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EDITORIAL

Reaching for new heights: *The Journal of Antibiotics* and NPG

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Sixty years since its first appearance, *The Journal of Antibiotics* (JA) is now taking proactive and innovative new steps to help place it firmly among the world's leading primary source journals. It is with great pleasure, and in expectation of unparalleled future growth and development, that I can confirm that JA will henceforth be published in collaboration with the renowned Nature Publishing Group (NPG). JA will thus be able to take its rightful place beside some of the world's premier scientific journals and publications. In a world witnessing unprecedented rates of scientific development and innovation, this new partnership with NPG will allow JA to fully extend both its reach and its influence on the global stage and in all aspects of antibiotics.

Looking back, the origins of the journal lay in the Japan Penicillin Association Corporation (JPAC), established in 1946, which began publishing the *Journal of Penicillin* a year later. In recognition of its growing importance and scope, the journal was renamed as *The Journal of Antibiotics* in 1948. Following suit, JPAC also changed its name to the Japan Antibiotic Research Association (JARA) in 1951, with JARA continuing to be responsible for the publication of the journal ever since.

In 1968, the Editorial Board of JA decided that the journal should expand its coverage and international stature, creating an English journal and recruiting new Board members from public and private institutions around the globe in the process. Close and sustained ties with industry were also established. The concept was to ensure that the results of the excellent research being conducted in Japan were conveyed to a far wider audience. In addition, English and science communication skills among Japanese researchers could be improved, especially with the active support and encouragement of international Editorial Board members. This has been a somewhat unique operating scenario, as is the fact that there has been a steady supply of funds donated by pharmaceutical company supporters to help meet the running costs of the journal.

JA has continuously been at the forefront of various areas of antibiotics research since its inception. Several original papers have been published on key drugs, such as pravastatin, tacrolimus (FK-506), sirolimus (rapamycin) and bleomycin. In addition, numerous papers have appeared with first reports of a plethora of important organic compounds of microbial origin that have entered into practical or commercial use in animal and/or human health, agriculture or as biochemical reagents (for example, pepsatin and staurosporine). Until recently, much of the material published was from the results of Japanese research work. It is now envisaged that, following the link with NPG, arguably the world's leader in science publishing, JA will adopt a truly global mantle, with an ever-increasing number of international contributors and readers. We specifically intend to place JA firmly in the vanguard of research publications focusing on antibiotics.

No journal can establish itself and thrive without the unswerving commitment and drive of talented and dedicated individuals. JA was fortunate in having such a man in the long-serving JARA Managing Director, the late Dr Yukimasa Yagaisawa, who expertly assumed and carried out the post and duties of Managing Editor of the journal. He, along with Dr Morimasa Yagisawa, was responsible for setting up and maintaining the journal's highly effective publication and administrative systems. The current Editorial Board members, journal staff and I will strive to build on their solid foundation and propel the journal to new levels of excellence and impact.

I would like to take this opportunity to express my immense gratitude to all those associated with JA, including researchers, readers, authors and all those involved in the various aspects of production and operation, for their support and encouragement through the years. In particular, I would like to reaffirm my sincere thanks to the many Friends of the Journal who have provided financial resources and support in recognition of the import and significance of the publication, and I hope that we can count on continued and increasing support in the foreseeable future. I humbly invite all to join me and my colleagues—at JA and at NPG—in an exciting journey to elevate the journal to greater heights in a new era of innovative research and development that will hopefully provide us all with new knowledge, better understanding, and new and novel antibiotics that are so desperately needed.

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EDITORIAL

The *Journal of Antibiotics* partners with Nature Publishing Group

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It is our great pleasure and honor to announce to our readers our new publishing partnership with Nature Publishing Group (NPG), a renowned international science publisher, to publish *The Journal of Antibiotics* (*JA*) from January 2009.

The new partnership between NPG and the Japan Antibiotics Research Association (JARA) to publish *JA* is an exciting development and will greatly enhance the visibility and impact of *JA*—and help to establish the journal as one of the leading bioactive and microbial natural products journals.

Under the leadership of Editor-in-Chief Dr Satoshi Ōmura, we foresee a close and fruitful collaboration among the vital elements of a successful journal. These elements include the Association and publishers, Editorial Board members, reviewers, and of course our authors. The outcome of this collaboration will ensure the high quality of *JA*, increase the international standing of the journal, and will, I believe, fulfill some of the aims of JARA: to serve the research community by publishing a journal with the highest quality and with high impact in the field of bioactive and microbial natural products research.

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REVIEW ARTICLE

Microbial drug discovery: 80 years of progress

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Microbes have made a phenomenal contribution to the health and well-being of people throughout the world. In addition to producing many primary metabolites, such as amino acids, vitamins and nucleotides, they are capable of making secondary metabolites, which constitute half of the pharmaceuticals on the market today and provide agriculture with many essential products. This review centers on these beneficial secondary metabolites, the discovery of which goes back 80 years to the time when penicillin was discovered by Alexander Fleming.
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INTRODUCTION

Back in 1928, Alexander Fleming[1] began the microbial drug era when he discovered in a Petri dish seeded with *Staphylococcus aureus* that a compound produced by a mold killed the bacteria. The mold, identified as *Penicillium notatum*, produced an active agent that was named penicillin. Later, penicillin was isolated as a yellow powder and used as a potent antibacterial compound during World War II. By using Fleming's method, other naturally occurring substances, such as chloramphenicol and streptomycin, were isolated. Naturally occurring antibiotics are produced by fermentation, an old technique that can be traced back almost 8000 years, initially for beverages and food production. Beer is one of the world's oldest beverages, produced from barley by fermentation, possibly dating back to the sixth millennium BC and recorded in the written history of ancient Egypt and Mesopotamia. Another old fermentation, used to initiate the koji process, was that of rice by *Aspergillus oryzae*. During the past 4000 years, *Penicillium roqueforti* has been utilized for cheese production, and for the past 3000 years soy sauce in Asia and bread in Egypt has represented examples of traditional fermentations.[2]

Natural products with industrial applications can be produced from primary or secondary metabolism of living organisms (plants, animals or microorganisms). Owing to technical improvements in screening programs, and separation and isolation techniques, the number of natural compounds discovered exceeds 1 million.[3] Among them, 50–60% are produced by plants (alkaloids, flavonoids, terpenoids, steroids, carbohydrates, etc.) and 5% have a microbial origin. Of all the reported natural products, approximately 20–25% show biological activity, and of these approximately 10% have been obtained from microbes. Furthermore, from the 22 500 biologically active compounds that have been obtained so far from microbes, 45% are produced by actinomycetes, 38% by fungi and 17% by unicellular bacteria.[3] The increasing role of microorganisms in the production of antibiotics and other drugs for treatment of serious diseases has been dramatic. However, the development of resistance in microbes and tumor cells has become a major problem and requires much research effort to combat it.

CHEMICALLY SYNTHESIZED DRUGS ORIGINATING FROM NATURAL PRODUCTS

Drugs of natural origin have been classified as (i) original natural products, (ii) products derived or chemically synthesized from natural products or (iii) synthetic products based on natural product structures. Evidence of the importance of natural products in the discovery of leads for the development of drugs for the treatment of human diseases is provided by the fact that close to half of the best selling pharmaceuticals in 1991 were either natural products or their derivatives.[4] In this regard, of the 25 top-selling drugs reported in 1997, 42% were natural products or their derivatives and of these, 67% were antibiotics. Today, the structures of around 140 000 secondary metabolites have been elucidated.

It is important to understand that many chemically synthesized drugs owe their origin to natural sources. Applications of chemically synthesized natural metabolites include the use of a natural product derived from plant salicyclic acid derivatives present in white willow, wintergreen and meadowsweet to relieve pain and suffering. Concoctions of these plants were administered by Hippocrates back in the year 500 BC, and even earlier in Egypt and Babylonia, for fever, pain and childbirth. Synthetic salicylates were produced initially by Bayer in 1874, and later in 1897, Arthur Eichengrun at Bayer discovered that an acetyl derivative (aspirin), reduced acidity, bad taste and stomach irritation. These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization (WHO) that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care.[5]

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Table 1 Anti-infective market in 2000[10]

Compounds	*Market (US$ billions)*
Cephalosporins	9.9
Penicillins	8.2
Other β-lactams	1.5
Antivirals excluding vaccines	10.2
Quinolones	6.4
Antifungals and antiparasitics	4.2
Aminoglycosides	1.8
Tetracyclines	1.4
Other antibacterials	6.1
Other anti-infectives	5.3
Total	55.0

Other synthesized compounds originating from natural products include a nonapeptide, designated teprotide, which was isolated from the venom of the Brazilian pit viper *Bothrops jararaca*.[6] This led to the design and synthesis of angiotensin-converting enzyme (ACE) inhibitors such as captopril, which was the first marketed, orally active ACE inhibitor.[7] Enalapril, another ACE inhibitor used in the treatment of cardiovascular disease, was approved for marketing by the Food and Drug Administration (FDA) in 1985.[6]

The alkaloid quinine, the active constituent of the 'fever tree' *Cinchona succirubra*, has been known for centuries by South American Indians to control malaria. During the twentieth century, massive programs to synthesize quinoline derivatives, based on the quinine prototype, were carried out. The first of the new quinolones to be used clinically as an antibacterial agent was nalidixic acid, which emerged as part of a large chemical synthesis program developed at the Sterling Winthrop Research Institute.[8,9] The program was begun when 7-chloro-1,4-dihydro-1-ethyl-4-oxoquinolone-3-carboxylic acid was obtained as a side product during purification of chloroquine and found to have antibacterial activity. The best compound found in the program was nalidixic acid, which had remarkable activity against Gram-negative bacteria and was shown to be an inhibitor of DNA gyrase. Its discovery led to a whole series of synthetic quinolone and fluoroquinolone antibiotics (pefloxacin, norfloxacin, ciprofloxacin, levofloxacin, ofloxacin, lomefloxacin, sparfloxacin, etc.), which have been very successful in medicine and have achieved major commercial success (Table 1). It is important to appreciate that all quinolones, though synthetic, are based on the structure of the natural plant product quinine.

Secondary metabolites have exerted a major impact on the control of infectious diseases and other medical conditions, and the development of pharmaceutical industry. Their use has contributed to an increase in the average life expectancy in the USA, which increased from 47 years in 1900 to 74 years (in men) and 80 years (in women) in 2000.[11] Probably, the most important use of secondary metabolites has been as anti-infective drugs. In 2000, the market for such anti-infectives was US$55 billion (Table 1) and in 2007 it was US$66 billion.

Table 1 shows that, among the anti-infective drugs, antivirals represent more than 20% of the market. Two antivirals that are chemically synthesized today were originally isolated from marine organisms. They are acyclovir (active against the herpes virus by inhibition and inactivation of DNA polymerase) and cytarabine (active against non-Hodgkin's lymphoma). Both compounds are nucleoside analog drugs, originally isolated from sponges.[12] Other antiviral applications of natural compounds are related to human immunodeficiency virus (HIV) treatment. In the pathogenesis of this disease, HIV-1, similar to other retroviruses, depends on its stable integration into the host genome to facilitate efficient replication of the viral RNA and maintenance of the infected state. Therefore, *de novo* viral DNA synthesized during reverse transcription is immediately integrated into the host cell DNA (through the integration step), allowing for further transcription of viral RNA. In the late phase of HIV viral replication, the large precursor polyprotein (gag-pol precursor, Pr 160) must be appropriately cleaved by a viral protease. The cleavage of the gag precursor protein of HIV is critical for the maturation and infectivity of the viral particle. Without the appropriate cleavage of the precursor polyproteins, non-infectious viral particles are generally produced. To confront this problem, a tremendous effort has been made at the US National Cancer Institute (NCI), in search of natural metabolites capable of inhibiting HIV reverse transcriptase and HIV protease. Chemically synthesized derivatives of these compounds are the main agents now used against HIV. Furthermore, reports have been published on natural product inhibitors of HIV integrase obtained from among the marine ascidian alkaloids; that is, the lamellarins (produced by the mollusk *Lamellaria* sp.), and from terrestrial plants (*Baccharis genistelloides* and *Achyrocline satureioides*). The most consistent anti-HIV activity was observed with extracts prepared from several *Baccharis* species.[13] In addition, NCI has been evaluating the HIV-1 inhibitory activity of pepstatin A, a small pentapeptide produced by several *Streptomyces* species. It contains a unique hydroxyamino acid, statine, that sterically blocks the active site of HIV-1 protease.[14,15]

REASONS FOR DEVELOPING NEW ANTIBIOTICS

New antibiotics that are active against resistant bacteria are required. Bacteria have lived on the Earth for several billion years. During this time, they encountered in nature a wide range of naturally occurring antibiotics. To survive, bacteria developed antibiotic resistance mechanisms. Therefore, it is not surprising that they have become resistant to most of the natural antimicrobial agents that have been developed over the past 50 years.[16] This resistance increasingly limits the effectiveness of current antimicrobial drugs. The problem is not just antibiotic resistance but also multidrug resistance. In 2004, more than 70% of pathogenic bacteria were estimated to be resistant to at least one of the currently available antibiotics.[17] The so-called 'superbugs' (organisms that are resistant to most of the clinically used antibiotics) are emerging at a rapid rate. *S. aureus*, which is resistant to methicillin, is responsible for many cases of infections each year. The incidence of multidrug-resistant pathogenic bacteria is increasing. The Infectious Disease Society of America (IDSA) reported in 2004 that in US hospitals alone, around 2 million people acquire bacterial infections each year (http://www.idsociety.org/Content.aspx?id=4682). *S. aureus* is responsible for half of the hospital-associated infections and takes the lives of approximately 100 000 patients each year in the USA alone.[18] The bacteria produce a biofilm in which they are encased and protected from the environment. Biofilms can grow on wounds, scar tissues and medical implants or devices, such as joint prostheses, spinal instrumentations, catheters, vascular prosthetic grafts and heart valves. More than 70% of the bacterial species producing such biofilms are likely to be resistant to at least one of the drugs commonly used in anti-infectious therapy.[14] In hospitals, there are also other examples of Gram-positive (*Enterococcus* and *Streptococcus*) and Gram-negative pathogens (*Klebsiella*, *Escherichia*, *Enterobacter*, *Serratia*, *Citrobacter*, *Salmonella* and *Pseudomonas*); these hospital-inhabiting microbes are

called 'nosocomial bacteria.' More than 60% of sepsis cases in hospitals are caused by Gram-negative bacteria.[14] Among them, *Pseudomonas aeruginosa* accounts for almost 80% of these opportunistic infections. They represent a serious problem in patients hospitalized with cancer, cystic fibrosis and burns, causing death in 50% of cases. Other infections caused by *Pseudomonas* species include endocarditis, pneumonia and infections of the urinary tract, central nervous system, wounds, eyes, ears, skin and musculoskeletal system. This bacterium is another example of a natural multidrug-resistant microorganism. Although many strains are susceptible to gentamicin, tobramycin and amikacin, resistant forms have also developed. These multidrug-resistant bacteria make hospitals "dangerous places to be, especially if you are sick, but even if not."[19]

Although we are seeing a steady increase in resistance in almost every pathogen to most of the current antibiotics over time, not all the antibacterial agents show the same rate of resistance development. For example, antimicrobials such as rifampicin, which targets single enzymes, are most susceptible to the development of resistance, whereas agents that inactivate several targets irreversibly generate resistance more slowly.

In addition to the antibiotic-resistance problem, new families of anti-infective compounds are needed to enter the marketplace at regular intervals to tackle the new diseases caused by evolving pathogens. At least 30 new diseases emerged in the 1980s and 1990s and they are growing in incidence. Emerging infectious organisms often encounter hosts with no prior exposure to them and thus represent a novel challenge to the host's immune system. Several viruses responsible for human epidemics have made a transition from animal host to humans and are now transmitted from human to human. HIV, responsible for the acquired immunodeficiency syndrome (AIDS) epidemic, is one example. Although it has not been proven, it is suspected that severe acute respiratory syndrome (SARS), caused by the SARS coronavirus, also evolved from a different species.[20]

In the early 1990s, after decades of decline, the incidence of tuberculosis began to increase. The epidemic took place owing to inadequate treatment regimens, a diminished public health system and the onset of the HIV/AIDS epidemic. The WHO has predicted that between 2000 and 2020, nearly 1 billion people will become infected with *Mycobacterium tuberculosis* and that this disease will cost the lives of 35 million people.

Sexually transmitted diseases have also increased during these decades, especially in young people (aged 15–24 years). The human papillomavirus, chlamydia, genital herpes, gonorrhea and HIV/AIDS are examples. HIV/AIDS has infected more than 40 million people in the world. Together with other diseases such as tuberculosis and malaria, HIV/AIDS accounts for over 300 million illnesses and more than 5 million deaths each year.

Additional evolving pathogens include the (i) Ebola virus, which causes the viral hemorrhagic fever syndrome with a resultant mortality rate of 88%; (ii) the bacterium *Legionella pneumophila*, a ubiquitous aquatic organism that lives in warm environments, which causes Legionnaire's disease, a pulmonary infection; (iii) the Hantavirus, which can infect humans with two serious illnesses: hemorrhagic fever with renal syndrome and Hantavirus pulmonary syndrome; (iv) at least three species of bacteria from the genus *Borrelia*, which cause Lyme disease, an emerging infection. In this case, the infection is acquired from the bite of ticks belonging to several species of the genus *Ixodes*. *Borrelia burgdorferi* is the predominant cause of Lyme disease in the US, whereas *Borrelia afzelii* and *Borrelia garinii* are implicated in most European cases. The disease presentation varies widely, and may include a rash and flu-like symptoms in its initial stage, followed by musculoskeletal, arthritic, neurologic, psychiatric and cardiac manifestations. In the majority of cases, symptoms can be eliminated with antibiotics, especially if the treatment begins early in the course of illness. However, late or inadequate treatment can lead to 'late-stage' Lyme disease that can be disabling and difficult to treat.[21] (v) Other evolving pathogens include the *Escherichia coli* 0157:H7 (enterohemorrhagic *E. coli*), a strain that causes colitis and bloody diarrhea by producing a toxin called Shiga toxin, which damages the intestines. It is estimated that this bacterium causes infection in more than 70 000 patients a year in the USA. Another example is (vi) *Cryptosporidium*, an obligate intracellular parasite commonly found in lakes and rivers. *Cryptosporidium parvum* is one of the common species affecting the digestive and respiratory organs. Intestinal cryptosporidiosis is characterized by severe watery diarrhea. Pulmonary and tracheal cryptosporidiosis in humans is associated with coughing and is frequently a low-grade fever. People with severely weakened immune systems are likely to have more severe and more persistent symptoms than healthy individuals.

In the developing world, nearly 90% of the infectious disease deaths are caused by six diseases or disease processes: acute respiratory infections, diarrhea, tuberculosis, HIV, measles and malaria. In both the developing and developed nations, the leading cause of death by a wide margin is acute respiratory disease. In the developing world, acute respiratory infections are attributed primarily to seven bacteria: *Bordetella pertussis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Chlamydia trachomatis*. In addition, the major viral causes of respiratory infections include respiratory syncytial virus, human parainfluenza viruses 1 and 3, influenza viruses A and B, as well as some adenoviruses. These diseases are highly destructive in economic and social as well as in human terms and cause approximately 17 million deaths per year, and innumerable serious illnesses besides affecting the economic growth, development and prosperity of human societies.[22] Morse[23] identified six general factors in the emergence of infectious diseases: ecological changes, human demographics and behavior, international travel, technology and industry, microbial adaptation and change, and breakdown in public health measures.[24]

One additional reason for developing new antibiotics is related to their own toxicity. As with other therapeutic agents, the use of antibiotics may also cause side effects in patients. These include mild reactions such as upset stomach, vomiting and diarrhea (cephalosporins, macrolides, penicillins and tetracyclines), rash and other mild and severe allergic reactions (cephalosporins and penicillins), sensitivity to sunlight (tetracyclines), nervousness, tremors and seizures (quinolones). Some side effects are more severe and, depending on the antibiotic, may disrupt the hearing function (aminoglycosides), kidneys (aminoglycosides and polypeptides) or liver (rifampin).

MOVES AGAINST ANTIBIOTIC RESISTANCE DEVELOPMENT IN BACTERIA

During recent decades, we have seen an increasing number of reports on the progressive development of bacterial resistance to almost all available antimicrobial agents. In the 1970s, the major problem was the multidrug resistance of Gram-negative bacteria, but later in the 1980s the Gram-positive bacteria became important, including methicillin-resistant staphylococci, penicillin-resistant pneumococci and vancomycin-resistant enterococci.[25] In the past, the solution to the problem has depended primarily on the development of novel

antimicrobial agents. However, the number of new classes of antimicrobial agents being developed has decreased dramatically in recent years.

The advent of resistant Gram-positive bacteria has been noticed by the pharmaceutical, biotechnology and academic communities. Some of these groups are making concerted efforts to find novel antimicrobial agents to meet this need. A new glycopeptide antibiotic, teicoplanin, was developed against infections with resistant Gram-positive bacteria, especially bacteria resistant to the glycopeptide vancomycin. In another instance, the approach involved the redesign of a mixture of two compounds, called streptogramin, into a new mixture, called pristinamycin, to allow administration of the drug parenterally and in higher doses than the earlier oral preparation.[26] The two components of streptogramin, quinupristin and dalfopristin, were chemically modified to allow intravenous administration. The new combination, pristinamycin, was approved by the FDA for use against infections caused by vancomycin-resistant *Enterococcus faecium*.

