model, where the primary binding site of tetracycline was determined at 2.3 Å resolution, and found to occur at the ribosome A-region, where the lower peripheral region of the molecule forms H-bonds and a metal bridge with ribosomal magnesium and key RNA nucleotide bases.[35]

Other mechanisms of tetracycline activity

It was also observed that some tetracyclines had diverse biochemical activities and that they could act as bactericidal agents, affecting cellular growth through membrane-mediated mechanisms. Ian Chopra showed that tetracyclines could diffuse across the outer cell membrane of Gram-negative bacteria, relying upon the electrochemical gradient of energized cells to partition into the cellular cytoplasm, with magnesium and other divalent metals playing a key role in antibiotic uptake into the cell and antibacterial activity.[36] Most tetracyclines acted as bacteriostatic or "typical" agents, as protein synthesis inhibitors against bacteria. But it was found that more lipophilic tetracyclines were "atypical," with a bactericidal mechanism that relied on membrane perturbation, affecting multiple pathways in cells, leading to cellular dysfunction.[37]

Now biology was showing the tetracyclines as a family to be chemically and biologically dynamic, with multiple mechanisms of activity and capable of interacting with multiple targets, either ribosomes or cellular membranes. Tetracycline structural dynamics were earlier evident and described in X-ray crystallography studies,[38] where it was found that they adapted and changed conformations through H-migrations or tautomerism that was environment dependent. In aqueous solution the tetracyclines could adopt multiple conformations, where both hydrophilic and zwitterionic forms predominated, while in lipid environs an uncharged and chemically neutral form prevailed. Modern computational analysis of the ionizing behavior and conformations showed that a single tetracycline may transpose into 64 different tautomeric substructures, with ten or more of them within $\leq$10 kcal separating their conformational changes in an aqueous environment.[39] Subsequent UV-Vis spectroscopic studies using Fourier techniques further demonstrated that the metal binding and proton ionizations of the tetracyclines were equally complex, where their conformations become dependent on pH and metal ion concentrations in a "chameleon-like" manner, with tetracycline adapting to its surrounding environment.[40]

Tetracycline resistance

Within one year after the discovery of Aureomycin, the first evidence of bacterial resistance to the drug was reported.[41] Other tetracycline-resistant clinical isolates appeared, especially intestinal organisms like *Shigella dysenteriae*. Also noted was the coexistence of tetracycline resistance and resistance to structurally different antibiotics, creating "multidrug resistant bacteria."[42] In the 1970s, many laboratories were beginning to understand the molecular biology and mechanisms of antibiotic resistance, where transferable extrachromosomal R factors were shown to alter bacterial physiology when mediating resistance phenotypes. One of the authors, Stuart Levy, with Laura McMurry at Tufts University School of Medicine, studied the cellular properties of a tetracycline-resistant *E. coli* bearing R-factor R222.[43] They found that cells bearing the plasmid showed a decreased uptake of tetracycline. This finding suggested that the R-factor specified a transport system for removing the drug from within the cell, thus rendering the host bacterium resistant. The efflux system they discovered was mediated by different resistance determinants and acted through different membrane-associated proteins called Tet proteins,[43] which they discovered and described earlier.[44] The protein and its activity represented the first demonstration of efflux as a mechanism of drug resistance and the first mechanism of resistance to the tetracyclines. Today antibacterial efflux as a mechanism of drug resistance is commonly found in all bacteria. It may be specific for a single drug, such as the tetracycline efflux system, or for multiple drugs through a multiprotein efflux mechanisms.[45]

A second type of tetracycline resistance was subsequently reported, a protein-based ribosomal protection mechanism, first found in streptococci[46] and later in anaerobic bacteria.[47] When tetracyclines bind to ribosomes they normally stop elongation of synthesizing proteins. However, ribosomal protection proteins, such as Tet(M) or Tet(O), interact with the ribosome causing the tetracycline to dislodge from the ribosome, thus protecting the bacterial cell from tetracycline's inhibitory activity, resulting in cellular growth.[48] Given the prevalence of resistance determinants in clinically studied pathogenic strains, tetracyclines useful in the future must have activity against strains harboring both efflux and ribosomal protection mechanisms.[49]

More recently, a third resistance mechanism has been described. Chemical inactivation of tetracycline was shown to be due to an oxygen-dependent flavin-monooxygenase enzyme encoded by the

previously described *tet*(X) gene.[50] Curiously, the oxygen-dependant determinant was first described in an anaerobe *Bacteroides*, and so its resistance activity was not noted in that bacterium, but only when the plasmid with the *tetX* gene was transferred to *E. coli*. Still, the work indicates that chemical inactivation of the tetracyclines is biologically possible in some species of microbes, although this degradative mechanism has not been reported to be of clinical relevance.