Additional moves against resistant microorganisms are the glycylcyclines developed to treat tetracycline-resistant bacteria. These modified tetracyclines show potent activity against a broad spectrum of Gram-positive and Gram-negative bacteria, including strains that carry the two major tetracycline-resistance determinants, involving efflux and ribosomal protection. Two of the glycylcyline derivatives, DMG-MINO and DMG-DMDOT, have been tested against a large number of clinical pathogens isolated from various sources. The spectrum of activity of these compounds includes organisms with resistance to antibiotics other than tetracyclines; for example, methicillin-resistant staphylococci, penicillin-resistant *S. pneumoniae* and vancomycin-resistant enterococci.[27] Tigecycline was approved by the FDA in 2005 as an injectable antibiotic.[28]

Among the novel class of antimicrobial agents used in treating resistance to Gram-positive infections, we can also mention the cyclic lipopeptide antibiotic daptomycin produced by *Streptomyces roseosporus*. This compound was approved in 2003 by the FDA for skin infections resulting from complications following surgery, diabetic foot ulcers and burns. It represents the first new natural antibiotic approved in many years. Its mode of action is distinct from any other approved antibiotic: it rapidly kills Gram-positive bacteria by disrupting multiple aspects of bacterial membrane function (by binding irreversibly to the bacterial cell membrane, causing membrane depolarization, destroying the ion concentration gradient and provoking the efflux of K^+). It acts against most clinically relevant Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsp. *equisimilis* and *Enterococcus faecalis*), and retains *in vitro* potency against isolates resistant to methicillin, vancomycin and linezolid. Traditionally, these infections were treated with penicillin and cephalosporins, but resistance to these agents became widespread.[29–32] Daptomycin seems to have a favorable side effect profile, and it might be used to treat patients who cannot tolerate other antibiotics.

Telithromycin, a macrolide antibiotic, is the first orally active compound of a new family of antibacterials named the ketolides. It shows potent activity against pathogens implicated in community-acquired respiratory tract infections, irrespective of their β-lactam, macrolide or fluoroquinolone susceptibility. Some of the microorganisms susceptible to this antibiotic are pneumococci, *H. influenzae* and *Moraxella catarrhalis*, including β-lactamase-positive strains. In addition, telithromycin has a very low potential for selection of resistant isolates or induction of cross-resistance found with other macrolides.[33]

Clavulanic acid, first detected in *Streptomyces clavuligerus*, contains a bicyclic β-lactam ring fused to an oxazolidine ring with an oxygen in place of a sulfur, a β-hydroxyethylidene substituent at C-2 and no acylamino group at C-6. It was first described in 1976 and shown to be a potent inhibitor of the β-lactamases produced by staphylococci and plasmid-mediated β-lactamases of *E. coli*, *Klebsiella*, *Proteus*, *Shigella*, *Pseudomonas* and *Haemophilus*. Although it is a broad-spectrum antibiotic, clavulanic acid possesses only very low antibacterial activity. Therefore, the molecule has been combined, as a β-lactamase inhibitor, with a variety of broad-spectrum semisynthetic penicillins. For example, when administered with amoxicillin, it is used for the treatment of infections caused by β-lactamase-producing pathogenic bacteria.[34] It has world sales of over US$1 billion, and in 1995 it was the second largest selling antibacterial drug. Clavulanic acid can also be combined with ticarcillin, which is a penicillin effective against organisms such as *E. coli*, *Proteus*, *Salmonella*, *Haemophilus*, *Pseudomonas* and *S. aureus*. It is normally used in hospitals for treating severe infections affecting blood or internal organs, bones and joints, upper or lower airways or skin and soft tissue. The combination extends ticarcillin antimicrobial activity by inhibiting the action of the β-lactamases produced by certain bacteria.

MOVES AGAINST RESISTANCE TO ANTIFUNGAL AGENTS

Mycosis is a condition in which fungi pass the resistance barriers of the human or animal body and establish infections. These organisms are harmless most of the time, but sometimes they can cause fungal infections. In most cases, these infections are not life threatening. However, when they are deeply invasive and disseminated, they lead to more serious infections, particularly in critically ill patients, elderly people and those who have conditions that affect the immune system (by disease or through the use of immunosuppressive agents). In addition, the use of antineoplastic and broad-spectrum antibiotics, prosthetic devices and grafts, and more aggressive surgery has increased invasive fungal infections. Patients with burns, neutropenia, pancreatitis or after organ transplantation (40% of liver transplants, 15–35% of heart transplants and 5% of kidney transplants) are also predisposed to fungal infection.[35] Approximately 40% of death from nosocomial infections are caused by fungi, and 80% of these are caused by *Candida* and *Aspergillus*, although *Cryptococcus* spp., *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp. and zygomycetes are increasingly involved.[36] Pulmonary aspergillosis is the main factor involved in the death of recipients of bone marrow transplants, and *Pneumocystis carinii* is the leading cause of death in AIDS patients from Europe and North America.[37]

The rising incidence of invasive fungal infections and the emergence of broader fungal resistance have led to the need for novel antifungal agents. Amphotericin B is the first-line therapy for systemic infection because of its broad spectrum and fungicidal activity. However, considerable side effects limit its clinical utility. Echinocandins are large lipopeptide molecules that inhibit the synthesis of 1,3-β-D-glucan, a key component of the fungal cell wall. Three echinocandins (caspofungin, micafungin and anidulafungin) have reached the market. Caspofungin is also known as pneumocandin or MK-0991. This compound was the first cell-wall-active antifungal approved as a new injectable antifungal; this was in 2000.[38] It irreversibly inhibits 1,3-β-D-glucan synthase, preventing the formation of glucan polymers and disrupting the integrity of fungal cell walls.[39] It is more active and less toxic than amphotericin B and shows a broad spectrum of activity against *Candida* (including fluconozole resistance), *Aspergillus*, *Histoplasma* and *P. carinii*, the major cause of HIV death. Micafungin is licensed for clinical use in Asian countries and in the US. This compound exhibits extremely potent antifungal activity against clinically important fungi, including *Aspergillus* and azole-resistant

strains of *Candida*. In animal studies, micafungin is as efficacious as amphotericin B with respect to improvement of survival rate. It is characterized by a linear pharmacokinetic profile and substantially fewer toxic effects. Anidulafungin is currently licensed in the US.[40]

Although several new antifungal drugs have been developed in the past 6 years, some patients remain resistant to treatments. The main reasons for this include intrinsic or acquired antifungal resistance, organ dysfunction preventing the use of some agents and drug interactions. In addition, some drugs penetrate poorly into sanctuary sites, including the eye and urine, and others are associated with considerable adverse events. However, there has been some progress. Posaconazole is a new member of the triazole class of antifungals. It has shown clinical efficacy in the treatment of oropharyngeal candidiasis and has potential as a salvage therapy for invasive aspergillosis, zygomycosis, cryptococcal meningitis and a variety of other fungal infections. It is available as an oral suspension and has a favorable toxicity profile. The wide spectrum of posaconazole activity in *in vitro* studies, animal models and preliminary clinical studies suggests that it represents an important addition to the antifungal armamentarium.[41]

ANTIBIOTICS WEAR MANY HATS

In addition to the screening programs for antibacterial activity, the pharmaceutical industry has extended these programs to other disease areas.[42,43] Microorganisms are a prolific source of structurally diverse bioactive metabolites and have yielded some of the most important products of the pharmaceutical industry. Microbial secondary metabolites are now being used for applications other than antibacterial, antifungal and antiviral infections. For example, immunosuppressants have revolutionized medicine by facilitating organ transplantation.[44] Other applications include antitumor drugs, enzyme inhibitors, gastrointestinal motor stimulator agents, hypocholesterolemic drugs, ruminant growth stimulants, insecticides, herbicides, coccidiostats, antiparasitics vs coccidia, helminths and other pharmacological activities. Further applications are possible in various areas of pharmacology and agriculture, developments catalyzed by the use of simple enzyme assays for screening before testing in intact animals or in the field.

Antitumor drugs

In the year 2000, approximately 10 million new cases of cancer were diagnosed in the world, resulting in 6 million cancer-related deaths. The tumor types with the highest incidence were lung (12.3%), breast (10.4%) and colorectal (9.4%).[45]

Microbial metabolites are among the most important of the cancer chemotherapeutic agents. They started to appear around 1940 with the discovery of actinomycin and since then many compounds with anticancer properties have been isolated from natural sources. More than 60% of the current compounds with antineoplasic activity were originally isolated as natural products or are their derivatives. Among the approved products deserving special attention are actinomycin D, anthracyclines (daunorubicin, doxorubicin, epirubicin, pirirubicin and valrubicin), bleomycin, mitosanes (mitomycin C), anthracenones (mithramycin, streptozotocin and pentostatin), enediynes (calcheamycin), taxol and epothilones.

Actinomycin D is the oldest microbial metabolite used in cancer therapy. Its relative, actinomycin A, was the first antibiotic isolated from actinomycetes. The latter was obtained from *Actinomyces antibioticus* (now *Streptomyces antibioticus*) by Waksman and Woodruff.[46] As it binds DNA at the transcription initiation complex, it prevents elongation by RNA polymerase. This property, however, confers some human toxicity and it has been used primarily as an investigative tool in the development of molecular biology. Despite the toxicity, however, it has served well against Wilms tumor in children.

The anthracyclines are some of the most effective antitumor compounds developed, and are effective against more types of cancer than any other class of chemotherapy agents.[47] They are used to treat a wide range of cancers, including leukemias, lymphomas, and breast, uterine, ovarian and lung cancers. Anthracyclines act by intercalating DNA strands, which result in a complex formation that inhibits the synthesis of DNA and RNA. It also triggers DNA cleavage by topoisomerase II, resulting in mechanisms that lead to cell death. In their cytotoxic effects, the binding to cell membranes and plasma proteins plays an important role. Their main adverse effects are heart damage (cardiotoxicity), which considerably limits their usefulness, and vomiting. The first anthracycline discovered was daunorubicin (daunomycin) in 1966, which is produced naturally by *Streptomyces peucetius*. Doxorubicin (adriamycin) was developed in 1967. Another anthracycline is epirubicin. This compound, approved by the FDA in 1999, is favored over doxorubicin in some chemotherapy regimens as it appears to cause fewer side effects. Epirubicin has a different spatial orientation of the hydroxyl group at the 4′ carbon of the sugar, which may account for its faster elimination and reduced toxicity. Epirubicin is primarily used against breast and ovarian cancer, gastric cancer, lung cancer and lymphomas. Valrubicin is a semisynthetic analog of doxorubicin approved as a chemotherapeutic drug in 1999 and used to treat bladder cancer.

Bleomycin is a non-ribosomal glycopeptide microbial metabolite produced as a family of structurally related compounds by the bacterium *Streptomyces verticillus*. First reported by Umezawa *et al.*[48] in 1966, bleomycin obtained FDA approval in 1973. When used as an anticancer agent (inducing DNA strand breaks), the chemotherapeutic forms are primarily bleomycins A2 and B2.

Mitosanes are composed of several mitomycins that are formed during the cultivation of *Streptomyces caespitosus*. Although the mitosanes are excellent antitumor agents, they have limited utility owing to their toxicity. Mitomycin C was approved by the FDA in 1974, showing activity against several types of cancer (lung, breast, bladder, anal, colorectal, head and neck), including melanomas and gastric or pancreatic neoplasms.[49] Recently, mitomycin dimers have been explored as potential alternatives for lowering toxicity and increasing efficiency.[50]

Mithramycin (plicamycin) is an antitumor aromatic polyketide produced by *Streptomyces argillaceous* that shows antibacterial and antitumor activity.[51] It is one of the older chemotherapy drugs used in the treatment of testicular cancer, disseminated neoplasms and hypercalcemia. It binds to G-C-rich DNA sequences, inhibiting the binding of transcription factors such as Sp1, which is believed to affect neuronal survival/death pathways. It may also indirectly regulate gene transcription by altering histone methylation. With repeated use, organotoxicity (kidney, liver and hematopoietic system) can become a problem.

Streptozotocin is a microbial metabolite with antitumor properties, produced by *Streptomyces achromogenes*. Chemically, it is a glucosamine-nitroso-urea compound. As with other alkylating agents in the nitroso-urea class, it is toxic to cells by causing damage to DNA, although other mechanisms may also contribute. The compound is selectively toxic to the β-cells of the pancreatic islets. It is similar enough to glucose to be transported into the cell by the glucose transport protein of these cells, but it is not recognized by the other glucose transporters. As β-cells have relatively high levels of glucose permease, the relative streptozotocin toxicity for these β-cells can be

explained.[52] In 1982, FDA granted approval for streptozotocin as a treatment for pancreatic islet cell cancer.

Pentostatin (deoxycoformycin) is an anticancer chemotherapeutic drug produced by *S. antibioticus*. It is classified as a purine analog, which mimics the nucleoside adenosine and thus tightly binds and inhibits adenosine deaminase (K_i of 2.5×10^{-12} M). It interferes with the cell's ability to process DNA.[53] Pentostatin is commonly used to treat hairy cell leukemia, acute lymphocytic leukemia, prolymphocytic leukemia (B- and T-cell origin), T-cell leukemia and lymphoma. However, it can cause kidney, liver, lung and neurological toxicity.[54] The FDA granted approval for pentostatin in 1993.

Calicheamicins are highly potent antitumor microbial metabolites of the enediyne family produced by *Micromonospora echinospora*. Their antitumor activity is apparently due to the cleavage of double-stranded DNA.[55] They are highly toxic, but it was possible to introduce one such compound into the clinic by attaching it to an antibody that delivered it to certain cancer types selectively. This ingenious idea of the Wyeth Laboratories avoided the side effects of calicheamicin. In this regard, gemtuzumab is effective against acute myelogenous leukemia (AML). Calicheamicin is bound to a monoclonal antibody against a transmembrane receptor (CD33) expressed on cells of monocytic/myeloid lineage. CD33 is expressed in most leukemic blast cells, but in normal hematopoietic cells the intensity diminishes with maturation. It was approved by the FDA for use in patients over the age of 60 years with relapsed AML who are not considered candidates for standard chemotherapy.[56]

A successful non-actinomycete molecule is taxol (paclitaxel), which was first isolated from the Pacific yew tree, *Taxus brevifolia*, but is also produced by the endophytic fungi *Taxomyces andreanae* and *Nodulisporium sylviforme*.[57] This compound inhibits rapidly dividing mammalian cancer cells by promoting tubulin polymerization and interfering with normal microtubule breakdown during cell division. The drug also inhibits several fungi (*Pythium*, *Phytophthora* and *Aphanomyces*) by the same mechanism. In 1992, taxol was approved for refractory ovarian cancer, and today it is used against breast and advanced forms of Kaposi's sarcoma.[58] A new formulation is available in which paclitaxel is bound to albumin. Taxol sales amounted to US$1.6 billion in 2006 for Bristol Myers-Squibb, representing 10% of the company's pharmaceutical sales and its third largest selling product. Currently, taxol production uses plant cell fermentation technology.

The epothilones (a name derived from its molecular features: epoxide, thiazole and ketone) are macrolides originally isolated from the broth of the soil myxobacterium *Sorangium cellulosum* as weak agents against rust fungi.[59] They were identified as microtubule-stabilizing drugs, acting in a similar manner to taxol.[60,61] However, they are generally 5–25 times more potent than taxol in inhibiting cell growth in cultures. Five analogs are now undergoing investigation as candidate anticancer drugs, and their preclinical studies have indicated a broad spectrum of antitumor activity, including taxol-resistant tumor cells. With the best currently available therapies, the median survival time for patients with metastatic breast cancer is only 2–3 years, and many patients develop resistance to taxanes or other chemotherapy drugs. One epothilone, ixabepilone, was approved in October 2007 by the FDA for use in the treatment of aggressive metastatic or locally advanced breast cancer no longer responding to currently available chemotherapies.[62] In tumor cells, *p*-glycoprotein reduces intracellular antitumor drug concentrations, thereby limiting access of chemotherapeutic substrates to the site of action. The epothilones are attractive because they are active against *p*-glycoprotein-producing tumors and have good solubility.[62] Epothilone B is a 16-membered polyketide macrolactone with a methylthiazole group connected to the macrocycle by an olefinic bond.

Testicular cancer is the most common cancer diagnosis in men between the ages of 15 and 35 years, with approximately 8000 cases detected in the United States annually.[63] The majority (95%) of testicular neoplasms are germ cell tumors, which are relatively uncommon carcinomas, accounting for only 1% of all male malignancies. Remarkable progress has been made in the medical treatment of advanced testicular cancer, with a substantial increase in cure rates from approximately 5% in the early 1970s to almost 90% today.[64,65] This cure rate is the highest of any solid tumor, and improved survival is primarily due to effective chemotherapy. A major advance in chemotherapy for testicular germ cell tumors was the introduction of cisplatin in the mid-1970s. Two chemotherapy regimens are effective for patients with a good testicular germ cell tumor prognosis: four cycles of etoposide and cisplatin or three cycles of bleomycin, etoposide and cisplatin.[66] Of the latter three agents, bleomycin and etoposide are natural products.

Enzyme inhibitors

Enzyme inhibitors have received increasing attention as useful tools, not only for the study of enzyme structures and reaction mechanisms but also for potential utilization in medicine and agriculture. Several enzyme inhibitors with various industrial uses have been isolated from microbes.[67] The most important are (1) clavulanic acid, the inhibitor of β-lactamases discussed above in the section 'Moves against antibiotic resistance development in bacteria,' and the statins, hypocholesterolemic drugs presented below in the section 'Hypocholesterolemic drugs.' Some of the common targets for other inhibitors are glucosidases, amylases, lipases, proteases and xanthine oxidase (XO).

Acarbose is a pseudotetrasaccharide made by *Actinoplanes* sp. SE50. It contains an aminocyclitol moiety, valienamine, which inhibits intestinal α-glucosidase and sucrase. This results in a decrease in starch breakdown in the intestine, which is useful in combating diabetes in humans.[68]

Amylase inhibitors are useful for the control of carbohydrate-dependent diseases, such as diabetes, obesity and hyperlipemia.[69,70] Amylase inhibitors are also known as starch blockers because they contain substances that prevent dietary starches from being absorbed by the body. The inhibitors may also be useful for weight loss, as some versions of amylase inhibitors do show potential for reducing carbohydrate absorption in humans.[71,72] The use of amylase inhibitors for the treatment of rumen acidosis has also been reported.[73] Examples of microbial α-amylase inhibitors are paim, obtained from culture filtrates of *Streptomyces corchorushii*,[74] and TAI-A, TAI-B, oligosaccharide compounds from *Streptomyces calvus* TM-521.[75]

Lipstatin is a pancreatic lipase inhibitor produced by *Streptomyces toxytricini* that is used to combat obesity and diabetes. It interferes with the gastrointestinal absorption of fat.[76] The commercial product is tetrahydrolipstatin, which is also known as orlistat.

In the pathogenic processes of some diseases, such as emphysema, arthritis, pancreatitis, cancer and AIDS, protease inhibitors are potentially powerful tools for inactivating target proteases. Examples of microbial products include antipain, produced by *Streptomyces yokosukaensis*, leupeptin from *Streptomyces roseochromogenes* and chymostatin from *Streptomyces hygroscopicus*.[70] Leupeptin is produced by more than 17 species of actinomycetes.[67]

XO catalyzes the oxidation of hypoxanthine to uric acid through xanthine. An excessive accumulation of uric acid in the blood, called hyperuricemia, causes gout.[77] The inhibitors of XO decrease the uric acid levels, which result in an antihyperuricemic effect. A potent

inhibitor of XO, hydroxyakalone, was purified from the fermentation broth of *Agrobacterium aurantiacum* sp. nov., a marine bacterial strain.[78]

Fungal products are also used as enzyme inhibitors against cancer, diabetes, poisonings, Alzheimer's disease, etc. The enzymes inhibited include acetylcholinesterase, protein kinase, tyrosine kinase, glycosidases and others.[79]

Immunosuppresants

Suppressor cells are critical in the regulation of the normal immune response. An individual's immune system is capable of distinguishing between native and foreign antigens and of mounting a response only against the latter. A major role has been established for suppressor T lymphocytes in this phenomenon. Suppressor cells also play a role in regulating the magnitude and duration of the specific antibody response to an antigenic challenge. Suppression of the immune response either by drugs or by radiation, to prevent the rejection of grafts or transplants or to control autoimmune diseases, is called immunosuppression.

A number of microbial compounds capable of suppressing the immune response have been discovered. Cyclosporin A was originally introduced as a narrow-spectrum antifungal peptide produced by the mold, *Tolypocladium nivenum* (originally classified as *Trichoderma polysporum* and later as *Tolypocladium inflatum*), by aerobic fermentation. Cyclosporins are a family of neutral, highly lipophilic, cyclic undecapeptides containing some unusual amino acids, synthesized by a non-ribosomal peptide synthetase, cyclosporin synthetase. Discovery of the immunosuppressive activity led to its use in heart, liver and kidney transplants and to the overwhelming success of the organ transplant field.[80] Cyclosporin was approved for use in 1983. It is thought to bind to the cytosolic protein cyclophilin (immunophilin) of immunocompetent lymphocytes, especially T lymphocytes. This complex of cyclosporin and cyclophilin inhibits calcineurin, which under normal circumstances is responsible for activating the transcription of interleukin-2. It also inhibits lymphokine production and interleukin release and therefore leads to a reduced function of effector T cells. Sales of cyclosporin A have reached US$1.5 billion per year.