The study and classification of tetracycline resistance mechanisms and their genetic bases, expression, and kinetics has resulted in the adoption of a systematic nomenclature[51] resulting from worldwide compilations of tetracycline-based resistance mechanisms that continue to be recognized. There are currently 46 different tetracycline resistance determinants.

The Tet repressor

Major insights into the molecular and structural biology of tetracycline resistance began in the 1980s and were realized through the work of Wolfgang Hillen and colleagues in Germany. They initially described the interaction of the tetracycline repressor protein Tet(R), with its DNA binding domains, and detailed the mechanisms of Tet protein expression at the biochemical level.[52] Hillen also showed that transposons encoding genetic control elements could control the efflux of tetracycline by sensing the drug via a repressor complex, resulting in DNA transcription and efflux protein expression. The structural attributes of the tetracycline-repressor-DNA binding interaction were further elaborated by X-ray crystallography to give a detailed look at the trans-activation process and the ensuing protein dynamics related to tetracycline resistance protein expression.[53]

While the biomechanics of the tetracycline resistance genetic machinery were being elaborated, Hiroshi Nikaido[54] and others, including the Levy laboratory, revealed further how antibiotic transport complexes functioned at the biochemical and structural level. Soon other families of efflux proteins across many bacterial species were described.[55] Currently, the study of antibiotic efflux proteins encompassing their genetics, mechanisms, and their inhibition has culminated with the description of the multidrug efflux systems of the resistance-nodule division (RND) family of export proteins found in clinically relevant *Pseudomonas aeruginosa* strains.[56] Bacteria possessing such efflux proteins are currently some of the most antibiotic-resistant organisms encountered clinically.

Tetracyclines in ancient history

While the fields of antibiotic and tetracycline resistance expanded rapidly during the 1980s, this time period also brought another surprising finding concerning the tetracyclines, one that appears to rewrite their history, changing the date of their discovery by almost 2000 years. Bioarchaeologist George Armelagos and his colleagues, then at the University of Amherst, Massachusetts, found tetracycline-like fluorescent bands in the bone of skeletal remains of a tribe from ancient Sudan dated from the Late Antiquity period, 350 A.D., from an area that was once known as Nubia.[57] Over 70 individuals from all age groups were studied, and they showed consistent and significant osteon bone labeling by tetracycline that only occurs with chronic and repeated tetracycline exposure.[58] These findings were derided by their peers, attributing the bone labeling to surface contamination by tetracycline-producing molds, but the team hypothesized that the Nubians routinely fermented grains as part of their diet-producing beer gruels, and that *Streptomyces* could have accidentally contaminated their vats, and once discovered by the Nubians, could have been purposefully propagated for their use.[59] More recently, the chemical identity of the bone fluorescent bands was first described by one of the authors, Mark Nelson, where the bone fluorophore was extracted and characterized by HPLC-mass spectrometry, and found to contain significant amounts of the antibiotic tetracycline.[60] These findings show that tetracyclines were produced by the ancient Nubians and could have been one of the first antibiotics produced via fermentation, predating penicillin by almost 2000 years. It was also evident that the fermentation of tetracyclines was not an isolated event, inasmuch as fluorophore-labeled ancient bone has been found in other local geographical areas and in other distant regions.[61]

Third-generation tetracyclines

In the late 1980s antibiotic resistance was becoming more prevalent in the clinic, prompting pharmaceutical companies, including Wyeth, formerly known as American Cyanamid, to re-enter or

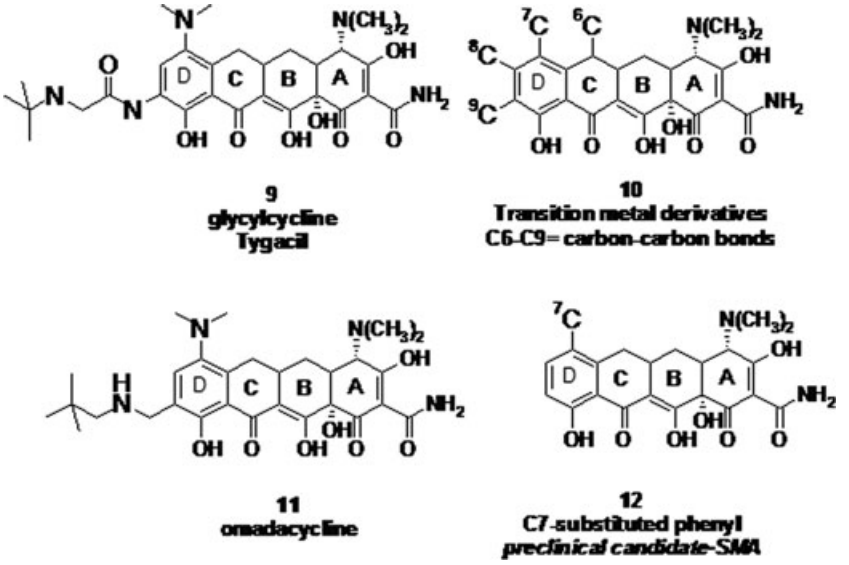

Figure 7. Third-generation tetracyclines.

expand their antibiotic discovery programs. Under the direction of chief scientist Frank Tally, with chemist Phaik-Eng Sum, and conducted in the same laboratories used by Duggar when he discovered Aureomycin 40 years earlier, Wyeth launched a program to chemically modify the minocycline scaffold, producing C9-amino derivatives possessing an amide functionality with a glycine subunit attached, generating hundreds of new analogs for assay and evaluation, and naming the series the glycylcyclines (see Fig. 7).[62] Many of the compounds were active against Gram-positive and Gram-negative bacteria, notably those with ribosomal protection or tetracycline efflux mechanisms. Soon their lead compound tigecycline[63] entered clinical studies and was approved by the FDA for hospital use in 2006. This was the first new third-generation tetracycline to enter the market in over 35 years and one specifically designed to confront tetracycline-resistant mechanisms.

Tigecycline today has been found to be clinically active within acceptable MIC ranges against Gram-positive isolates obtained globally, including resistant organisms, such as MRSA (methicillin-resistant *S. aureus*), vancomycin-resistant enterococci, and penicillin-resistant *S. pneumoniae*.[64] Against Gram-negative pathogens, particularly *Enterobacteriaceae* and *Acinetobacter*, tigecycline also shows clinical utility, inhibiting growth in 98% and 94% of overall species in an MIC_{90} determination.[65] The clinical uses of tigecycline may also expand into community-acquired bacterial pneumonia, as it is found to be potent both *in vitro* and *in vivo* in patients,[66] while also harboring potent activity against *C. difficile* anaerobes, both *in vitro* and *in vivo*.[67] In addition to its growth-inhibitory activity against *Clostridium* species, tigecycline and the tetracyclines in general also inhibit the production of bacterial exotoxin A, an important feature for the tetracycline family of antibiotics.

Our laboratories, in the late 1980s at Tufts University School of Medicine, began to screen tetracycline derivatives as inhibitors of the tetracycline efflux proteins, with the goal of blocking the efflux mechanism of resistance, allowing a clinically used tetracycline to enter the cell and inhibit protein synthesis and growth. While these studies detailed the structure–activity relationships for inhibiting efflux proteins and their mechanism,[68,69] we became focused on synthesizing more potent third-generation tetracycline derivatives by themselves; in 1996, Paratek Pharmaceuticals, Inc. of Boston was launched. We began applying transition metal–based chemistry to the tetracyclines and generated many new classes of compounds that possessed antibiotic activity (Fig. 7, structure 10),[70] testing thousands of compounds against tetracycline-susceptible and -resistant

bacteria in animal models of infectious disease. In another semisynthetic series, we generated a 9-aminomethyl aromatic D-ring group intermediate of minocycline that was subsequently modified to yield 9-alkylaminomethyl minocycline analogues with improved activity against a broad spectrum of tetracycline-susceptible and -resistant bacteria.[71] One compound was chosen for its superior activity, lack of toxicity, and oral bioavailability and now is named omadacycline. This compound is a new third-generation tetracycline that has completed Phase II clinical trials against acute bacterial skin and skin structure infections (ABSSSI) and is useful in IV to oral step-down therapy.[72] Omadacycline is currently in the beginning of Phase III clinical studies against ABSSSIs.