Other important transplant agents include sirolimus (rapamycin) and tacrolimus (FK506), which are produced by actinomycetes. Rapamycin is especially useful in kidney transplants as it lacks the nephrotoxicity seen with cyclosporin A and tacrolimus. It is a macrolide, first discovered in 1975 as a product of *S. hygroscopicus*, and was initially proposed as an antifungal agent. However, this was abandoned when it was discovered that it had potent immunosuppressive and antiproliferative properties. This compound binds to the immunophilin FK506-binding protein (FKBP12), and this binary complex interacts with the rapamycin-binding domain and inactivates a serine-threonine kinase termed the mammalian target of rapamycin. The latter is known to control proteins that regulate mRNA translation initiation and G1 progression.[81] The antiproliferative effect of rapamycin has also been used in conjunction with coronary stents to prevent restenosis, which usually occurs after the treatment of coronary artery disease by balloon angioplasty. Rapamycin also shows promise in treating tuberous sclerosis complex (TSC), a congenital disorder that leaves sufferers prone to benign tumor growth in the brain, heart, kidneys, skin and other organs. In a study of rapamycin as a treatment for TSC, University of California, Los Angeles (UCLA) researchers observed a major improvement in mice regarding retardation related to autism.[82]

As rapamycin has poor aqueous solubility, some of its analogs, RAD001 (everolimus), CCI-799 (tensirolimus) and AP23573 (ARIAD), have been developed with improved pharmaceutical properties. Everolimus is currently used as an immunosuppressant to prevent the rejection of organ transplants. Although it does not have FDA approval in the USA, it is approved for use in Europe and Australia, and phase III trials are being conducted in the US. Everolimus may have a role in heart transplantation as it has been shown to reduce chronic allograft vasculopathy in such transplants.[83] Everolimus is also used in drug-eluting coronary stents as an immunosuppressant to prevent rejection. CCI-779 is a rapamycin ester that can be converted to rapamycin *in vivo*. RAD001 is a rapamycin analog currently being investigated in phase II trials for recurrent endometrial cancer as a single agent, and in phase I/II trials for the treatment of glioblastoma in combination with the inhibitor of certain epidermal growth factor receptor and vascular endothelial growth factor receptor family members.[84] AP23573 is a novel non-prodrug rapamycin analog with a nonlinear pharmacokinetic behavior that has demonstrated antiproliferative activity against several human tumor cell lines *in vitro* and against experimental tumors *in vivo*.[85] This agent is currently under evaluation in phase I–II trials, including patients with different tumors. Two additional small-molecule rapamycin analogs, AP23841 and AP23675, are currently in preclinical development for the treatment of bone metastases and primary bone cancer.[86]

Tacrolimus (FK506) was discovered in 1987 in Japan.[87] It is produced by *Streptomyces tsukubaensis*. However, its use was almost abandoned because of dose-associated toxicity. Dr Thomas Starzl (University of Pittsburgh) rescued it by using lower doses, realizing that it was approximately 100 times more active as an immunosuppressive than cyclosporin A.[88] It was introduced in Japan in 1993, and in 1994 it was approved by the FDA for use as an immunosuppressant in liver transplantation. Furthermore, its use has been extended to include bone marrow, cornea, heart, intestines, kidney, lung, pancreas, trachea, small bowel, skin and limb transplants, and for the prevention of graft-vs-host disease. Topically, it is also used against atopic dermatitis, a widespread skin disease. In the laboratory, tacrolimus inhibits the mixed lymphocyte reaction, the formation of interleukin-2 by T lymphocytes, and the formation of other soluble mediators, including interleukin-3 and interferon γ. Recently, it has been reported that tacrolimus inhibits tumor growth factor-β-induced signaling and collagen synthesis in human lung fibroblastic cells. This factor plays a pivotal role in tissue fibrosis, including pulmonary fibrosis. Therefore, tacrolimus may be useful for the treatment of pulmonary fibrosis, although its use in the acute inflammatory phase may exacerbate lung injury.[89]

Hypocholesterolemic drugs

Atherosclerosis is generally viewed as a chronic, progressive disease characterized by the continuous accumulation of atheromatous plaque within the arterial wall. The past two decades have witnessed the introduction of a variety of anti-atherosclerotic therapies. The statins form a class of hypolipidemic drugs used to lower cholesterol by inhibiting the enzyme HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway of cholesterol biosynthesis. Inhibition of this enzyme in the liver stimulates low-density lipoprotein (LDL) receptors, resulting in an increased clearance of LDL from the bloodstream and a decrease in blood cholesterol levels. Through their cholesterol-lowering effect, they reduce the risk of cardiovascular disease, prevent stroke and reduce the development of peripheral vascular disease.[90] In addition, they are anti-thrombotic and anti-inflammatory.

Currently there are a number of statins in clinical use. The entire group of statins reached an annual market of nearly US$30 billion before it became a generic pharmaceutical. The first member of the group (compactin; mevastatin) was isolated as an antibiotic product of *Penicillium brevicompactum* and later from *Penicillium citrinum*. Although not of commercial importance, compactin's derivatives achieved overwhelming medical and commercial success. An ethylated form, known as lovastatin (monacolin K; mevinolin), was isolated in the 1970s in the broths of *Monascus ruber* and *Aspergillus terreus*.[91] Lovastatin, the first commercially marketed statin, was approved by the FDA in 1987. A semisynthetic derivative of lovastatin is simvastatin, a major hypocholesterolemic drug, selling for US$7 billion per year before becoming generic. Another statin, pravastatin (US$3.6 billion per year), is made through different biotransformation processes from compactin by *Streptomyces carbophilus*[92] and *Actinomadura* sp.[93] Other genera involved in the production of statins are *Doratomyces*, *Eupenicillium*, *Gymnoascus*, *Hypomyces*, *Paecilomyces*, *Phoma*, *Trichoderma* and *Pleurotus*.[94] A synthetic compound, modeled from the structure of the natural statins, is atorvastin, which has been the leading drug of the entire pharmaceutical industry in terms of market share (approximately US$14 billion per year) for many years.

Insecticides

An insecticide is a pesticide used against insects in all developmental forms. They include ovicides and larvicides used against the eggs and larvae of insects, respectively. Insecticides are used in agriculture, medicine, industry and households. The use of insecticides is believed to be one of the major factors behind the increase in agricultural productivity in the twentieth century.

Synthetic insecticides pose some hazards, whereas natural insecticides offer adequate levels of pest control and pose fewer hazards. Microbially produced insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low. Compared with other commonly used insecticides, they are safe for both the pesticide users and consumers of treated crops. The action of microbial insecticides is often specific to a single group or species of insects, and this specificity means that most microbial insecticides do not naturally affect beneficial insects (including predators or parasites of pests) in treated areas.

The spinosyns (A83543 group) are a group of natural products produced by *Saccharopolyspora spinosa* that were discovered in 1989. The researchers isolated spinosyn A and D, as well as 21 minor analogs. They are active on a wide variety of insect pests, especially lepidopterans and dipterans, but do not have antibiotic activity.[95] The compounds attack the nervous system of insects by targeting two key neurotransmitter receptors, with no cross-resistance to other known insecticides. The spinosyns are a family of macrolides with 21 carbon atoms, containing four connected rings of carbon atoms at their core to which two deoxysugars (forosamine and 2,3,4, tri-*O*-methylrhamnose, which are required for bioactivity) are attached. Novel spinosyns have been prepared by biotransformation, using a genetically engineered strain of *Saccharopolyspora erythraea*.[96] A mixture of spinosyn A (85%) and D (15%) (spinosad) is being produced through fermentation and was introduced to the market in 1997 for the control of chewing insects on a variety of crops. Spinosyn formulations were recently approved for use on organic crops and for animal health applications.

Recently, a new naturally occurring series of insect-active compounds was discovered from a novel soil isolate, *Saccharopolyspora pogona* NRRL30141. [97] The culture produced a unique family of over 30 new spinosyns. They have a butenyl substitution at the 21 position on the spinosyn lactone and are named butenyl-spinosyns or pogonins.

Herbicides

Herbicides are chemicals marketed to inhibit or interrupt normal plant growth and development. They are widely used in agriculture, industry and urban areas for weed management. Approximately 30 000 kinds of weeds are widely distributed in the world; yield losses caused by 1800 kinds of weeds are approximately 9.7% of total crop production every year.[98] Herbicides provide cost-effective weed control with a minimum of labor. Most are used on crops planted in large acreages, such as soy, cotton, corn and canola.[99] There are numerous classes of herbicides with different modes of action, as well as different potentials for adverse effects on health and the environment. Over the past century, chemical herbicides, used to control various weeds, may have caused many serious side effects, such as injured crops, threat to the applicator and others exposed to the chemicals, herbicide-resistant weed populations, reduction of soil and water quality, herbicide residues and detrimental effects on non-target organisms.[100] For example, alachlor and atrazine were reported to cause cancer in animal tests. With increasing global environmental consciousness, bioherbicides, which are highly effective for weed control and environmentally friendly as well, are very attractive both for research and for application. Microbial herbicides can be divided into microbial preparations (microorganisms that control weeds) and microbially derived herbicides.

The first microbial herbicide was independently discovered in Germany and Japan. In 1972, the Zähner group in Germany isolated phosphinothricin tripeptide, a peptide antibiotic consisting of two molecules of L-alanine and one molecule of the unusual amino acid L-phosphinothricin; that is, *N*(4[hydroxyl(methyl)phosphinoyl]homoalanyl)alanylalanine. They isolated it from *Streptomyces viridochromogenes* as a broad-spectrum antibacterial including activity against *Botrytis cinerea*.[101] In Japan, it was discovered at the Meiji Seiki laboratories in 1973 from *S. hygroscopicus* and named bialaphos.[102] The bioactive L-phosphinothricin is a structural analog of glutamic acid, acting as a competitive inhibitor of glutamine synthetase, and has bactericidal (Gram-positive and Gram-negative bacteria), fungicidal (*B. cinerea*) and herbicidal properties.[103] Glufosinate (DL-phosphinothricin) (without Ala-Ala) was developed as a herbicide. Therefore, the agent acts as a herbicide with or without Ala-Ala. Bialaphos has no influence on microorganisms in the soil and is easily degraded in the environment, having a half-life of only 2 h. This low level of environmental impact is of great interest to environmentalists.

Antiparasitics and ruminant growth stimulants

In 2006, the global animal health market was valued at US$16 billion, of which 29% was derived from parasiticides. Parasites are organisms that inhabit the body and benefit from a prolonged, close association with the host. Antiparasitics are compounds that inhibit the growth or reproduction of a parasite; some antiparasitics directly kill parasites. In general, parasites are much smaller than their hosts, show a high degree of specialization for their mode of life and reproduce more quickly and in greater numbers than their hosts. Classic examples of parasitism include the interactions between vertebrate hosts and such diverse animals as tapeworms, flukes, *Plasmodium* species and fleas. Parasitic infections can cause potentially serious health problems and even kill the host. Parasites mainly enter the body through the mouth, usually through ingestion of tainted food or drink. This is a very common problem in tropical areas, but is not limited to those regions. There are 3200 varieties of parasites in four major

categories: Protozoa, Trematoda, Cestoda and Nematoda. The major groups include protozoans (organisms having only one cell) and parasitic worms (helminths). Each of these can infect the digestive tract, and sometimes two or more can cause infection at the same time. The WHO reported that approximately 25% of the world's population is infected with roundworms. In addition, a major agricultural problem has been the infection of farm animals by worms.

The predominant type of antiparasitic screening effort over the years was the testing of synthetic compounds against nematodes, and some commercial products did result. Certain antibiotics were also shown to possess antihelmintic activity against nematodes or cestodes, but these failed to compete with the synthetic compounds. Although Merck had earlier developed a commercially useful synthetic product, thiabendazole, they had enough foresight to examine microbial broths for antihelmintic activity, and found a non-toxic fermentation broth that killed the intestinal nematode *Nematosporoides dubius* in mice. The *Streptomyces avermitilis* culture, isolated by Ōmura and coworkers at the Kitasato Institute in Japan,[104] produced a family of secondary metabolites (eight compounds) with both antihelmintic and insecticidal activities. These compounds, named 'avermectins,' are pentacyclic, 16-membered macrocyclic lactones, that harbor a disaccharide of the methylated sugar, oleandrose, with exceptional activity against parasites, especially Nemathelminthes (nematodes) and arthropod parasites (10 times higher than any known synthetic antihelmintic agent). Surprisingly, avermectins lack activity against bacteria and fungi, do not inhibit protein synthesis and are not ionophores. Instead, they interfere with neurotransmission in many invertebrates, causing paralysis and death by neuromuscular attacks.[105]

The annual market for avermectins surpasses US$1 billion. They are used against both nematode and arthropod parasites in sheep, cattle, dogs, horses and swine. A semisynthetic derivative, 22,23-dihydroavermectin B1 ('ivermectin') is 1000 times more active than thiabendazole and is a commercial veterinary product. The efficacy of ivermectin has made it a promising candidate for the control of human onchocerciasis and human strongyloidiasis.[106] Another avermectin, called doramectin (or cyclohexyl avermectin B1), produced by 'mutational biosynthesis' was commercialized for use by food animals.[107] A semisynthetic monosaccharide derivative of doramectin called selamectin is the most recently commercialized avermectin, and is active against heartworms (*Dirofilaria immitis*) and fleas in companion animals. Although the macrocyclic backbone of each of these molecules (ivermectin, doramectin and selamectin) is identical, there are different substitutions at pharmacologically relevant sites such as C-5, C-13, C-22,23 and C-25.[108] The avermectins are closely related to the milbemycins, a group of non-glycosidated macrolides produced by *S. hygroscopicus* subsp. *Aureolacrimosus*.[109] These compounds possess activity against worms and insects.

Coccidiostats are used for the prevention of coccidiosis in both extensively and intensively reared poultry. Coccidiosis is the name given to a common intestinal disease caused by the invading protozoan parasites of the genus *Eimeria* that affects several different animal species (cattle, dogs, cats, poultry, etc.). The major damage is caused by the rapid multiplication of the parasite in the intestinal wall and the subsequent rupture of the cells of the intestinal lining, leading to high mortality and severe loss of productivity. Coccidia are obligate intracellular parasites that show host specificity; only cattle coccidia will cause disease in cattle; other species-specific coccidia will not.

For many years, synthetic compounds were used to combat coccidiosis in poultry; however, resistance developed rapidly. A solution came on the scene with the discovery of the narrow-spectrum polyether antibiotic monensin, which had extreme potency against the coccidian.[110] Made by *Streptomyces cinnamonensis*, monensin led the way for additional microbial ionophoric antibiotics, such as lasalocid, narasin and salinomycin. All are produced by various *Streptomyces* species. They form complexes with the polar cations K^+, Na^+, Ca^{2+} and Mg^{2+}, severely affecting the osmotic balance in the parasitic cells and thus causing their death.[111] The widespread use of anticoccidials has revolutionized the poultry industry by reducing the mortality and production losses caused by coccidiosis. Of great interest was another extremely valuable application of monensin; that is, growth promotion in ruminants. Synthetic chemicals had been tested for years to inhibit wasteful methane production by cattle and sheep and increase fatty acid formation (especially propionate) to improve feed efficiency; however, they failed. The solution was monensin, which became a major success as a ruminant growth enhancer.[110]

For more than 40 years, certain antibiotics have been used in food-animal production to enhance feed utilization and weight gain.[112] From a production standpoint, feed antibiotics have been consistently shown to improve animal weight gain and feed efficiency, especially in younger animals. These responses are probably derived from an inhibitory effect on the normal microbiota, which can lead to reduced intestinal inflammation and improved nutrient utilization.[113] Pigs in the USA are exposed to a great variety of antibiotics. These include β-lactam antibiotics (including penicillins), lincosamides and macrolides (including erythromycin and tetracyclines). All these groups have members that are used to treat infections in humans. In addition, bacitracin, flavophospholipol, pleuromutilins, quinoxalines and virginiamycin are utilized as growth stimulants. Flavophospholipol and virginiamycin are also used as growth promoters in poultry.

As described above, cattle are also exposed to ionophores such as monensin to promote growth. The Animal Health Institute of America[114] has estimated that without the use of growth-promoting antibiotics, the USA would require an additional 452 million chickens, 23 million more cattle and 12 million more pigs to reach the levels of production attained by the current practices.

Considering that animal health research and the development of new anti-infective product discovery have decreased, the discovery of new antibiotics has decreased over the past 15 years, with few new drug approvals.[115] Therefore, it will be incumbent on veterinary practitioners to use the existing products in a responsible manner to ensure their longevity. It remains to be seen what effects the dearth of new antibiotics for veterinary medicine will have on the future practice of veterinary medicine, production agriculture, food safety and public health.[116]

Since the 1999 EU decision to prohibit antibiotic use for food-animal growth promotion, four antibiotic growth promoters have been banned, including the macrolide drugs tylosin and spiramycin.[117] Although macrolides are no longer formally used as 'growth promoters,' their use under veterinary prescription has risen from 23 tons in 1998 to 55 tons in 2001, which suggests that more of them are being used now than before the prohibition.

Gastrointestinal motor stimulators

It is well known that the most effective route for feeding is via the gastrointestinal tract. Many critically ill patients who accept early feeding improve their health. In some post-operative patients, gastric stasis and excessive volumes in the stomach increase the risk of aspiration and subsequent pneumonia. On account of the importance of achieving early and adequate nutritional intake, it is common practice in many intensive care units to use drugs to improve gastrointestinal motility.

Erythromycin is a macrolide antibiotic with a broad spectrum of activity. It is well recognized that when prescribed, either intravenously or orally, it causes side effects, such as diarrhea, nausea and vomiting. These side effects are, in part, due to the action of erythromycin at motilin receptors in the gut. This makes this antibiotic very attractive to be used in ill patients with gastrointestinal motility problems. There have been some developments on erythromycin analogs that lack antibiotic action but retain action at motilin receptors. These have been named 'motilides.'[118,119] Recently, an orally active erythromycin-derived motilin receptor agonist (mitemcinal) has been tested in patients with idiopathic and diabetic gastroparesis. In both cases, an improvement of gastroparetic symptoms was observed.[120]

FINAL COMMENTS

The 80-year contribution of microorganisms to medicine and agriculture has been overwhelming. However, antibiotic resistance in microbes has created a dangerous situation and the need for new antibiotics is clear. Unfortunately, most of the large pharmaceutical companies have abandoned the search for new antimicrobial compounds. Owing to the economics, they have concluded that drugs directed against chronic diseases offer a better revenue stream than do antimicrobial agents, as for the latter the length of treatment is short and government restriction is likely. Some small pharmaceutical and biotechnology companies are developing antibiotics, but most depend on venture capital rather than sales income, and with the present regulations, they face huge barriers to enter the market. These barriers were raised with the best intentions of ensuring public safety, but will have the opposite effect if they terminate antibiotic development while resistance continues to increase.[121] However, there are some bright possibilities. One of the most promising is the utilization of uncultivated microorganisms. Considering that 99% of the bacteria and 95% of the fungi have not been cultivated in the laboratory, putting efforts into finding means to grow such microorganisms are proceeding and succeeding.[122] Furthermore, researchers are now extracting bacterial DNA from soil and marine habitats, cloning large fragments into, for example, bacterial artificial chromosomes, expressing in a host bacterium and screening the library for new antibiotics. This metagenomic effort is allowing access to a vast untapped reservoir of genetic and metabolic diversity,[123,124] which could result in the discovery of new and useful natural products.[125] In addition to these two relatively new techniques, the chemical and biological modification of old antibiotics could still supply new and powerful drugs. These comments also apply to non-antibiotics such as antitumor agents and other microbial products.

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REVIEW ARTICLE

Chemical biology of natural indolocarbazole products: 30 years since the discovery of staurosporine

Hirofumi Nakano and Satoshi Ōmura

Staurosporine was discovered at the Kitasato Institute in 1977 while screening for microbial alkaloids using chemical detection methods. It was during the same era that protein kinase C was discovered and oncogene v-src was shown to have protein kinase activity. Staurosporine was first isolated from a culture of *Actinomyces* that originated in a soil sample collected in Mizusawa City, Japan. Thereafter, indolocarbazole compounds have been isolated from a variety of organisms. The biosynthesis of staurosporine and related indolocarbazoles was finally elucidated during the past decade through genetic and biochemical studies. Subsequently, several novel indolocarbazoles have been produced using combinatorial biosynthesis. In 1986, 9 years since its discovery, staurosporine and related indolocarbazoles were shown to be nanomolar inhibitors of protein kinases. They can thus be viewed as forerunners of today's crop of novel anticancer drugs. The finding led many pharmaceutical companies to search for selective protein kinase inhibitors by screening natural products and through chemical synthesis. In the 1990s, imatinib, a Bcr-Abl tyrosine kinase inhibitor, was synthesized and, following human clinical trials for chronic myelogenous leukemia, it was approved for use in the USA in 2001. In 1992, mammalian topoisomerases were shown to be targets for indolocarbazoles. This opened up new possibilities in that indolocarbazole compounds could selectively interact with ATP-binding sites of not only protein kinases but also other proteins that had slight differences in ATP-binding sites. ABCG2, an ATP-binding cassette transporter, was recently identified as an important new target for indolocarbazoles.
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Keywords: biosynthesis; indolocarbazole; protein kinase; staurosporine; topoisomerase

INTRODUCTION

The discovery of medically useful natural products has heralded hitherto unimagined possibilities in the chemotherapy of human and animal diseases.[1,2,3]

It is well known that important medical compounds, such as penicillin, cyclosporine A and lovastatin, were only developed as drugs once their key properties were recognized, more than 10 years after their initial discovery.[4] Similarly, in the case of staurosporines, their crucial protein kinase inhibitory properties were only identified a decade or so after their initial discovery.[5]

In 1986, 9 years after the isolation of staurosporine from a streptomyces, the related natural indolocarbazole products, staurosporine and K252, were shown to be nanomolar inhibitors of protein kinases, offering tremendous promise for drug development.[6] The reports led many pharmaceutical companies to begin searching for selective protein kinase inhibitors through natural product screening and chemical synthesis, with the result that, during the 1990s, protein kinases became the second most important drug target after G-protein-coupled receptors.[7]

In parallel with the development of indolocarbazoles as anticancer drugs targeting protein kinases, mammalian DNA topoisomerase I was shown to be a new target for indolocarbazoles by Yamashita *et al.*[8] Thereafter, many antitumor indolocarbazoles have been synthesized, as DNA topoisomerases were known to be targets for antitumor drugs such as camptothecin and VP-16. DNA topoisomerases alter DNA topology by transiently breaking and re-sealing one strand of DNA through a covalent protein–DNA intermediate.[9] In 1996, it was shown that topoisomerase I has an intrinsic protein kinase activity (Topo I kinase) required for phosphorylation of the SR (serine arginine-rich) protein required for splicing.[10]

The action of indolocarbazole derivatives on topoisomerase indicated that these compounds may selectively interact with ATP-binding sites of not only protein kinases but also other proteins. As an example of this, during the current decade, it was shown that ABCG2, an ABC transporter with importance in drug resistance, oral drug absorption and stem cell biology, could be a key new target for indolocarbazoles.

This review outlines the pivotal pioneering studies relating to the discovery, biosynthesis and biological activities of natural indolocarbazole products.

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PRODUCING ORGANISM

Staurosporine was discovered in 1977 in a culture of an actinomycete (*Streptomyces* strain AM-2282^T) while screening for microbial alkaloids using chemical detection methods[11]. The strain AM-2282^T (NRRL 11184, ATCC 55006) has been renamed through repeated revisions of the taxonomy of soil *Actinomyces* as *Streptomyces staurosporeus* AM-2282^T in 1977, *Saccharothrix aerocolonigenes* subsp. *staurosporea* AM-2282^T in 1995[12] and *Lentzea albida* in 2002.[13] Over the past 30 years, staurosporine and related natural indolocarbazole compounds have been isolated from several *actinomycetes* (including *Streptomyces*, *Saccharothrix*, *Lentzea*, *Lechevalieria*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Actinomadura* and *Micromonospora*) as well as from myxomycetes (slime molds) and cyanobacteria (Figure 1).

Staurosporine derivatives have also been isolated from marine invertebrates, such as sponges, tunicates, bryozoans and mollusks. However, it remains unknown whether invertebrates actually have genes for indolocarbazole biosynthesis, as many natural products from marine invertebrates are produced by associated microorganisms.[14]

Interestingly, half of the 14 indolocarbazole-producing strains deposited in the global culture collection have been isolated from Japanese soils. In the 1980 s, fermentation broths of 5163 new Japanese soil isolates were tested and five *Streptomyces* were found to produce staurosporine, together with new analogs (UCN-01 and UCN-02 (stereo-isomers of 7-hydroxy staurosporines)). In other words, ca 0.1% of newly isolated soil actinomycetes were shown to produce staurosporine using a fixed culture condition.[15]

In 1993, staurosporine and K252a were shown to inhibit *in vitro* phosphorylation of crude extracts from *Streptomyces griseus* and also from a staurosporine-producing *Streptomyces* sp.[16] Although staurosporine did not show significant antibacterial activity, it was shown to affect cell differentiation processes in *Streptomycetes*, such as pigment production and spore formation, depending on the AfsK family serine/threonine protein kinases involved. Later, on the basis of genome sequence analysis of *Streptomyces avermitilis* in 2001[17] and *Streptomyces coelicolor* in 2002,[18] it was revealed that more than 30 protein kinase genes are coded in these organisms. Further research is needed to determine the exact role and impact of staurosporine on differentiation of producing strains and microorganisms in soil.

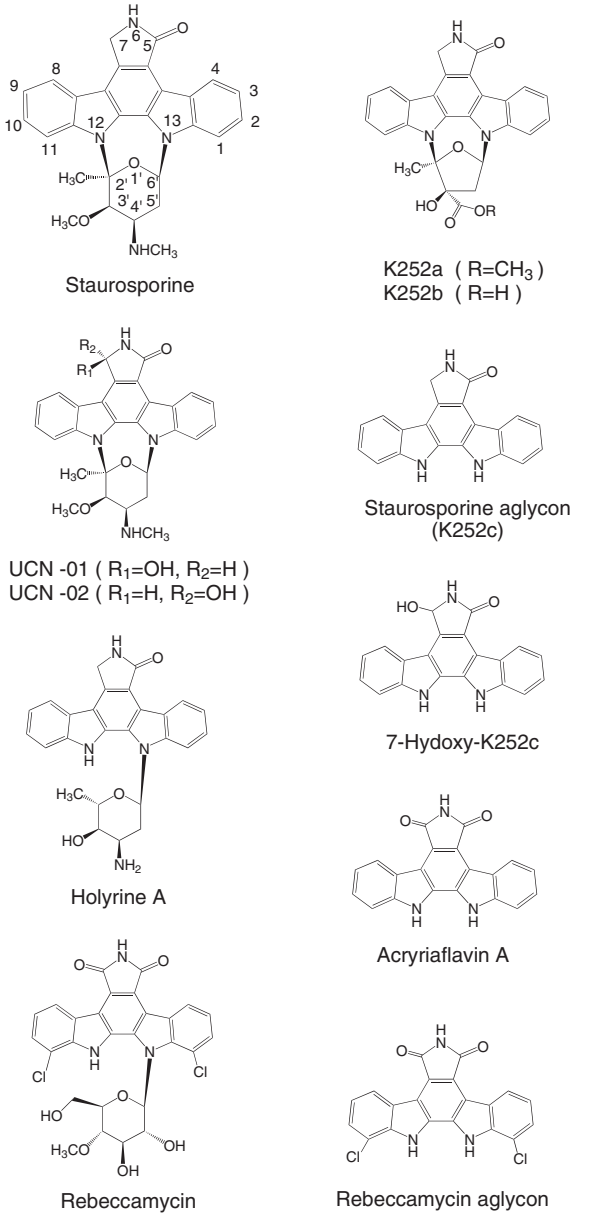


Figure 1 Chemical structures of staurosporine and related indolocarbazoles isolated from culture broths.

BIOSYNTHESIS OF STAUROSPORINE

Biosynthetic studies carried out in the 1980s and 1990s using isotope-labeled precursors showed that the indolocarbazole structure of staurosporine is derived from two molecules of tryptophan, and that the sugar moiety is derived from glucose and methionine. Cloning of the biosynthetic genes of staurosporine was triggered in 2000 by identification of the *ngt* gene encoding *N*-glycosyltransferase. Ohuchi *et al.*[19] heterologously expressed the *ngt* transferase gene from *Lechevalieria aerocolonigenes*, a rebeccamycin producer, in *Streptomyces lividans* and showed that *ngt* is responsible for N-glycosylation of the indolocarbazole chromophore. Starting from the *ngt* gene, whole biosynthetic gene clusters of staurosporine and rebeccamycin have been cloned by Onaka *et al.*[20,21] and Sanchez *et al.*[22] To date, structures of the accumulated products from 18 single-gene disruption mutants of staurosporine and rebeccamycin biosynthesis gene clusters have been identified (Figure 2).

Studies of these accumulated products and the gene function predicted by the amino-acid sequence database searches have revealed the biosynthetic pathway of staurosporine and rebeccamycin.[14,23] (Figure 3).

In staurosporine biosynthesis, staO initiates synthesis by catalyzing L-tryptophan to the imine form of indole-3-pyruvic acid (IPA imine) and staD, and then catalyzes the coupling of two IPA imines to yield chromopyrrolic acid. Formation of the indolocarbazole core of staurosporine is catalyzed by staP, which converts chromopyrrolic acid into three indolocarbazole compounds, staurosporine aglycone (K252c), 7-hydroxy-K252c and acryriaflavin A, by intramolecular C–C bond formation and oxidative decarboxylation. Crystallography of P450 staP revealed that a heme of staP removes two electrons from the indole ring to generate an indole cation radical, and intramolecular radical coupling then forms the C–C bond to yield the indolocarbazole core.[24] The presence of staC predominantly directs the formation of K252c. staG catalyzes N-glycosidic bond formation between N-13 and C-6′ and then staN, a P450 homolog, catalyzes an additional C–N bond formation between N-12 and C-2′. These two enzymes convert K252c to 3′-*O*-demethyl, 4′-*N*-demethyl-staurosporine through holyrine A and holyrine B. staMA catalyzes *N*-methylation

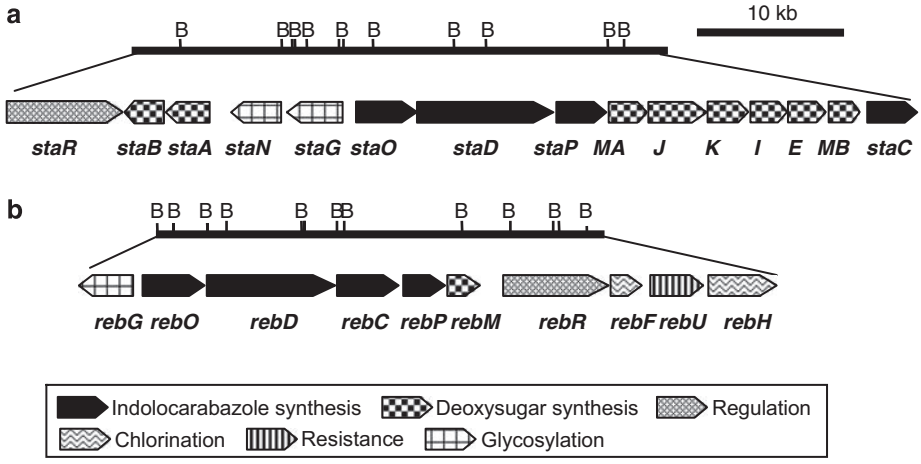


Figure 2 The gene cluster encoding biosynthesis of staurosporine and rebeccamycin. Restriction and organization chromosomal map of the sta locus (**a**) from *Streptomyces* TP-A0274 and reb locus (**b**) from *Lechevalieria aerocolonigenes* ATCC39243. 'B' indicates the *Bam*HI sites (adapted from Onaka[23]).

of 3′-*O*-demethyl, 4′-*N*-demethyl-staurosporine and staMB catalyzes *O*-methylation, which results in the formation of staurosporine.

In rebeccamycin biosynthesis, conversion of L-tryptophan to 7-chloro-L-tryptophan is the first step, the reaction being catalyzed by a two-component (halogenase rebH and flavin reductase rebF) enzyme. Subsequently, rebO and rebD catalyze 7-chloroindole-3-pyruvic acid imine formation, and coupling of two imines yields 11,11-dichlorochromopyrrolic acid.

The genes involved in the main pathway of indolocarbazole structure formation in staurosporine and rebeccamycin showed striking similarity between *staO*, *staD*, *staP*, *staC* and *staG*, and *rebO*, *rebD*, *rebP*, *rebC* and *rebG*, respectively. The formation of chromopyrrolic acid or 11,11-dichlorochromopyrrolic acid, key intermediates of indolocarbazole biosynthesis, is catalyzed by staD or rebD. It is noteworthy that the staD family includes only two homologs, rebD and VioB, which are involved in violacein biosynthesis. The staD family is a new type of hemoprotein with a novel structure and function.[25,26]

N-glycosidic bond formation between the N-12 and C-1′ positions is catalyzed by staG or rebG *N*-glycosyltransferases. *rebG* is the same gene that was cloned in 2000 by Ohuchi *et al.* as *ngt*, which can catalyze the N-glycosylation of the indolocarbazole chromophore. In the staurosporine structure, there exists an additional, unusual C–N bond between the N-13 and C-6′ positions. Onaka *et al.* showed through gene disruption and bioconversion experiments that staN, a P450 homolog, is responsible for this unusual C-N bond formation. StaN was the first example used to show that the P450 homolog is involved in N-glycosidic bond formation. Deletion of *staG* abolished glycosylation and led to accumulation of K252c, whereas deletion of *staN* resulted in the production of holyrine A. Salas *et al.* also showed the function of staN in C–N bond formation by heterologous expression of the *staN* gene.[27]

COMBINATORIAL BIOSYNTHESIS OF STAUROSPORINE ANALOGS

Single-gene disruption studies and biochemical characterization of enzymes of staurosporine and rebeccamycin biosynthesis gene clusters have revealed the entire biosynthetic pathways of staurosporine and related indolocarbazole compounds. In addition, the entire sets of both staurosporine or rebeccamycin biosynthetic gene clusters were heterologously expressed in *Streptomyces lividans* by Onaka *et al.*[20] and in *Streptomyces albus* by Salas and collaborators,[14,28] which resulted in the production of staurosporine or rebeccamycin in the surrogate hosts.

During and based on the above studies, new analogs of staurosporine and rebeccamycin have been identified as follows (Figure 4):

1. Intermediates of biosynthetic pathways

Although the majority of compounds identified as intermediates of the indolocarbazole biosynthetic pathways were previously isolated from culture broth as minor components, 11,11-dichlorochromopyrrolic acid was a new discovery that arised from use of an *rebP* disrupted mutant.

2. New analogs produced by heterologous gene expression

10-Hydroxy-staurosporine aglycone (10-hydroxy K252c) was produced in a *staG*-deficient strain by heterologous gene expression of *ToxA* gene, a tryptophan hydroxylase from *Streptomyces mobaraense* FERM BP-2785, which produces BE-13793C (1,11-dihydroxy, 7-oxostaurosporine aglycone). 3′-demethyl-3′-acetylstaurosporine was isolated from a *staMA*-blocked mutant.

3. Adaptability of enzymes in indolocarbazole biosynthetic pathways to unnatural substrates

Replacing sugar moiety: staG was shown to accept a variety of sugar derivatives. It has been used successfully for producing novel glycosylated indolocarbazoles consisting of L-rhamnose, L-olivose, L-digitoxose or D-olivose by Salas *et al.* In contrast to staG, no sugar derivative other than NDP-D-glucose has been reported as a substrate for rebG.

Tryptophan halogenase: rebF/rebH, tryptophan halogenase in rebeccamycin biosynthesis, accepts chloride and bromide ions. rebO could also use 1-methyl, 5-methyl and 5-fluoro-L-tryptophan as substrates.

4. Analogs with different oxidation states at the C-7 position

Many analogs with a different oxidation state at the C-7 position of the pyrrole ring in the indolocarbazole chromophore have been isolated. rebP and staP were functionally equivalent, and any of the cytochrome *P*450 enzymes could be responsible for the decarboxylative oxidation of a chromopyrrolic acid intermediate into indolopyrrolocarbazole aglycone. A mixture of the three indolopyrrolocarbazole chromopyrrolic acids, which differ in their oxidation states at the C-7 position, were produced in the absence of staC or rebC. Interestingly, rebC and staC determine different oxidation states in the final product. Addition of *staC* produced the single product K252c, whereas acryriaflavin A was produced by *rebC*.

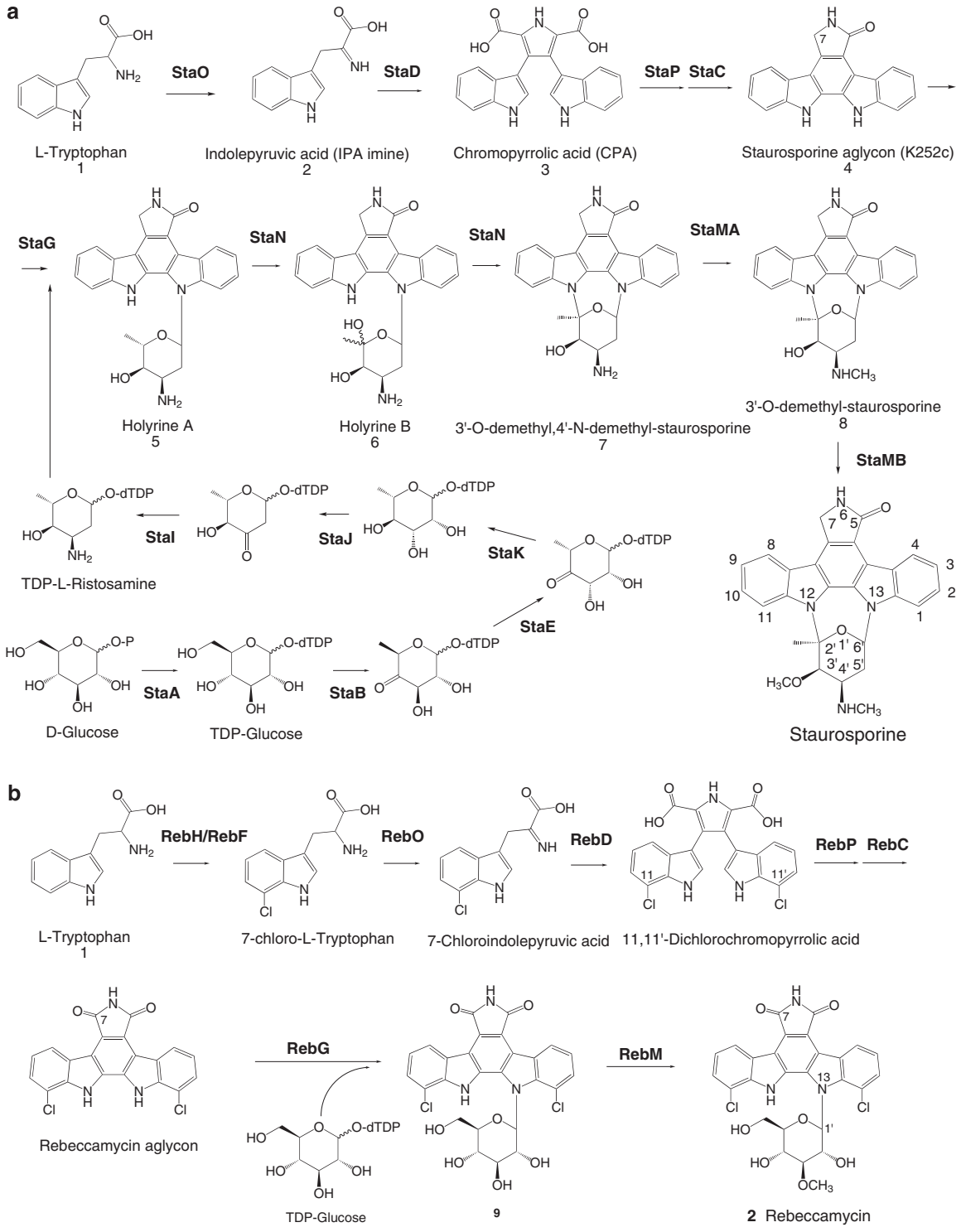


Figure 3 The proposed overall biosynthetic pathways of staurosporine (**a**) and rebeccamycin (**b**) (modified from Onaka[23]).

BIOLOGICAL ACTIVITIES

Inhibition of mammalian protein kinases

In the mid-1980s, staurosporine and the related indolocarbazole K252 were shown to be potent inhibitors of protein kinases by Kyowa Hakko Co. (Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan)[29,30] Following the discovery that staurosporine was a nanomolar inhibitor of protein serine/threonine kinases such as protein kinase C and also protein tyrosine kinase v-src[31] (Figure 5), many laboratories and pharmaceutical companies sought selective protein kinase inhibitors by chemical synthesis or screening of new natural products. 7-Hydroxystaurosporine (UCN-01) was identified during microbial screening for selective protein kinase C inhibitor.[32] UCN-01 showed antitumor activity in mouse tumor models and thus entered clinical studies.[33]

However, it was difficult to screen selective inhibitors against human protein kinases in the mid-1980s until bacculovirus expression systems for large-scale production of human proteins were developed

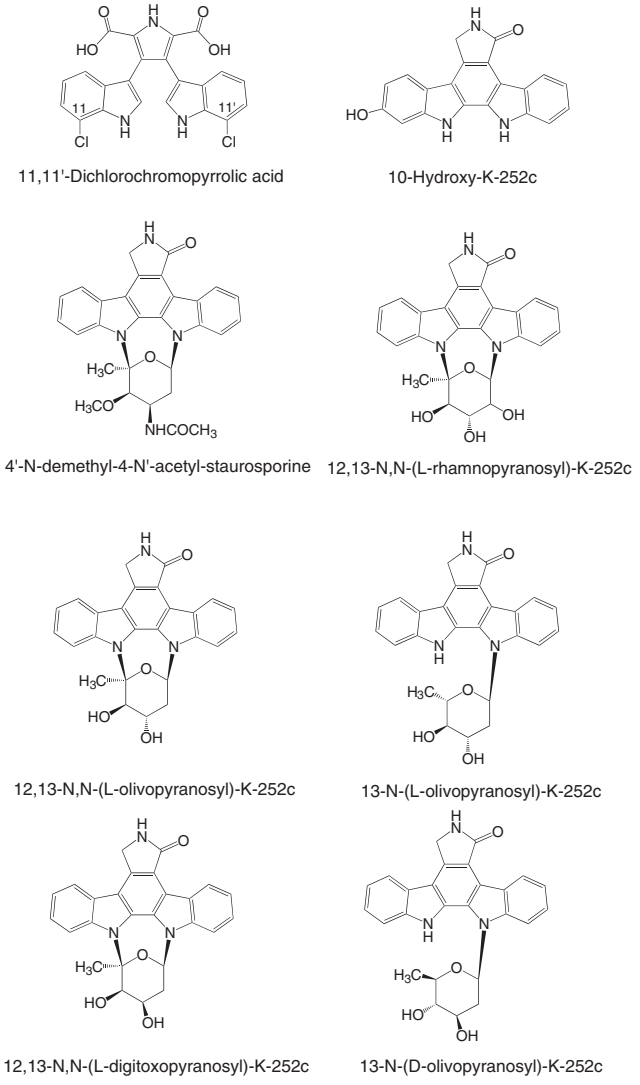


Figure 4 Chemical structures of new indolocarbazoles produced by combinatorial biosynthesis.

in the late 1980s. Researchers in Ciba-Geigy (Novartis International AG, Basel, Switzerland) started a protein kinase project in the mid-1980s and, in the early 1990s, a synthetic inhibitor named CGP5714B (later named imatinib, structure in Figure 6) was discovered. In 1996, CGP5714B was shown to be a potent and selective inhibitor in colony formation assay against tumor cells from chronic myelogenous leukemia in which Bcr-Abl tyrosine kinase is activated.[34] Imatinib (Gleevec) entered human clinical trials on chronic myelogenous leukemia in 1998 and was approved for use in 2001 in the USA.