Additionally, the Paratek tetracycline semisynthesis collection has generated numerous preclinical nonantibacterial compounds potentially useful in mammalian disease states. For example, a 7-phenyl derivative (Fig. 7, structure 12), has been shown to correct genetic splicing defects in cellular and animal models of spinal muscular atrophy.[73]

More recent developments have involved the total synthesis of tetracycline derivatives. Harvard University chemist Andrew Myers and his colleagues reported a facile and versatile route to produce numerous novel tetracyclines at positions that are not semisynthetically feasible.[74] Their pathway chose a conjunctive approach, coupling a BA ring fragment with a DC ring fragment, and generating the tetracycline DCBA ring system and novel compounds of varying antibacterial activity. In the past and over several decades, the total synthesis efforts of Conover and Woodward,[75] then Drueckheimer,[76] among others, were successful in generating several compounds of which only one entered clinical trials, and it was soon abandoned presumably because of toxicity issues.[77] Currently, this pathway of tetracycline synthesis has been used by Tetraphase Pharmaceuticals of Watertown, Massachusetts, and has resulted in the generation of a C7-fluorotetracycline derivative coded TP-434, which is currently entering Phase II clinical trials against pathogens and indications related to intra-abdominal infections.[78]

Tetracyclines and genetic engineering

Studies of the tetracycline repressor protein and its genetics and properties have resulted in a genetic and biochemical tool that today is regarded as the most often used genetic switching system, both *in vitro* and *in vivo*, for the expression of target proteins. It also affects the turning on and off of cellular phenotypes in both prokaryotic and eukaryotic cells. The Tet-On-Tet-Off genetic construct was reported in 1996 by Hermann Bujard and Manfred Gossen,[79] and operates using doxycycline and other tetracyclines as an effector to express desired genetic constructs under responsive conditional operators. Today, there are hundreds of reports in the literature across many different biology and medical disciplines with numerous reports and reviews on its usefulness in genetic engineering and controlling gene expression.[80–83] The doxycycline–Tet repressor interaction is used to affect a genetic construct controlling its expression, in the case of the Tet-On system, while the Tet-Off system works in opposite fashion, turning off gene expression upon removal of doxycycline. Gene products, cellular phenotypes, cellular pathways, and organismal physiology can be studied on the molecular, biochemical, physiological, and even neurobehavioral level, using doxycycline or other tetracycline analogs of higher repressor affinity.[84,85]

The nonantibiotic uses of the tetracyclines

In 1983 another scientific front evolved from the tetracycline family that underscores their inherent potential as mammalian therapeutics. This time it was outside of antibacterial chemotherapy and in the field of inflammation. Observations of gingival tissue treated by tetracyclines by Lorne Golub and Nungaravam Ramamurthy showed that minocycline possessed inherent and extremely potent anti-inflammatory activity that was independent of its antibacterial action, inhibiting collagenase activity in germ-free rats.[86] They hypothesized that other inflammatory diseases could similarly be affected, and Robert Greenwald soon expanded their observations to osteoarthritis and rheumatoid arthritis in animal models and found similar results.[87] It was further determined that the tetracyclines also affected matrix-metalloprotease (MMP) enzyme activity in cells, also implicated in inflammation-based diseases, and that they acted upon specific MMP isozymes expressed during the course and progression of different disease states.[88] One compound, doxycycline, was found to be a potent inhibitor of degradative MMP enzymes both *in vitro* and *in vivo*, and was approved by the FDA in 2001 as a low-dose

Table 2. Nonantibiotic uses of the tetracyclines in inflammation-based disease states

Therapeutic properties[90]	Angiogenesis effects[100]
Anti-inflammation review[91]	Multiple sclerosis–clinical effects[101]
Aneurysm effects[92]	Rheumatoid arthritis effects[102]
Hypoxia-ischemia[93]	Pain-nocioception effects[103]
Neuroprotective effects[94]	Autism disorders–Fragile X[104]
Antidiabetes properties[95]	
Microglial effects[96]	
Cytokine effects[97]	
Multiple sclerosis effects[98]	
Artheriosclerosis effects[99]	

formulation at 20 mg/day for the treatment of adult periodontitis.[89]

Since the nonantibiotic tetracyclines were described almost 30 years ago, there has been an explosive increase in the numbers of reports on the anti-inflammatory, antineurodegenerative, and mammalian activity of the tetracyclines. A chosen list of reviews and targets of tetracycline activity is provided in Table 2. Comparisons of the literature of the antibiotic versus nonantibiotic tetracyclines clearly indicate that in the future the uses of the tetracyclines will continue to grow in the areas of inflammation, neuroinflammation, and neurodegeneration and their use in chemotherapeutic intervention.