Following the success of imatinib, EGF receptor tyrosine kinase inhibitors such as gefitinib (Iressa) and erlotinib (Tarceva) were approved for the treatment of lung cancer. Consequently, protein kinases have become the second most important drug targets after G-protein-coupled receptors.[7]

Staurosporine-related structures (for example, indolocarbazole aglycone, diindolylmaleimides and dianilinophthalamides) have been derivatized and developed into selective inhibitors of pharmacologically interesting targets. The structure of several representative compounds evaluated in clinical studies on cancer, such as midostaurin (CGP41251), lestaurtinib (CEP-701, KT-5555) and enzaistaurin (LY317815), are shown in Figure 6.[7,14,35,36]

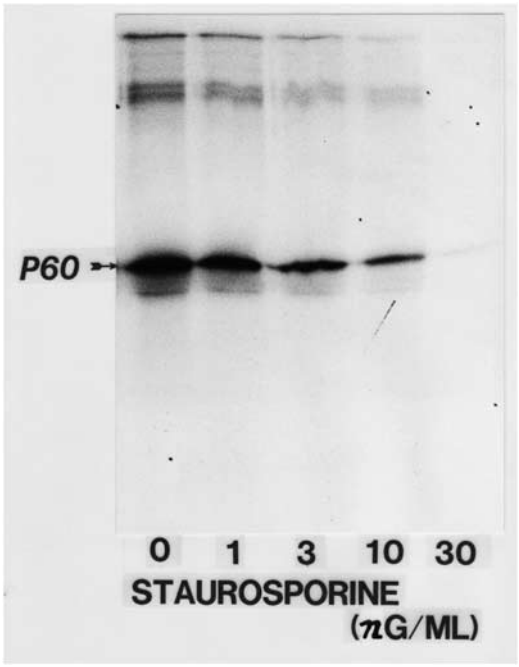


Figure 5 Inhibition of tyrosine-specific protein kinase activity of $p60^{v-src}$ by staurosporine. $p60^{v-src}$ was immunoprecipitated from the lysates of chicken embryo fibroblasts infected with Raus sarcoma virus by anti-p60 serum. The immunoprecipitates were incubated with kinase assay buffer 820 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$ and 10 μCi [γ-^{32}P]ATP for 30 min at 20°C. Staurosporine was dissolved, diluted in dimethyl sulfoxide (DMSO) and added to the assay buffer at the concentrations indicated. The reaction products were analyzed on SDS-10% acrylamide gel. The dried gel was exposed to X-ray film for 90 min (adapted from Nakano *et al.*[31]).

In addition to antitumor activity, staurosporine and K252a derivatives possess many other useful pharmacological properties. The γ-lactone form of staurosporine was shown to have antiplatelet aggregation activity, although it has more than 100-fold lower activity against protein kinase C and smooth muscle contraction.[37] A series of 3,9-disubstituted K252a derivatives have been synthesized and evaluated for neurotrophic activity. KT-7515 (CEP1347), an ethylthiomethyl derivative of K252a, showed reduced kinase inhibitory properties for *trk* A, PKC and PKA while enhancing neurotrophic activity.[38]

Staurosporine was shown to inhibit many protein serine-threonine kinases and tyrosine kinases, and has been used as the reference compound for various protein kinase assays. In 2002, based on human genome sequence data, 518 protein kinase genes were identified (about 1.7% of the total number of human structure genes).[39] Figure 7 shows the IC_{50} values of staurosporine against 235 protein kinases assayed using kinases produced by bacculovirus expression systems.[40] Figure 8 shows the inhibitory profiles of imatinib and gefitiniib, determined under the same assay conditions.[40] The inhibitory profile of imatinib is not directly linked to the position of protein kinases on the evolutionary dendrogram; it exhibits comparable efficiencies at inhibiting PDGFR and KIT, which are more divergent from the Abl tyrosine kinase than the SRC subfamily, while also exhibiting low inhibition potency with regard to the tyrosine kinase activity of the SRC family.

The crystal structures of staurosporine and protein kinases, including PKC isozymes, cyclin-dependent kinases and EGF receptor tyrosine kinase, showed that several binding sites of staurosporine overlap with those of ATP.[41] Further, comparison of the crystal structures of staurosporine and UCN-01 in complex with the kinase domain of CHK1 (cell cycle checkpoint kinase-1) and PDK1

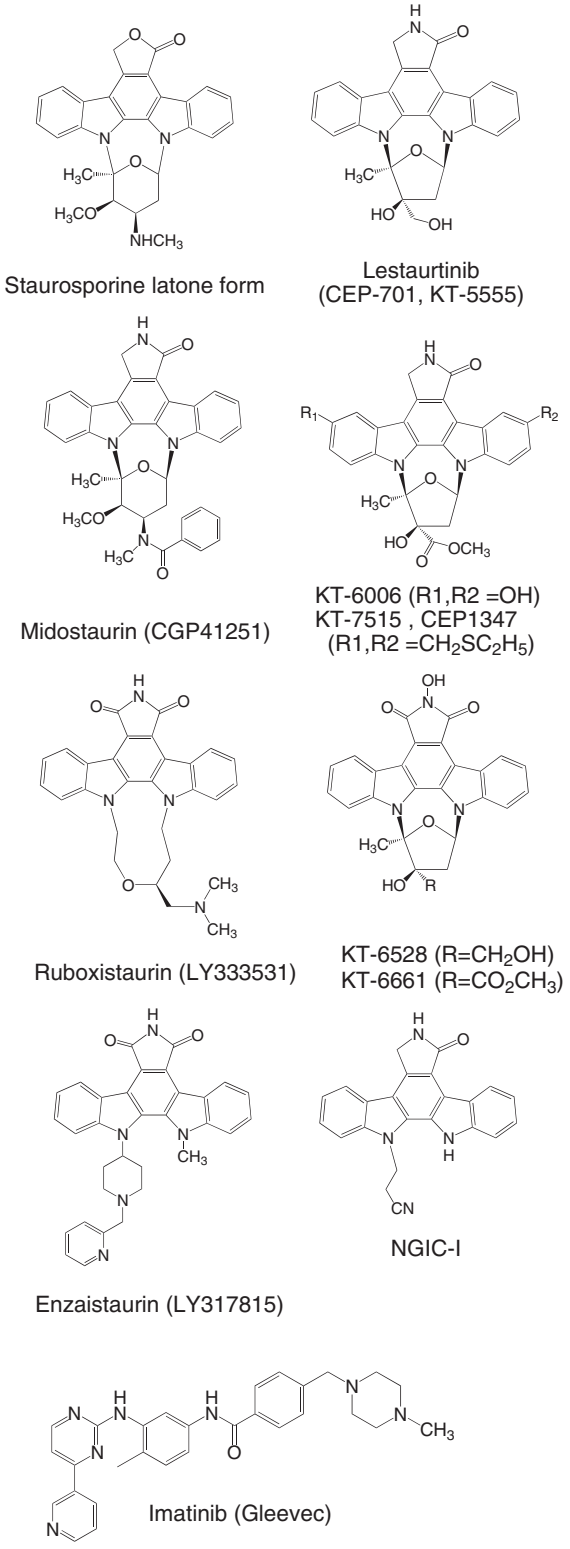


Figure 6 Chemical structures of some synthetic indolocarbazole derivatives and mimics that stimulate interest in drug discovery targeting protein tyrosine kinases and topoisomerases.

(3-phosphoinositide-dependent protein kinase-1) showed that the 7-hydroxy group of UCN-01 generates additional hydrogen-bonding interactions with active site residues, directly to threonine or serine and indirectly through an ordered water[42] (Figure 9).

In 2004, the crystal structure of Gleevec bound to Syk (Spleen tyrosine kinase) revealed that imatinib binds Syk in a novel, compact *cis* conformation that differs from the binding mode observed with unphosphorylated Abl, the imatinib-sensitive form of Abl. The *cis* conformation of imatinib bound to Syk closely resembles the structure of staurosporine bound to Syk. The pyridine, pyrimidine and benzene rings of imatinib closely correspond with all the three coplanar arms of staurosporine[43] (Figure 10).

Anti-malarial and anti-trypanosomal drug targeting protein kinases in protozoa

New drugs and molecular targets are needed against human parasitic protozoa, such as *Plasmodium falciparum* and *Trypanosoma brucei*, due to the increased resistance to existing drugs.[44] The clinical success of protein kinase inhibitors against various human diseases stimulated a search for protein kinases in human parasite protozoa. Prior to the genome sequence analysis of the human malarial parasite *P. falciparum*, several plasmodial protein kinases were identified through homology with mammalian protein kinases. The three-dimensional structure of PfPK5, a cyclin nuclear division cycle-dependent kinase (CDK) in *P. falciparum*, has been identified. Staurosporine inhibits PfPK5 with an IC_{50} 1000 nM less potent than that of the CDK2 inhibitors NU6102 (215 nM) and purvalanol B (130 nM).[45]

In 2004, completion of the whole genome sequence of *P. falciparum* revealed profound divergences between kinomes of the parasite and those of its host. About one-third of the 85 *Plasmodium* protein kinases are 'orphans,' which do not cluster with any of the protein kinase families established from any mammalian or yeast kinomes. These may turn out to represent very attractive targets for novel, parasite-specific protein kinase inhibitors. In addition, homology of ortholog genes showed 40–60% similarity between the host and the parasite; hence, it will be highly beneficial to identify protein kinase inhibitors with 'parasite versus host' selectivity targeting protein kinases that are essential for parasite development.[46]

In 2005, the sequenced genomes of three human trypanosomatid protozoa, *Leishmania major*, *T. brucei* and *Trypanosoma Cruzi*, showed that they encode 179, 156 and 171 eukaryotic protein kinases, respectively (about one-third of the human complement).[47]

Activity against microbial and viral protein kinases

Staurosporine was shown to have antifungal activity, but to lack significant antibacterial activity. However, in the 1990s, many protein Ser/Thr kinases were identified in prokaryotes and viruses, including several pathogenic organisms. Reports suggested that protein kinases in pathogenic mycobacteria could be valuable new therapeutic targets for antituberculosis drug discovery.[48] In 2007, K252a, K252b and staurosporine were shown to inhibit a mycobacterial protein kinase PknB with IC_{50} values of 96, 106 and 600 nM, respectively. The PknB gene is strictly conserved in all known mycobacterial genomes and some related actinomycetes. PknB is a receptor-like transmembrane protein, with an extracellular signal sensor domain and an intracellular kinase domain that shares similarity with eukaryotic protein kinases. Staurosporine and K252a were found to inhibit the growth of *Mycobacterium tuberculosis* H37Rv at 5–50 μM, whereas K252b failed to inhibit the growth.[49]

The protein kinases were considered to be evolutionarily segregated into eukaryotic serine/threonine/tyrosine kinases and prokaryotic histidine kinase. However, genome sequences of bacteria revealed an abundance of serine/threonine/tyrosine protein kinases (for example, in pathogenic strains; 13 in *M. tuberculosis* and 5 in *Corynebacterium diphtheriae*.[50] In addition, phosphoproteome analysis using high-

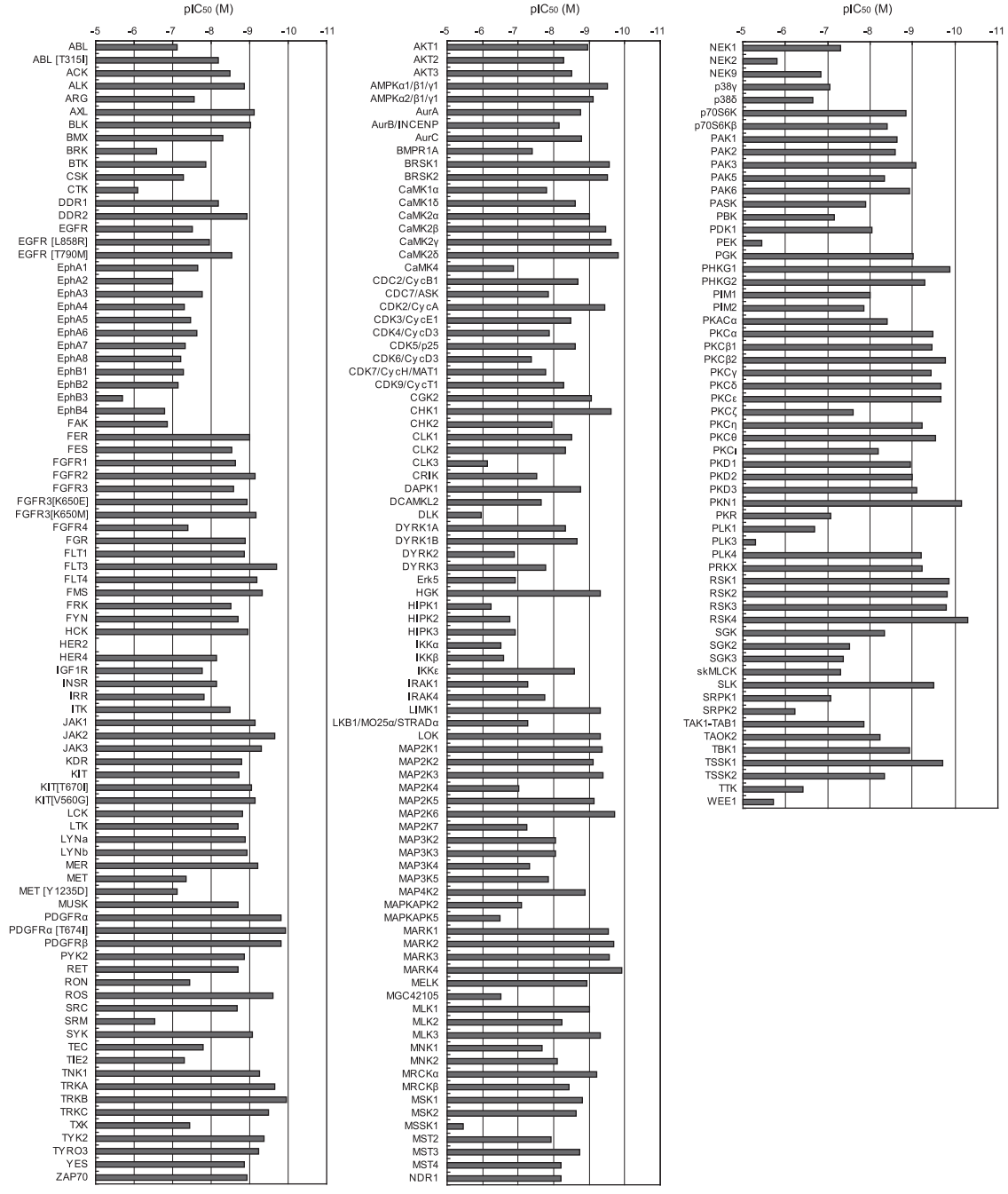


Figure 7 IC_{50} of staurosporine against 235 protein kinases (by Carna Bioscience Inc., Kobe, Japan). Inhibition profiles were assayed by mobility shift assay using kinases produced as N-terminal glutathione *S*-transferase (GST) fusion protein by bacculovirus expression systems and purified by glutathione sepharose chromatography. The kinase reaction takes place in a 384-well plate. A capillary sipper transfers the sample into the microchip, which serves as a separation device for the substrate, and the phosphorylated product is detected through laser-induced fluorescence. The substrate design and peak separation condition of the substrate and products are critical and were optimized for each kinase. Assay conditions for each kinase are published in a kinase profiling book by Carna Bioscience Inc.: http://www.carnabio.com/output/pdf/ProfilingProfilingBook_ja.pdf.

resolution mass spectrometry showed that the ratio of phosphotyrosine, phosphothreonine and phosphoserine is 10:20:70 in *Bacillus subtilis*, similar to the ratio of 2:12:86 in human cells as determined by the same method.[51]

Indolocarbazoles also possess antiviral properties, including activity against the human immunodeficiency virus, cytomegalovirus and Epstein–Barr virus.[52–54] Several indolocarbazoles inhibit the protein kinase activity of pUL97, encoded by human cytomegalovirus, NGIC-I being the most effective, exhibiting an IC_{50} of 42 nM, and an IC_{50} of 38 nM in an antiviral assay using green fluorescent protein expressing recombinant human cytomegalovirus.

Action on topoisomerases

DNA topoisomerases have been shown to be important targets for antitumor drugs and antibacterial agents. Both antitumor drugs in eukaryotes and quinolone antibiotics in prokaryotes stabilize

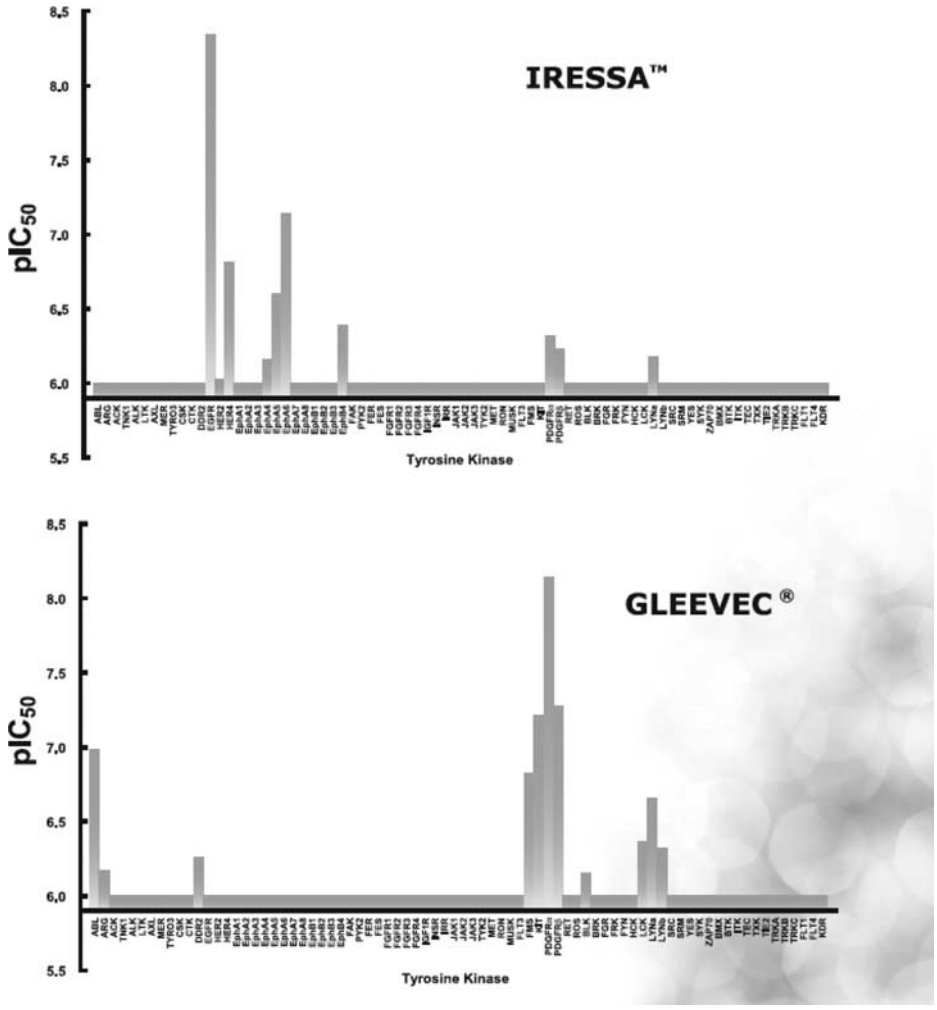


Figure 8 Kinase profiling results of imatinib (Gleevec) and gefitinib (Iressa) (by Carna BioScience).[40] Adapted from a publication in 'Screening-Trends in Drug Discovery' (journal issued by GIT Verlag GmbH & Co. KG).

DNA-cleavable complexes with the topoisomerases of target cells. In 1992, Yamashita *et al.* showed that K252 derivatives, such as KT-6006, KT-6528 and KT-6661 (structures in Figure 6) induce a DNA-cleavable complex with mammalian topoisomerase I.[8] These semisynthetic derivatives of K252a were potent inducers of DNA-cleavable complexes with topoisomerase I, whereas rebeccamycin was a weak inducer in the same assay (Figure 11).

Many antitumor indolocarbazoles targeting topoisomerase have since been synthesized. Indolocarbazoles are now the most advanced non-camptothecin topoisomerase I inhibitors in clinical development.[55] In 1996, topoisomerase I was shown to have an intrinsic protein kinase activity (Topo I kinase). Topo I kinase is required for phosphorylation of the SR protein that functions in ESE (exonic splicing enhancer)-dependent splicing.[10] Human topoisomerase I exhibits at least two different conformations: (1) as drug targets in the form of a complex with substrates of the kinase reaction and (2) a topoisomerase I–DNA complex. Based on the common structure of ternary trapping by small molecules of the intermediate protein complex, such as a topoisomerase I–DNA covalent complex, Pommier *et al.* proposed the 'interfacial inhibitor paradigm.' This paradigm described for topoisomerase I inhibitors can be generalized to cover a variety of natural products that trap macromolecular complexes that undergo catalytic conformational changes providing hotspots for drug binding.[56] Staurosporine and related indolocarbazoles represent natural products that can trap several of these interfacial macromolecular complexes.

CONCLUSION

We have reviewed several key components of the chemistry and biology of natural indolocarbazole products elucidated during the three decades since the discovery of staurosporine in 1977. Over the same timeframe, remarkable progress has been made in molecular and cellular biology, such as molecular target identification of human diseases, genome-based research on the human genome and analysis of crystal structures of drug–protein complexes using human proteins produced by bacculovirus–insect cell expression systems. Staurosporine and related natural indolocarbazole products have attracted not only scientists in drug discovery and development but also chemists and biologists engaged in biosynthetic gene discovery, combinatorial biosynthesis and cell biology. Owing to space constraints, we have not been able to describe an important activity of staurosporine, namely the induction of cell death (apoptosis). To our surprise, a search for 'staurosporine' plus 'apoptosis' on 'Google scholar' resulted in 18 300 hits. Staurosporine shows an extremely strong cytotoxic activity in some cases (4 pM, Hela-S3, 72 h exposure) and induces apoptosis. Several tumor cell lines are completely resistant to different anticancer

drugs, but remain sensitive to staurosporine-induced apoptosis.[57] Further research on the use of staurosporine derivatives in anticancer therapy is clearly necessary, and the molecular mechanism of staurosporine-induced apoptosis remains to be determined.[58]

In the mid-2000s, nearly 30 years after the discovery of staurosporine, it was revealed that ABCG2, an ABC transporter with importance in cancer drug resistance, oral drug absorption and stem cell biology, is a promising new target for indolocarbazoles such as UCN-01 and bisindolylmaleimide.[59,60] Consequently, we predict that a library of compounds derived from staurosporine and related indolocarbazoles will have huge potential for inhibition of new targets with ATP-binding domains, in addition to mammalian protein kinases, topoisomerases and ABC transporters.

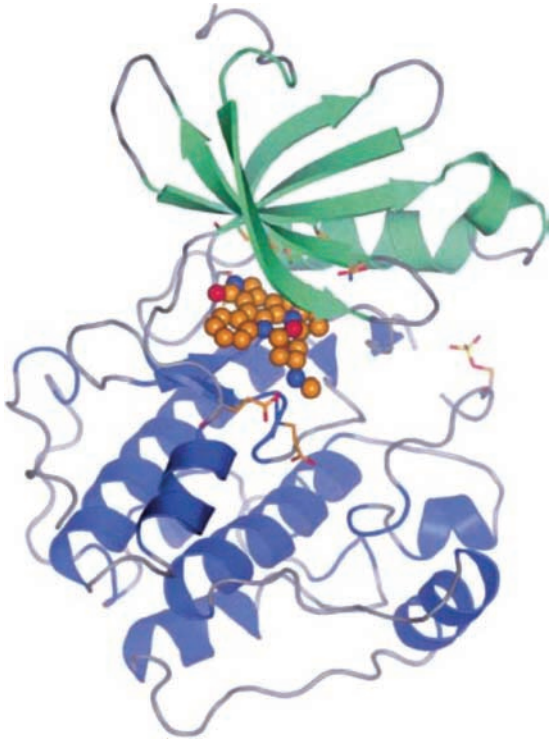


Figure 9 Overview of PDK1 kinase domain bound to staurosporine. The two lobes (in green (N-terminal lobe) and blue (C-terminal lobe)) of the kinase enclose the inhibitor molecule (shown by the orange spheres). The phosphorylated T-loop (shown as a stick representation with a yellow phosphorus atom and red oxygen atoms) lacks residues, due to disorder. Side chains interacting with the inhibitor molecule are shown as a stick representation with green carbon atoms (adapted from Komander *et al.*[42]). See online version for color figure.

Clearly, staurosporine and related natural products have a supreme ternary structure helping in their interaction with several important drug target proteins. They represent a splendid and highly adaptable

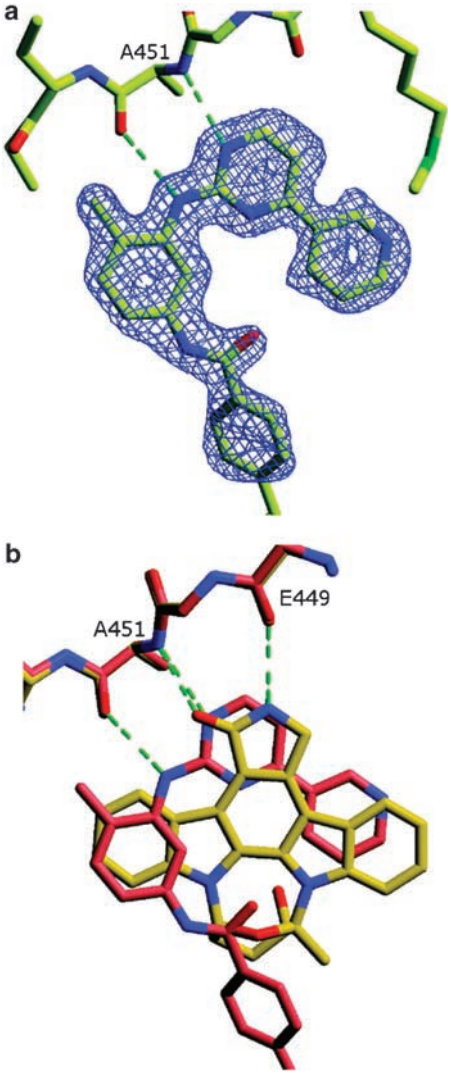
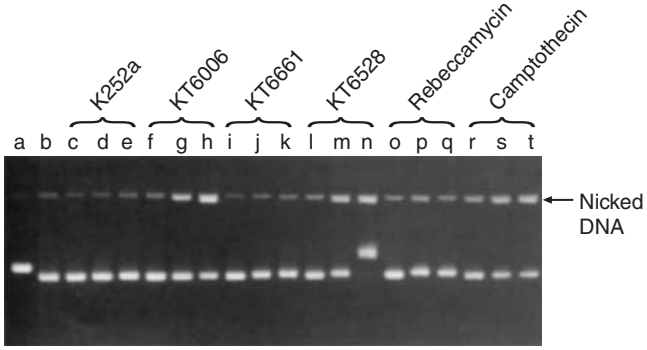


Figure 10 Structure of imatinib (Gleevec) bound to Syk (spleen tyrosine kinase) bearing a striking resemblance to the structure of staurosporine. (**a**) Binding of imatinib to Syk. Ligand difference in electron density. (**b**) Binding of imatinib and staurosporine to Syk. Imatinib (yellow) binding to Syk in the compact *cis* conformation mimics the structure and binding mode of staurosporine (pink). The key residues are labeled and hydrogen bonds are denoted with dashed lines (adapted from Atwell *et al.*[43]). See online version for color figure.

Figure 11 Mammalian DNA topoisomerase I-mediated DNA cleavage activities of indolocarbazole derivatives. *Methods*: In DNA cleavage assay, 0.48 μg of supercoiled pBR322 DNA was incubated with 100 U of topoisomerase I in the presence of drugs (lanes c–t) followed by SDS/proteinase K treatment, and was then analyzed on an agarose gel containing 0.5 $\mu g ml^{-1}$ ethidium bromide. Lane a, CCC-DNA control; lane b, no drug; lanes c–e, K252a; lanes f–h, KT6006; lanes i–k, KT6661; lanes l–n, KT6528; lanes o–q, rebeccamycin; lanes r–t, camptothecin. Drug concentrations were (lanes c, f, i, l, o and r) 0.5, (lanes d, g, j, m, p and s) 5 and (lanes e, h, k, n, q and t) 50 μM. *Results*: KT6006 induced topoisomerase I-mediated DNA cleavage in a dose-dependent manner at drug concentrations up to 50 μM, whereas DNA cleavage induced by KT6528 was saturated at 5 μM. The maximal amount of nicked DNA produced by KT6006 was more than 50% of substrate DNA, which was comparable to that of camptothecin (adapted from Yamashita *et al.*[8]).



gift from nature, which can be manipulated by human endeavor to bestow immeasurable benefit for human health.

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REVIEW ARTICLE

Micafungin: a sulfated echinocandin

Seiji Hashimoto

Micafungin is the second approved antifungal agent in the echinocandin series and is now used worldwide in chemotherapy for life-threatening fungal infections. It is water-soluble and is semi-synthesized from the acylated cyclic hexapeptide FR901379, a natural product from the fungus *Coleophoma empetri* F-11899, through enzymatic deacylation of FR901379, followed by chemical reacylation with the optimized *N*-acyl side chain. The water solubility of micafungin is ascribed to a sulfate moiety in the molecule. This feature differentiates micafungin from other echinocandin members. Micafungin is a potent inhibitor of 1,3-β-glucan synthase, an enzyme necessary for cell-wall synthesis of several fungal pathogens.
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Keywords: antifungal; echinocandin; FK463; 1,3-β-glucan synthase; micafungin

INTRODUCTION

Fungal infections cause not only superficial diseases such as athlete's foot and onychomycoses, but also life-threatening diseases. Serious deep-seated fungal infections caused by *Candida* spp., *Aspergillus* spp. and *Cryptococcus neoformans* are a threat to human health. Incidences of these systemic fungal infections have increased significantly over the past few years. The major reasons for this dramatic increase are the extensive use of broad-spectrum antibiotics and the growing number of immunocompromised patients with acquired immunodeficiency syndrome (AIDS), cancer and transplants.[1,2]

In the mid 1900s, few compounds, such as polyenes (for example, nystatin and amphotericin B) and flucytosine, were available for antifungal chemotherapy. Although the development of azole drugs started in the early 1970s, only a limited number of antifungal agents were available for treatment of life-threatening fungal infections. Moreover, the existing agents had disadvantages, such as the significant nephrotoxicity of amphotericin B[3] and the emergence of resistance to the azoles.[4] To overcome these defects, lipid formulations of polyenes were developed to reduce toxicity, and new triazoles (for example, voriconazole, ravuconazole and posaconazole) were developed to improve the antifungal spectra or susceptibility to azole-resistant isolates.[5] Despite a number of therapeutic advancements, there was a need to develop a new class of antifungal agents with novel mechanisms of action.

The echinocandins were a new class of antifungal drugs developed for the first time since azoles. The first launched echinocandin was caspofungin acetate (Merck & Co. Inc. (Merck), Readington, NJ, USA), followed by micafungin (Fujisawa Pharmaceutical Co., Ltd, now Astellas Pharma Co., Ltd, Fujisawa, Japan) and anidulafungin (Vicuron Pharmaceuticals Inc., now Pfizer Inc., New York, NY, USA), which was originally developed by Eli Lilly and Company (Indianapolis, IN, USA) (Lilly) as LY 303366 and subsequently licensed to Vicuron (formerly Versicor) as VER-002. The approved echinocandins are synthetically modified lipopeptides that originate from natural compounds produced by filamentous fungi. The original anidulafungin, caspofungin and micafungin compounds were echinocandin B from *Aspergillus nidulans var. echinulatus*,[6] pneumocandin B0 from *Glarea lozoyensis*[7] and FR901379 from *Coleophoma empetri*,[8] respectively.

Although natural echinocandins have potent antifungal activity *in vitro*, their structures were chemically altered to improve their absorption, distribution, metabolism and excretion characteristics. Such operations were initiated by Lilly on echinocandin B to yield cilofungin.[9] This compound was subjected to Phase II clinical trials, but was abandoned due to toxicity. Further modification of the structure by converting the phenolic hydroxyl to a sodium phosphate ester produced the more soluble prodrug LY307853, which resulted in the active form, LY303366.[10] Merck has produced MK-0991 using pneumocandin B0 as the starting material.[11] MK-0991 likewise possesses increased water solubility. Other reviews on echinocandins or individual antifungal agents have reported their usefulness in clinical practice.[12–16] This review describes the discovery and development of micafungin, focusing on the chemical diversity of echinocandins.

DISCOVERY OF FR901379

The seed compounds of micafungin, FR901379 and two related compounds (FR901381 and FR901382), were discovered at Fujisawa Pharmaceutical Co., Ltd in 1989 from the screening of approximately 6000 microbial broth samples (Figure 1). These new compounds were categorised as members of the echinocandin class of lipopeptides. Echinocandin B, pneumocandin B0 and other echinocandin lipopeptides are structurally characterized by a cyclic hexapeptide acylated with a long side chain, and have an excellent anti-*Candida* activity

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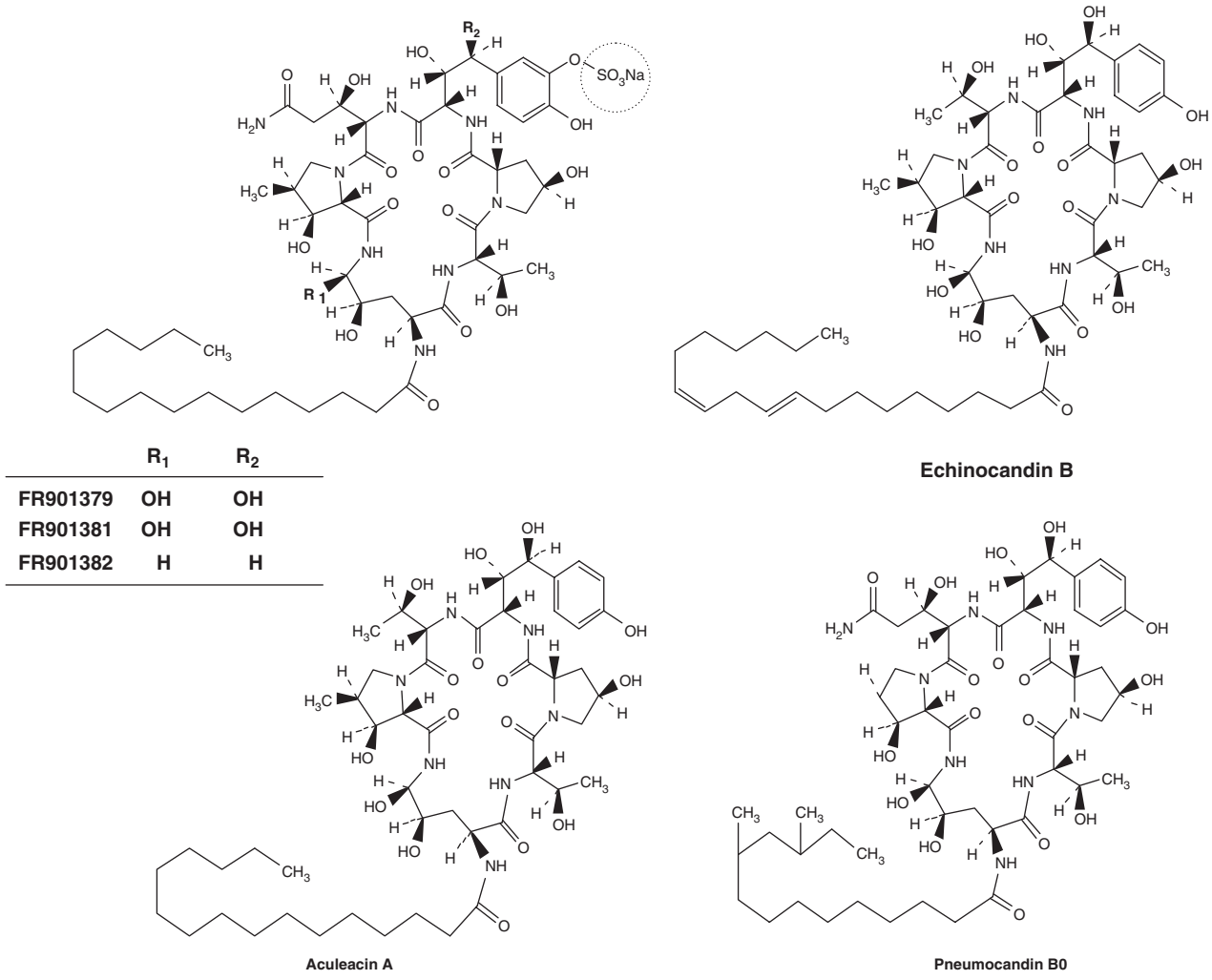


	R_1	R_2
FR901379	OH	OH
FR901381	OH	OH
FR901382	H	H

Figure 1 Structures of echinocandins.

attributed to selective inhibition of 1,3-β-glucan synthesis, although their intrinsic water insolubility is a major barrier for drug development.[17–19] However, FR901379 and related compounds showed both high water solubility and a strong antifungal effect on *Candida* spp.[20] The structural difference between FR901379 and the other echinocandins is that FR901379 has a sulfate moiety in its molecule (Figure 1, circled). This residue was speculated to be the basis for the high water solubility of FR901379 (soluble in water even at a concentration of 50 mg ml^{-1}, a concentration at which other compounds have low solubility). To probe this hypothesis, FR901379 was digested with aryl sulfatase from *Aerobacter aerogenes* (Table 1), after which the water solubility of the desulfated molecule (FR133302) was decreased to 1 mg ml^{-1}, even though the inhibitory activity on 1,3-β-glucan synthase did not decrease markedly.[21] This result indicated that the excellent water solubility of FR901379 was attributed to the sulfate moiety in its structure.

The IC_{50} value of FR901379 on 1,3-β-glucan synthase is 0.7 μg ml^{-1}, which is superior to that of echinocandin B (Table 1). The *in vitro* antifungal activity of FR901379 and related compounds against both *Candida albicans* and *A. fumigatus* indicates a higher potency than that of aculeacin A (Table 2); however, it is only weakly active against *A. fumigatus.* None of these compounds show antifungal activity against *C. neoformans.* Table 3 shows the therapeutic effect of FR901379 in a murine *C. albicans* infection model in which drugs were administered s.c. for four consecutive days. FR901379 and related compounds significantly prolonged the survival of infected mice. FR901379 was the most potent compound, with an ED_{50} value of 2.7 mg kg^{-1} 14 days after the infection. This value was almost comparable to that of fluconazole. In spite of its potent antifungal activity and its good water solubility, FR901379 could not be developed further because of class-specific reticulocyte lysis at low concentrations (Table 4), although the lytic activity of FR901379 was weaker than that of amphotericin B.

The producer strain of FR901379, identified as *C. empetri* F-11899, was originally isolated from a soil sample collected at Iwaki City, Fukushima Prefecture, Japan. Its morphological characteristics were determined on the basis of cultures on sterilized azalea leaf affixed to a Miura's LCA plate, because the strain produced conidial structures on the leaf segment alone.

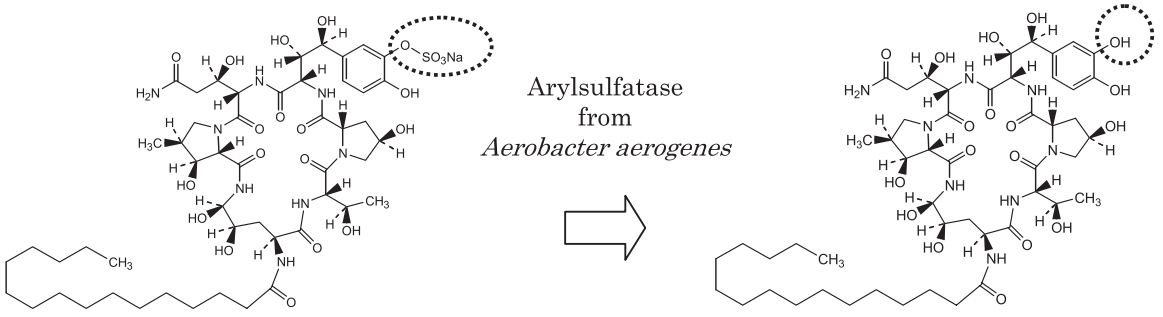
GENERATION OF THE LEAD COMPOUND, FR131535

When FR901379 was discovered by Fujisawa, both Merck and Lilly had already investigated this area of echinocandins. However, FR901379 had two advantages over other analogs. One was its excellent intrinsic water solubility, and the other was that the hemolytic activity of echinocandins was reduced by substituting the acyl side chain. As

Table 1 Water solubility and inhibitory effect of echinocandins on 1,3-β-glucan synthase

Compound	*Solubility in water (mg ml^{-1})*	*Inhibition of 1,3-β-glucan synthase IC_{50} (μg ml^{-1})*
FR901379	>50	0.7
FR133302	1	1.3
Echinocandin B	0.008	2.6
Cilofungin	0.1	nt

Abbreviation: nt, not tested.



FR901379 FR133302

Table 2 *In vitro* antifungal activity of FR901379 and related compounds

Test organism	*IC_{50} (μg ml^{-1})*			
	FR901379	*FR901381*	*FR901382*	*Aculeacin A*
Candida albicans FR578	0.008	0.008	0.008	0.008
C. albicans FP582	0.025	0.015	0.03	0.06
C. albicans FP629	0.008	0.004	0.008	0.015
C. albicans FP633	0.025	0.025	0.03	0.06
C. tropicalis YC118	0.025	0.05	0.015	0.31
C. krusei YC109	0.16	0.16	0.16	0.62
C. utilis YC123	0.03	0.003	0.003	0.06
Aspergillus fumigatus FD050	1.9	1.6	0.62	2.5
A. niger ATCC9642	0.03	0.03	0.03	2.5
Cryptococcus neoformans YC203	>2.5	>2.5	>2.5	>2.5

Table 3 *In vivo* efficacy in a neutropenic mouse model of disseminated candidiasis

Compound	*ED_{50} (mg kg^{-1})*
FR901379	2.7
Aculeacin A	6.4
Fluconazole	4.5

Infection: *Candida albicans* FP633.

other compounds were not as soluble, Fujisawa's researchers focused on side-chain replacement, while keeping the sulfate group intact. Considering that the hemolytic activity may be related to the long alkyl side chain, they attempted to reduce the hemolytic activity of FR901379 by substituting the side chain, as Lilly's researchers had.[22]

FR901379 was treated with acylase from *Actinoplanes utahensis* to remove the palmitoyl group to yield FR179642, and a new acyl side chain was prepared starting from 1-bromooctane and 4-hydroxybenzoic acid. Thus, 2,4,5-trichlorophenyl 4-(*n*-octyloxy) benzoate was obtained from 4-(*n*-octyloxy) benzoic acid and 2,4,5-trichlorophenol using *N*, *N*′-dicyclohexylcarbodiimide (DCC) in ether. The reacylation of FR179642 by the 2,4,5-trichlorophenyl active ester method yielded FR131535 (Figure 2).[23] The water solubility of FR131535 was as high as that of FR901379, even after replacement of the acyl side chain. FR131535 inhibited 1,3-β-glucan synthase prepared from *C. albicans* 6406 with an IC_{50} value of 2.8 μg ml^{-1} in a non-competitive manner (Ki 4.0 mM) and exhibited potent activity against a variety of fungal species by the micro broth dilution method. The protective efficacy of FR131535 administered s.c. against a murine systemic infection with *C. albicans* was reported to be superior to those of echinocandin B and cilofungin, and almost as potent as fluconazole. Furthermore, FR131535 showed potent *in vivo* activity against *A. fumigatus*: the ED_{50} value of FR131535 was improved dramatically from 70 to 4.3 mg kg^{-1} (Table 5). Thus, FR131535 was the first echinocandin to show an effective anti-*Aspergillus* activity. The hemolytic activity of FR131535 had also been greatly reduced compared with that of FR901379 (Table 5). These findings led to extensive research on the optimization of the acyl side chain.

Table 4 Hemolytic activity of FR901379

Compound	*MLC[a] (μg ml^{-1})*
FR901379	62
Aculeacin A	31
Echinocandin B	125
Amphotericin B	8

[a]Minimum lytic concentration.