The future of the tetracyclines

The medicinal chemistry and synthesis of newer analogues have recently undergone a renaissance, whereby new semisynthetic derivatives as well as totally synthetic compounds are now being studied and used against resistant bacteria. Newer and more potent antibacterial compounds can be expected, and the tetracyclines will continue to be useful in the treatment of infectious diseases, as tetracycline-resistant pathogens continue to evolve and the numbers of useful antibiotics in the clinic decrease. The tetracyclines will also continue to be useful against other microbial diseases, made more amenable to treatment with specific and targeted tetracycline molecules, and in an era where new discoveries are needed. New third-generation derivatives have been designed to be more potent, especially against bacteria possessing ribosomal protection and efflux mechanisms, and the increase in potency of the tetracyclines over time is paralleled in other structural classes of antibiotics. In the future, more potent derivatives will be designed with activity against resistant strains as resistance mechanisms evolve and emerge in the clinic.

Now, scientific advances have revealed the biological activity of the nonantibiotic tetracyclines, which in time will also become more common in the clinic, as their targets, disease phenotypes, and clinical candidates are studied against inflammation, neurodegeneration, and in genetic diseases that will be treatable by tetracycline analogs. These new uses of an established family of therapeutics, and the ability to remove antibiotic activity chemically while maintaining cellular and clinical efficacy also suggest that someday anti-inflammatory and nonantibiotic tetracyclines will have increased utility in treating inflammation-based disease states.

Connections and conclusion

There are many scientific disciplines that have benefited from the discovery of the tetracyclines, and advances in soil science, biochemistry, and X-ray crystallography have been made during their discovery. These advances highlight the connections and scientific achievements that occurred throughout the history of the tetracyclines. For example, the hydrogenation reaction pioneered by Conover, while generating the first semisynthetic tetracycline, eventually led to radiolabeled tetracyclines, which then were used in studies of the ribosome and the discovery of the tetracycline efflux system, resulting in a scientific front in antibiotic resistance that continues to the present day.

In another instance, the studies of the process of infection in mammalian diseases led by observation to the discovery of the tetracycline anti-inflammatory activity by Golub, and the ensuing explosion in research dedicated to understanding the scope and mechanisms of their action in human disease states. All of these events were interconnected by a single experiment performed over a half-century ago—a hydrogenation reaction.

One last example of a circular connection that is inextricably linked by tetracycline research is also evident. Antibiotic resistance and the discovery of their genetic mechanisms ushered in the discovery of the Tet repressor and its function by Hillen, which eventually evolved into the Tet-On-Off

Figure 8. The gravesite of Benjamin Minge Duggar in Oak Hill Cemetery, Nyack, New York.

genetic switch pioneered by Bujard and Gossen. Now these methods are used in laboratories worldwide, helping in the discovery of novel therapeutics, the elaboration of disease pathways, new drug targets, and their therapeutic intervention, including in the area of antibiotic discovery and antibiotic resistance.

This review aims to highlight the human efforts and the spirit of discovery that have driven past tetracycline discovery and research. Unfortunately, not all the events, people, details, or even molecules could be included, but all efforts are appreciated. Research is documented in the literature for future generations of scientists and clinicians to explore and discover for the future advancement of science and medicine.

At Oak Hill Cemetery in Nyack, New York sits a simple granite gravestone inscribed "Benjamin Minge Duggar, 1872–1956 Scientist, Teacher, Humanitarian" with its lunette engraving composed of two fruiting mushrooms, instead of the usual gravestone symbols (Fig. 8). For a scientist who discovered from soil one of the most important medical therapeutics of the past century, his gravestone memorial to the soil and earth is a lasting and fitting tribute to a great scientific pioneer from the antibiotic era that ushered in a new beginning in the fight against diseases that plague humankind.

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Conflicts of interest

Some of the research described was performed by employer Paratek Pharmaceuticals, Inc.

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