LEAD COMPOUND OPTIMIZATION TO FK463[24]

As the acyl side chain substitution led to the expansion of the antifungal spectrum to include *Aspergillus* spp., the relationship between the side-chain lipophilicity and antifungal activity was

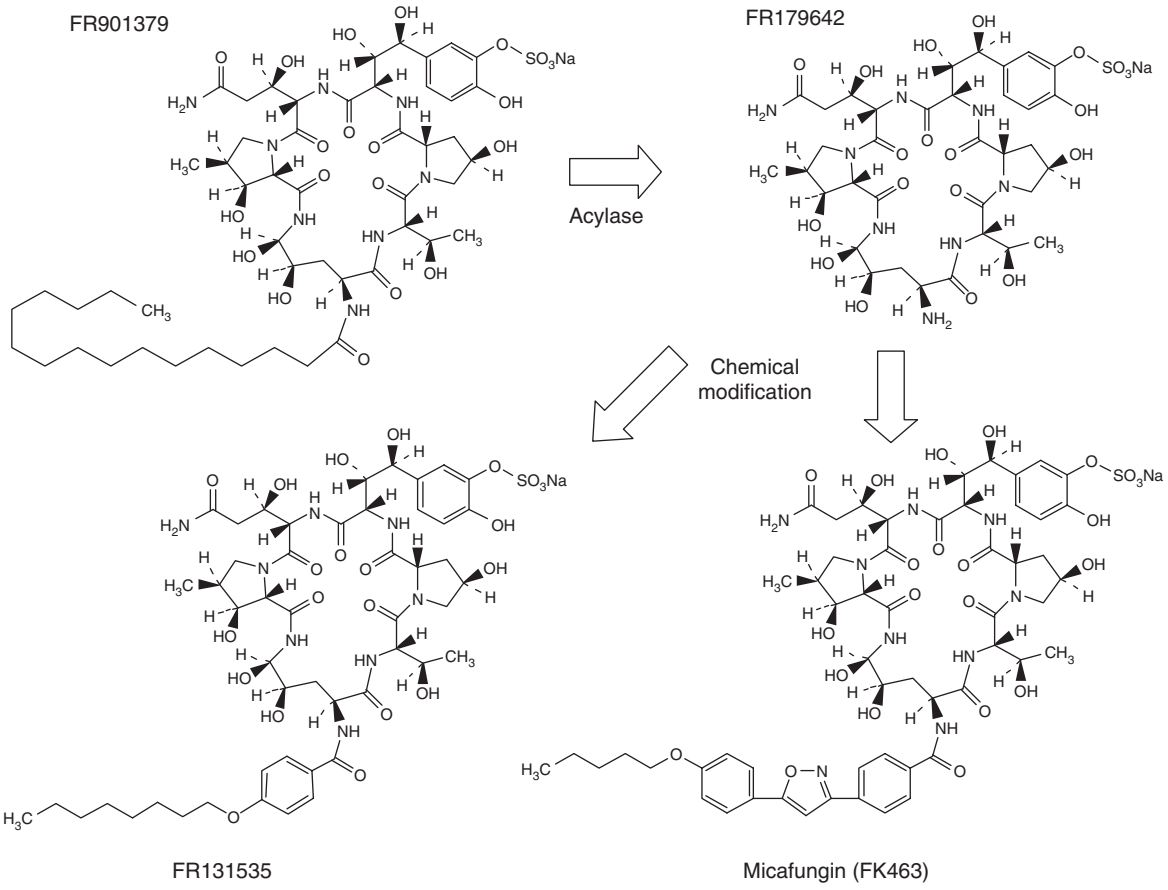
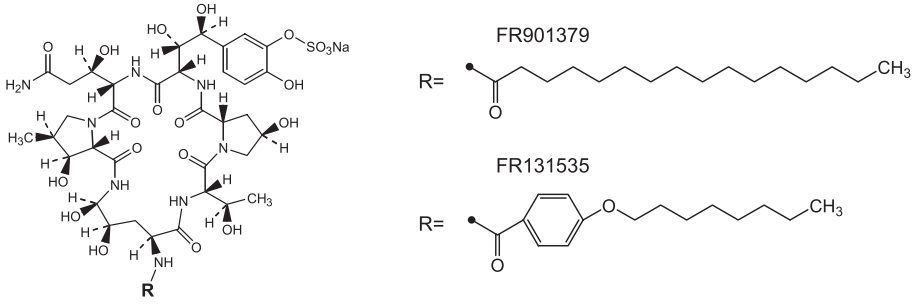


Figure 2 Semi-synthesis of FR131535 and micafungin (FK463).

Table 5 Influence of acyl side chain on MIC, ED_{50} and hemolysis



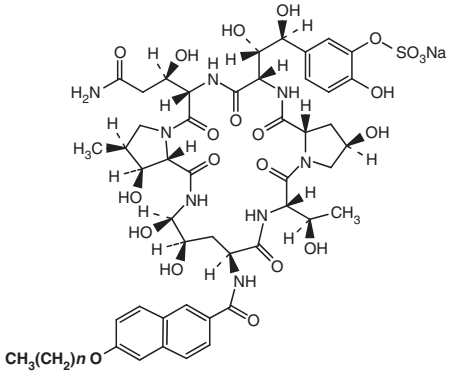
Compound	*Candida albicans FP633*		*Aspergillus fumigatus FP1305*	*Hemolysis*
	MIC ($\mu g\,ml^{-1}$)	*ED_{50}* ($mg\,kg^{-1}$)	*ED_{50}* ($mg\,kg^{-1}$)	*LC_{30}*[a] ($mg\,ml^{-1}$)
FR901379	0.2	1.8	70	0.456
FR131535	0.78	3.7	4.3	>8

[a]Lytic concentration 30%.

examined.[25,26] Naphthalene side chains, which are compact and amenable to modulation of lipophilicity, were chosen as the initial acyl side chains to be substituted. Meanwhile, the relationship between antifungal activities and hemolysis was examined by changing the lipophilicity by varying the length of the alkyl chains. The lipophilicity increase resulted in improved anti-*Candida* activity, which was most potent with an octyloxy group (n=7; Figure 3a). Hemolytic activity was enhanced in proportion to the length of the alkyl chains. To make the analog design more quantitative, the ClogP value [octanol–water partition coefficient (calculated value)] of the side chain was used to evaluate the correlation with anti-*Candida* activity. The strongest *in vivo* effect was observed in compounds with ClogP values of

a

n	C. albicans *FP633* MIC (mg ml^{-1})	*Hemolysis* (% at 2 mg ml^{-1})
3	12.5	1
5	0.78	5
6	0.39	–
7	0.1	34
9	0.2	100
11	0.78	100



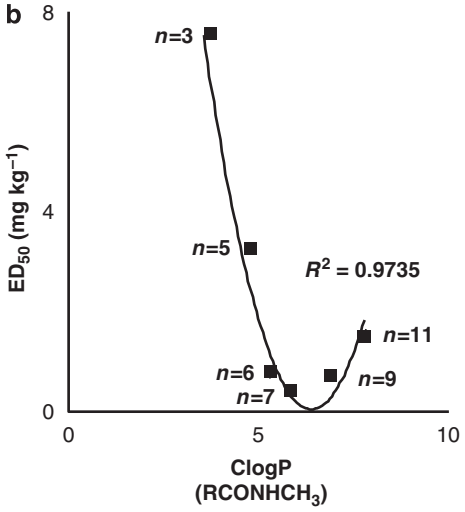
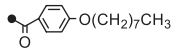
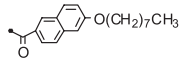
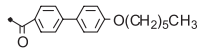
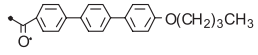
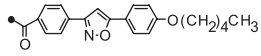


Figure 3 Effect of lipophilicity on MIC, hemolysis and ED_{50}: (**a**) length of alkyl chain; (**b**) ClogP of the side chain determined for $CH_3(CH_2)_nCONHCH_3$.

Table 6 Key compounds in optimization for side chain

Compound	*Side chain*	*ClogP*	*Candida albicans FP633*		*Aspergillus fumigatus FP1305*		*Hemolysis*	
			MIC (μg ml^{-1})	*Serum MIC* (μg ml^{-1})	*ED$_{50}$*[a] (mg kg^{-1})	*ED$_{50}$* (mg kg^{-1})	*LC$_{30}$*[a] (mg ml^{-1})	*At 1 mg ml^{-1} (%)*
FR131535		4.77	0.78	25	1.5–4.3	4.31	>10	<20
1		5.80	0.1	6.25	0.742	0.788	10	
2		5.37	0.2	6.25	0.658		1.74	
3		6.14	0.025	1.56	0.447		0.37	79
Micafungin		5.32	0.05	1.56	0.658	0.228		<20

[a]Lytic concentration 30%.

approximately 6; therefore, the side chains were thereafter prepared by adjusting the ClogP value to approximately 6 (Figure 3b).

Conversion of a single benzene ring of FR131535 into a naphthalene ring improved anti-*Candida* activity, and further introduction of aromatic rings into the side chain was also effective (Table 6). Anti-*Candida* activity was improved as the number of benzene rings increased, and compound **3** showed the strongest MIC. Furthermore, compound **1**, which had a naphthalene ring, showed a large improvement in anti-*Aspergillus* activity when compared with FR131535. However, even though compound **3** showed the lowest MIC value, its *in vivo* efficacy (ED_{50} 0.447 mg kg^{-1}) was only slightly better than that of compound **2** (ED_{50} 0.658 mg kg^{-1}), which had a 10-fold greater MIC. This apparent large discrepancy was reduced using serum MIC values that were measured in a medium supplemented with mouse serum. A good correlation between the *in vivo* effect (ED_{50}) and *in vitro* activity (serum MIC) was obtained.[27] The marked difference in the MIC values was deduced to result from a decreased concentration of the free form due to binding of the compound to serum protein. On the basis of the observed correlation, prediction of *in vivo* effects by measuring the serum MIC of the compound became more feasible, and structure–activity relationships were established rapidly. Consequently, compound **3**-type derivatives with three linearly linked aromatic rings revealed strong anti-*Candida* and anti-*Aspergillus* activities. However, the hemolytic activity of these analogs was still evident. This was overcome by converting the central benzene ring of compound **3** into various heterocycles. Initially, hemolytic

activity reduction was difficult because FR901379 derivatives have an amphiphilic structure and surfactant activity. The difference in the amount of unsaturated fatty acids between erythroid and eukaryotic cell membranes was noted, which led to the hypothesis that reduction of hemolytic activity might be possible by decreasing the linearity of the acyl side chains. As expected, the introduction of a heterocycle into the acyl side chain resulted in a marked lowering of hemolytic activity without a decrease in the potent antifungal activity of compound **3**. Finally, the cyclic peptide nucleus FR179642, obtained by enzymatic cleavage of the natural product FR901379, was reacylated with an optimal side chain containing an isoxazole ring to yield FK463 (later named micafungin) (Figure 2).

PRE-CLINICAL STUDY OF FK463

Micafungin is a non-competitive inhibitor of 1,3-β-glucan synthase, a key enzyme necessary for the synthesis of 1,3-β-glucan, that is a main component of fungal cell walls.[28–30] Fungal cells unable to synthesize 1,3-β-glucan lose the rigidity required to resist osmotic pressure, resulting in cell lysis. This mechanism is common to the echinocandin class of antifungal agents. Most fungal cell walls are composed of chitin, glucan and mannoprotein, although the quantity and relative content of each component depend on fungal species. The multi-enzyme complex 1,3-β-glucan synthase is composed of the catalytic subunit FKS1p/FLs2p and the regulatory subunit Rho1p GTPase.[31] The potent inhibitory effect of micafungin on the activity of 1,3-β-glucan synthase was determined in membrane preparations of both *C. albicans* and *A. fumigatus* by measuring incorporation of [^{14}C]UDP-glucose into a trichloroacetic-acid-insoluble precipitate. The short-term exposure of *C. albicans* to 0.1–0.5 mg ml^{-1} micafungin induced abnormal swelling and irregular shape of fungal cells, a reported feature resulting from inhibition of cell wall synthesis.[32]

Micafungin shows potent fungicidal activity against clinically isolated yeast species of *Candida*, including *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata* and *C. krusei*, and against *C. parapsilosis*, *C. lusitaniae* and *C. guilliermondii* with slightly higher MIC_{90} values.[33–35] No cross-resistance to fluconazole-resistant clinical isolates of *Candida* has been observed.[36] Characteristically, micafungin has potent *in vitro* inhibitory activity against *Aspergillus* species at lower concentrations than amphotericin B and itraconazole, but micafungin was not fungicidal against *Aspergillus* spp.[37] There are no reports on acquired resistance to micafungin in susceptible yeast species in clinical practice, except for a case of resistance developed in *C. parapsilosis*.[38] Repeated passage of *C. albicans* in sub-inhibitory concentrations of micafungin resulted in negligible changes in MIC values, which suggests a low possibility of developing resistance in a clinical setting.

Among the compounds prepared in the optimization study, micafungin had the strongest *in vivo* effects against *Candida* spp. and *Aspergillus* spp. The efficacy of micafungin was determined in neutropenic murine models of disseminated candidiasis and aspergillosis in comparison with amphotericin B and fluconazole.[39] As shown in Table 7, the ED_{50} values of micafungin against disseminated infections with *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei* ranged from 0.14–0.77 mg kg^{-1}. Although micafungin efficacy was 1.4–3.1 times inferior to that of amphotericin B (0.09–0.26 mg kg^{-1}), it was 9.6 $\geqslant$77 times superior to that of fluconazole. The ED_{50} of micafungin against disseminated *C. parapsilosis* infection was 1.0 mg kg^{-1}, which was 11 times superior to that of fluconazole (10.9 mg kg^{-1}) and 18 times inferior to that of amphotericin B (0.06 mg kg^{-1}). Micafungin was also effective against disseminated *A. fumigatus* infection, with ED_{50} values in the range of 0.25–0.50 mg kg^{-1}. The efficacies of micafungin were 1.7–2.3 times inferior to those of amphotericin B (0.11–0.29 mg kg^{-1}) and more than 80 times superior to those of fluconazole. These results indicated that micafungin would be a potent parenteral therapeutic agent for disseminated candidiasis and aspergillosis.

Table 7 ***In vivo*** **efficacy of micafungin in a neutropenic mouse model of disseminated candidiasis and aspergillosis**

Organism	ED_{50} (mg kg^{-1})		
	Micafungin	*Fluconazole*	*Amphotericin B*
C. albicans FP633	0.18	1.54	0.07
C. glabrata 16011	0.18	4.89	0.10
C. tropicalis 16004	0.35	7.2	0.21
C. krusei 15001	1.61	>20.0	0.71
C. parapsilosis 16005	3.21	4.57	0.08
C. guilliermondii 13003	0.77	6.27	0.32
A. fumigatus TIMM0063	0.33	>20.0	0.25
A. fumigatus IFM40835	0.26	>20.0	0.25
A. fumigatus IFM40836	0.45	>20.0	0.46

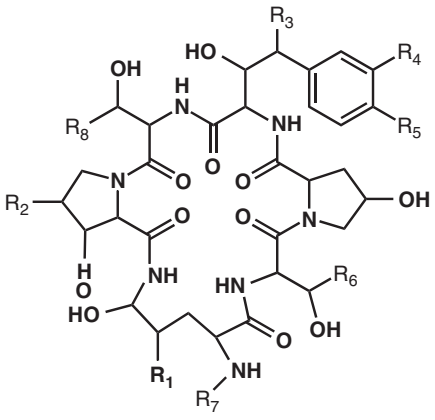
CLINICAL STUDY OF FK463

The clinical efficacy of micafungin was evaluated in the treatment and prophylaxis of *Candida* and *Aspergillus* infections. A dose–response trial and two randomized double-blinded trials were first conducted to evaluate the safety and efficacy of micafungin in the treatment of esophageal candidiasis in patients with AIDS.[40–42] A total of 120 patients with endoscopically proven esophageal candidiasis were included in the initial study, with an i.v. micafungin dosing of 12.5, 50, 75 and 100 mg day^{-1}. Clinical improvement was endoscopically noted in all patients, especially those who received 75 or 100 mg day^{-1}. Symptoms improved or resolved within 3–5 days of treatment in the majority of patients, irrespective of the underlying CD4+ lymphocyte count. The efficacies of i.v. micafungin and i.v. fluconazole were compared in a randomized, double-blind, dose–response study. In all, 245 patients with a prior diagnosis of AIDS/human immunodeficiency virus (HIV) infection and esophageal candidiasis confirmed by endoscopy and culture were randomized to receive micafungin (50, 100 or 150 mg day^{-1}) or fluconazole (200 mg day^{-1}). Both agents were administered once daily by 1-h i.v. infusion for 14–21 days and the response was endoscopicallly determined. Cure rates were 68.8, 77.4 and 89.8% with 50, 100 and 150 mg of micafungin, respectively. The average cure rate with two higher doses of micafungin (83.5%) was comparable to that of 200 mg of fluconazole (86.7%). This study confirmed the efficacy of micafungin in treatment of esophageal candidiasis in HIV-positive patients.

Other treatment and prophylaxis trials against candidemia, invasive candidiasis and aspergillosis were conducted to assess the efficacy, adverse effects, pharmacokinetics, drug–drug interactions and dosing of micafungin. A convenient dosing regime, an excellent safety profile and remarkably few drug interactions were observed, indicating that micafungin is a potent weapon in antifungal therapy.

OTHER SULFATED ECHINOCANDINS

The acyl side chain of echinocandin has been readily replaced by established semi-synthesis methods; however, modification of the cyclic peptide by chemical synthesis is difficult. As an alternative, Fujisawa's researchers attempted to obtain structural diversity in the cyclic peptide by extensive screening of fungal products. A number of



	R1	R2	R3	R4	R5	R6	R7	R8
FR901379	OH	CH_3	OH	OSO_3H	OH	CH_3	Palmitoyl	CH_2CONH_2
FR901381	OH	CH_3	H	OSO_3H	OH	CH_3	Palmitoyl	CH_2CONH_2
FR901382	H	CH_3	H	OSO_3H	OH	CH_3	Palmitoyl	CH_2CONH_2
FR220897	OH	H	H	OH	OSO_3H	CH_3	Palmitoyl	CH_2CONH_2
FR220899	OH	CH_3	H	OH	OSO_3H	CH_3	Palmitoyl	CH_2CONH_2
FR209602	OH	CH_3	H	OSO_3H	OH	H	Palmitoyl	CH_2CONH_2
FR209603	OH	H	H	OSO_3H	OH	H	Palmitoyl	CH_2CONH_2
FR209604	H	CH_3	H	OSO_3H	OH	H	Palmitoyl	CH_2CONH_2
FR190293	OH	CH_3	OH	OSO_3H	OH	CH_3	Branched acyl	CH_2CONH_2
FR227673	OH	CH_3	OH	OSO_3H	OH	CH_3	Branched acyl	CH_2CONH_2
Echinocandin B	OH	CH_3	OH	H	OH	CH_3	Linoleoyl	CH_3
Aculeacin Ay	OH	CH_3	OH	H	OH	CH_3	Palmitoyl	CH_3
Pneumocandin B0	OH	H	OH	H	OH	CH_3	Branched acyl	CH_2CONH_2
Mulundocandin	OH	CH_3	OH	H	OH	CH_3	Branched acyl	H

Figure 4 Summary of the structure of echinocandins.

sulfated echinocandins, which differ not only in the amino acid constituents of the cyclic peptide but also in the acyl chain, have been isolated (Figure 4). FR209602, FR209603, FR209604, FR220897 and FR220899 have different methylation and hydroxylation modifications of amino acids in the cyclic peptide, with the same palmitoyl acyl chain as FR901379,[43,44] whereas FR190293 and FR227673 have the same cyclic peptide as FR901379, with different branched acyl side chains.[45] Furthermore, the sulfated hydroxyl residue presents at a different position in FR209602, FR209603 and FR209604 than those in other sulfated echinocandins. The strain producing FR209602, FR209603 and FR209604, identified as *C. crateriformis* no. 738, also belongs to the same genus as that producing FR901379. The strain producing FR220897 and FR220899, identified as *C. empetri*, belonged to the same species as that producing FR901379. On the contrary, FR190293 and FR227673 were produced from non-*Coleophoma* strains; that is, *Tolypocladium parasiticum* and *Chalara* sp., respectively. Whereas structural diversity in cyclic peptides was observed among the compounds produced by the three strains belonging to the *Coleophoma* genus, chemical diversity in the acyl side chain was exhibited by non-*Coleophoma* species.

Considering other echinocandins, we have proposed a classification model for the echinocandin-producing strains, based on the chemical diversity of their product: type I as a sulfated echinocandin-producing strain and type II as a non-sulfated echinocandin-producing strain (Table 8).[46] Type I has been further classified into two sub-groups. Type IA is a coelomycetes group that produces a sulfated cyclic peptide nuclear structure with a palmitoyl chain. The strains belonging to this group form conidial structures only on leaf segments. Type IB, a hyphomycetes group, produced FR190293 and FR227673, which comprise the same peptide nuclear structure as FR901379, with a variety of branched acyl side chains. These strains form hyphal conidia in a general medium. Type II includes strains (for example, *Aspergillus* spp. and *G. lozoyensis*) that produce non-sulfated echinocandins, such as echinocandin B, aculeacin A, mulundocandin and pneumocandin B0. Type I fungi may have a specific enzyme, aryl sulfotransferase, to sulfate a phenolic hydroxyl residue in the cyclic peptide structure. This putative classification model may help in elucidating the molecular evolution of echinocandins, as well as the phylogenetics of the strains producing them.

PROSPECTS

A novel series of sulfated echinocandins has been reviewed in this paper. The compounds are highly soluble in water, even at a concentration of 50 mg ml^{-1}. Their structural and physico-chemical properties enabled the efficient development of micafungin. Thus, chemical diversity in screening sources is a key factor in successful

Table 8 A classification model for echinocandin-producing microorganisms

Class	*Sub group*	*Compound*	*Producing strain*
Type I sulfated		FR901379 FR901381 FR901382	*Coleophoma empetri*
	A	FR209602 FR209603 FR209604	*C. crateriformis*
		FR220897 FR220899	*C. empetri*
	B	FR190293 FR227673	*Tolypocladium parasiticum* *Chalara* sp.
Type II non-sulfated		Aculeacin Ar Echinocandin B Pneumocandin B0 Mulundocandin	*Aspergillus japonicus var. aculeatus* *A. nidulans var. echinulatus* *Glarea lozoyensis* *A. sydowi*

drug discovery and subsequent development. In the case of microbial screening, such diversity can be achieved by suitable selection of producing strains and culture conditions. Chemical diversity in the echinocandin family has been observed not only in the cyclic peptide nuclear structure but also in the acyl side chain moiety. Interestingly, various fungi produce common structures, such as an acylated cyclic hexapeptide, with some modifications. Thus far, the mechanism for the biosynthesis of echinocandins has not been elucidated, although it is likely to include a non-ribosomal peptide synthetase to produce the cyclic peptide core, with a fatty acid or polyketide synthase to produce the acyl side chain. An increase in echinocandin chemical diversity may be realized using genetic engineering of the biosynthetic genes.

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ORIGINAL ARTICLE

Modification of the antibiotic olivomycin I at the 2′-keto group of the side chain. Novel derivatives, antitumor and topoisomerase I-poisoning activity

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A novel way of chemical modification of the antibiotic olivomycin I at the 2′-keto group of the side chain of the aglycone moiety was developed. Reaction of olivomycin I with the carboxymethoxylamine hemihydrochloride gave the key intermediate, 2′-carboxymethoxime-olivomycin I, which was further reacted with different amines in the presence of benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate to give the corresponding amides. The antiproliferative and topoisomerase I (Topo-I)-poisoning activities of the novel derivatives were examined. One of the novel derivatives showed a marked inhibitory activity against Topo-I, a pronounced antitumor activity in *in vivo* experiments on mice bearing leukemia P-388 and lower toxic side effects compared with the parent olivomycin I.
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Keywords: antitumor activity; aureolic acid antitumor antibiotics; olivomycin I; topoisomerase I

INTRODUCTION

The aureolic acid family of antitumor antibiotics includes a group of clinically active agents such as olivomycin I (olivomycin A), mithramycin, and also chromomycin A_3 and durhamycin.[1] The antibiotics of the aureolic acid family interact with the DNA minor groove in high-GC-content regions in a nonintercalative way and with the requirement for Mg^{2+} ions.[2]

The antitumor antibiotic olivomycin I was discovered at the Gause Institute of New Antibiotics, Moscow.[3] Comparative study of the antitumor action of olivomycin I and chromomycin A_3 in *in vivo* experiments on murine lymphosarcoma LY01 revealed that the chemotherapeutic index (LD_{50}/DIT_{50}) of olivomycin I is more favorable (2.35) than that of chromomycin A_3 (0.99).[4] A similar study on the inhibitory effect of olivomycin I, chromomycin A_3 and mithramycin against transplantable murine leukemia La showed that a similar antitumor effect (an increase in the lifespan of mice by 25%) can be achieved at lower doses of olivomycin I than those for the other aureolic acid antibiotics studied.[4] Clinical investigations of mithramycin and olivomycin I showed that these antibiotics give favorable results in treatment of testicular tumors. It was shown that these antibiotics exhibit side effects such as gastrointestinal, hepatic, renal and bone marrow toxicities.[5] The major clinically limiting toxicity of mithramycin was a hemorrhagic diathesis associated with a precipitous thrombocytopenia. It is of considerable interest that hemorrhagic diathesis was not observed after administration of olivomycin I.[4]

As olivomycin I possesses the best chemotherapeutic index among the aureolic acid antibiotics, it can be considered as the best scaffold for the development of novel semisynthetic aureolic acid analogs with increased therapeutic indices and lower toxicity compared with the parent antibiotic.

Here we describe chemical modifications of olivomycin I at the 2′-keto group of the side chain of the aglycone moiety. The antiproliferative and topoisomerase I (Topo-I)-poisoning activities of the novel derivatives (**2–7**) were tested. One of the novel derivatives showed pronounced antitumor activity in *in vivo* experiments on mice bearing lymphocyte leukemia P-388, together with lower toxicity to animals compared with the parent olivomycin I.

RESULTS

Chemistry

We developed a novel method of chemical modification of olivomycin I (**1**) based on the introduction of a carboxyl group into the molecule of the antibiotic. Reaction of olivomycin I (**1**) with carboxymethoxylamine gave the key intermediate, 2′-carboxymethoxime-olivomycin I (**2**) (Scheme 1), which was further reacted with different amines in the presence of PyBOP to give the corresponding amides **3–7**. The resulting compounds were purified by column chromatography on silica gel.

TLC and HPLC analyses showed that compounds **2–7** were homogeneous. ^{1}H- and ^{13}C-NMR investigations of **2** identified all

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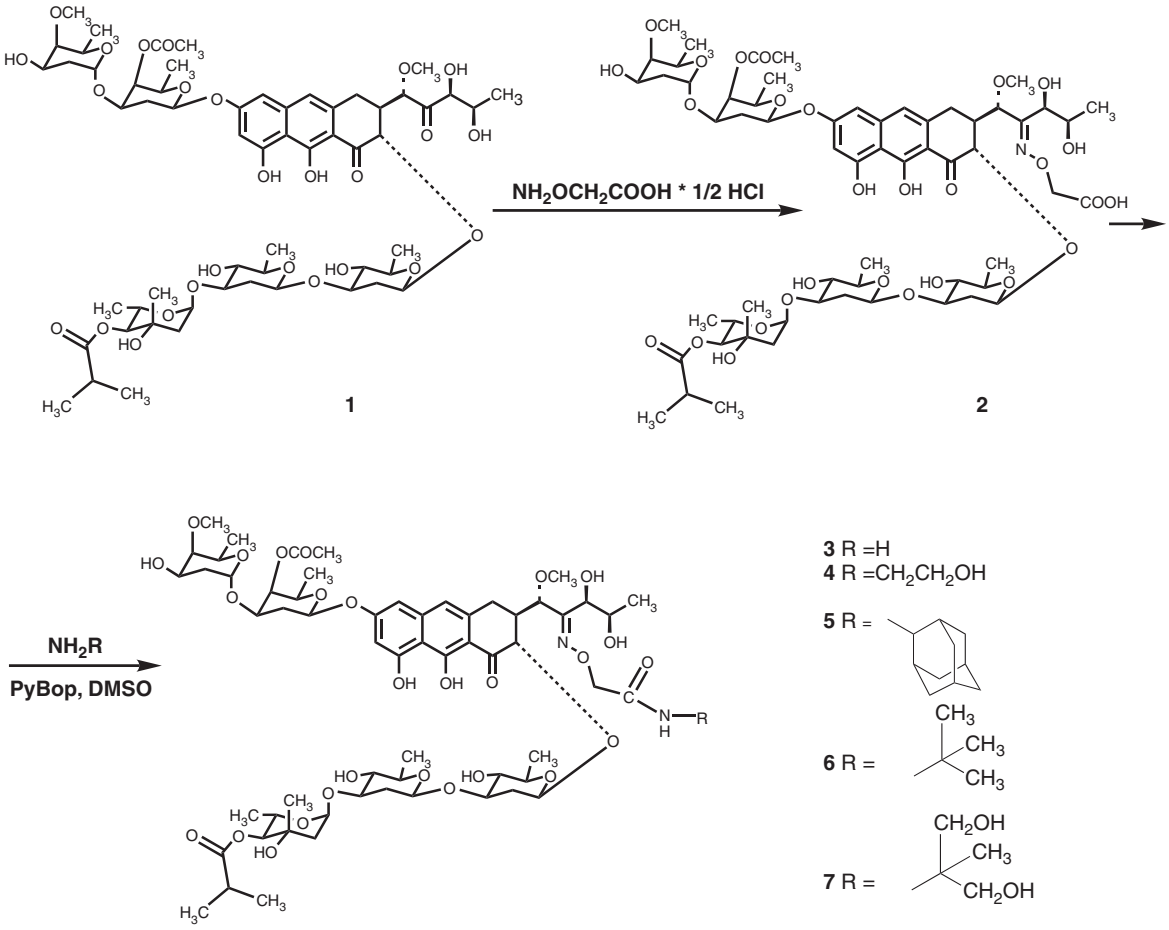


Scheme 1

the signals of hydrogen and carbon atoms in aglycone and carbohydrate moieties. The structures of all new derivatives were supported by MS data and by ^{1}H-NMR spectra (Scheme 1).

Biological testing

The cytotoxicity of the compounds in comparison with the parent olivomycin I was tested. Cells were incubated with drugs for 48–72 h to ensure the completion of late events in cell death. Table 1 shows the comparative potencies of these compounds against the wild-type murine leukemia L1210 cells, the human leukemia cell line K562 and the human malignant T-lymphocyte Molt4/C8 and CEM cells.

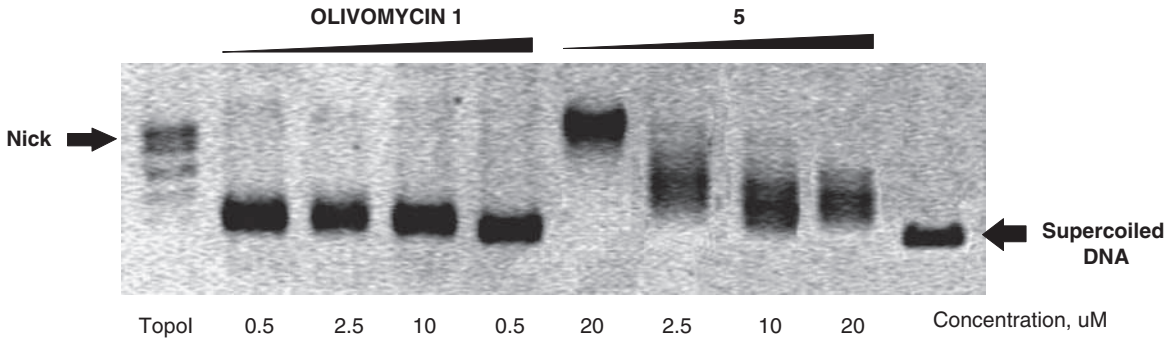
All novel derivatives (**2**–**7**) caused cell death at higher concentrations than olivomycin I. Remarkably, amides with the bulky hydrophobic substituents (adamantyl- **5**; *tert*-butyl-, **6**) showed antiproliferative activity that was at an IC_{50} of only one order of magnitude higher (for L1210: 0.19 and 0.20 μM, correspondingly) than that of olivomycin I, but at a markedly lower IC_{50} than that of 2′-carboxymethoxime-olivomycin I (**2**) or the amides with small or hydrophilic substituents **3**, **4** and **7** (IC_{50} for L1210: 6.5–20 μM).

To identify tentative intracellular targets important for cytotoxicity of olivomycin I and its novel derivatives (**2**–**7**), we tested these compounds for their ability to modulate Topo-I activity *in vitro*. Olivomycin I (**1**) and all novel derivatives (**2**–**7**) were potent Topo-I inhibitors at all concentrations investigated (0.5–20 μM) (data not shown). Figure 1 shows the results of electrophoretic analysis of the relaxation products of the Topo-I-dependent supercoiled DNA relaxation in the absence and presence of the antibiotics olivomycin I and **5**. In the absence of antibiotics (track of Topo-I), the reaction led to a set of topoisomers and the disappearance of the supercoiled form of DNA. This effect was revealed by the presence of residual amounts of rapidly migrating topoisomers. Olivomycin I (**1**) inhibited Topo-I

Table 1 Toxicity of olivomycin I (1) and its derivatives (2–7) against murine leukemia L1210 and human erythroleukemia K562 cells, and human malignant T-lymphocyte Molt4/C8 and CEM cells

	IC_{50}[a] (μM)			
Compound	*K562*	*L1210*	*Molt4/C8*	*CEM*
Olivomycin I (**1**)	0.025 ± 0.002	0.034 ± 0.002	0.0040 ± 0.0004	0.0025 ± 0.0000
2	>3.2	6.5 ± 2.8	1.8 ± 1.4	2.3 ± 1.6
3	>3.2	3.6 ± 3.1	1.0 ± 0.1	0.82 ± 0.19
4	>3.2	18 ± 5	3.7 ± 0.8	6.4 ± 0.0
5	ND	0.19 ± 0.01	0.026 ± 0.007	0.018 ± 0.002
6	0.025 ± 0.002	0.20 ± 0.01	0.033 ± 0.003	0.032 ± 0.001
7	2.47 ± 1.8	20 ± 11	3.1 ± 2.3	4.5 ± 1.2

Abbreviation: ND, not determined.
[a]IC_{50}—the concentration that caused 50% growth inhibition after 48 h (L1210) or 72 h (other cell lines) of exposure.



Nick – open circular DNA; Track TopoI – enzyme topoisomerase I without compounds; other tracks – topoisomerase I + compounds.

Figure 1 Electrophoretic analysis of the relaxation products of the Topo-I-dependent supercoiled DNA relaxation in the absence and presence of the antibiotics examined. Nick—open circular DNA; Track Topo-I—enzyme topoisomerase I without compounds; other tracks—topoisomerase I-positive compounds.

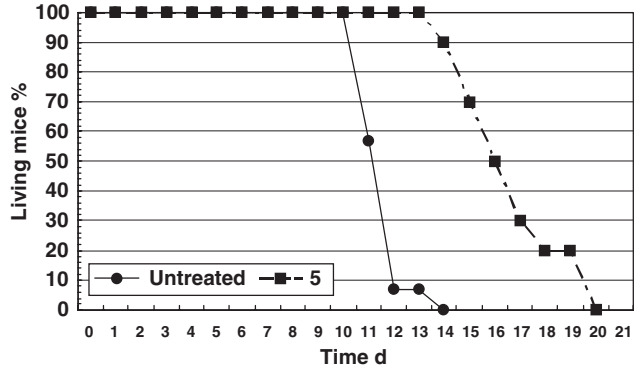


Figure 2 Antitumor activity of olivomycin derivative **5** in male mice ((B6D2F1) with leukemia P-388 on quintuple i.p. injection 72 h after implantation of tumor) at dose of 5 mg kg^{-1} daily.

activity at all concentrations investigated (0.5–20 μM); compound **5** was less active but still a potent Topo-I inhibitor—the rapidly migrating topoisomers were observed on the track starting at the compound concentration of 2.5 μM.

When **5** was i.p. injected to mice with leukemia P-388 72 h after i.p. implantation of tumor, 62% increase of lifespan (ILS) at the dose of 50 mg kg^{-1} was achieved.

Compound **5** did not show any cumulative toxic effects at the quintuple doses of 5 and 10 mg kg^{-1} (25 and 50 mg kg^{-1} total dose, respectively), whereas all of the mice that received **1** (5 mg kg^{-1} daily) had drug-related toxic death after the third injection (data no shown). All mice that received daily doses of 5 and 10 mg kg^{-1} had tumor-related death. The ILS was 43% compared with the group of untreated mice (Figure 2). Quintuple injections of **5** at 20 mg kg^{-1} per dose (100 mg kg^{-1} total dose) resulted in drug-related death of 15% of the mice (data not shown).

DISCUSSION

In most antitumor antibiotic families, extensive efforts have been made to prepare different semisynthetic derivatives and analogs. However, no systematic efforts have been made for the aureolic acid-type antibiotics. In the past decades, a number of aureolic acid analogs were obtained from genetically engineered mutant producers showing distinct structural changes.[5–8] This has yielded in some information on the structure–activity relationship of this type of compounds.

It is known that deoxysugars are necessary for stabilizing the complex of the antibiotic with DNA, and that their structures influence the sequence specificity of binding. Earlier, we have shown that olivomycin I in nanomolar concentrations induces apoptosis of human tumor cells and inhibits p53-dependent transcription of the reporter gene, whereas its aglycone olivine has no cytotoxicity and does not block transcription.[9] Whereas modifications of the carbohydrate and aglycone moieties decreased biological activity significantly, changes in the side chain were more promising.[5,8]

In an effort to find new analogs with improved pharmacological properties, we developed a novel method of chemical modification of the aureolic acid antibiotic olivomycin I on the side chain of the aglycone moiety. The key intermediate, 2′-carboxymethoxime-olivomycin I (**2**), contains a carboxyl group that allowed further modifications. A series of amides of **2** was thus prepared. Studies of the antiproliferative activity of the novel compounds on different tumor cell lines revealed that the structure of the amine residue greatly influenced the eventual cytotoxicity of the new derivatives. Thus, by varying the amine introduced into the acylation reaction with (**2**), one can obtain olivomycin I derivatives with different antiproliferative activities. Moreover, for the first time it was shown that olivomycin I and its novel derivatives (**2**–**7**) are potent Topo-I poisons. It is known that DNA topoisomerases, which solve topological problems created by the double-helical structure of DNA in essential cellular processes, are the targets of many therapeutic agents.[10] Known Topo-I inhibitors, particularly camptothecin and its analogs, inhibit Topo-I activity by trapping a reversible Topo-I–DNA cleavable complex. Some undesirable problems (that is, rapid diffusion of the drug from the Topo-I–DNA complex and tumor resistance to these drugs) justify the importance of a search for other types of drugs that act on Topo-I by a different mode of action.[11] The discovery in this study that olivomycin I and its analogs are effective Topo-I poisons and are endowed with antitumor activity and lesser side effects in mice than the parent drug extends the potential of this class of antibiotics as novel drug candidates for further (pre)clinical investigations.

MATERIALS AND METHODS

General experimental procedures

Olivomycin I was produced at the pilot plant of the Gause Institute of New Antibiotics. Carboxymethoxylamine hemihydrochloride and benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from Acros (Geel, Belgium). All the other reagents and solvents were purchased from Aldrich (St Louis, MO, USA) and Merck (Darmstadt, Germany). Reaction products were purified by column chromatography on Merck silica gel G60 (0.040–0.063 μM). Reaction progress, column eluates and all final samples were analyzed by TLC and HPLC. TLC was performed on Merck $G60F_{254}$-precoated plates in the following system (system A): $CHCl_3$–MeOH–HCOOH, 9:1:0.05. The spots were detected visually and with an ultraviolet lamp at 254 nm. Melting points were determined on Buchi SMP-20 and are uncorrected. HPLC analyses were performed on a Shimadzu HPLC LC 50 instrument equipped with a Diaspher C18 column (4.0×250 mm, 5 μk; BioChem Mack, Moscow, Russia) and a variable-wavelength ultraviolet detector set at 274 nm with an injection volume of 10 μl. Elutions were carried out at a flow rate of 110 μl min^{-1} using a 0.01-N H_3PO_4–MeCN mixture, pH 2.6, at 20 °C using a gradient of MeCN from 40 to 80%. The sample concentration was 0.05–0.2 mg ml^{-1}. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR-400 spectrometer at 400 MHz using the DQCOSY method. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR-400 NMR spectrometer at 35 °C and referenced to TMS observing protons at 400 MHz and ^{13}C at 100 MHz. Mass spectra determined by electrospray ionization (ESI) were recorded on a Finnigan MAT 900S spectrometer (Brucker, Bremen, Germany) and those determined by matrix-assisted laser desorption/ionization (MALDI) were recorded on Brucker BIFLEX III (Brucker). The data for the predominant monoisotope peak are presented. All solutions were dried over sodium sulfate and evaporated at reduced pressure on a Buchi rotary evaporator at a temperature below 35 °C.

^{1}H- and ^{13}C-NMR spectra of olivomycin I ($CDCl_3$ and DMSO-d_6) fully corresponded to the ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$ and DMSO-d_6) described in the literature.[12,13]

2′-(Carboxymethoxime)-olivomycin I (2)

Carboxymethoxylamine hemihydrochloride (4.5 mg, 0.42 mmol) was added to the solution of olivomycin I (100 mg, 0.084 mmol) in MeOH (3 ml). The reaction mixture was kept at 37 °C for 50 h. It was evaporated and the crude residue was purified by column chromatography on silica gel; elution was carried out with $CHCl_3$–MeOH–HCOOH (9:1:0.05). The resulting fractions were combined and evaporated to a small volume. Addition of petroleum ether gave a precipitate, which was filtered off, washed with petroleum ether and dried in vacuum to yield **2** as an amorphous powder (53 mg, 50%). Rf 0.42 (system A), HPLC at room temperature (Rt) 12.38 min, m.p. 140–142 °C (decomposition). MALDI-MS: calculated MW for $C_{60}H_{87}NO_{28}$ 1269.54, observed MW 1292.63 $(M+Na)^+$.

The ^{1}H-NMR spectrum (DMSO-d_6) of **2** corresponded to the ^{1}H-NMR spectrum of olivomycin I,[13] except for the presence of the signal at 4.63 p.p.m. (s, 2H, $-OCH_2$).

The ^{13}C-NMR spectrum (DMSO-d_6) of **2** corresponded to the ^{13}C-NMR spectrum (DMSO-d_6) of olivomycin I,[12] except for the absence of the signal at 211.81 p.p.m. (2′-carbonyl group), and the presence of signals at 171.203 p.p.m. (−COOH), 158.815 p.p.m. (−C=N−) and 70.45 p.p.m. ($-OCH_2$).

2-Adamantylamide of 2′-(carboxymethoxime)-olivomycin I (5)

2-Adamantylamine hydrochloride (14.6 mg, 0.078 mmol) was added to the solution of 2′-(carboxymethoxime)-olivomycin I (**2**) (50 mg, 0.039 mmol) in MeOH (3 ml). Et_3N was added to the reaction mixture to adjust the pH to 8–8.5 and afterwards PyBOP (30 mg, 0.058 mmol) was added portion-wise. The reaction mixture was stirred at 22 °C for 2 h. The reaction mixture was evaporated and the crude residue was purified by column chromatography on silica gel; elution was carried out first with $CHCl_3$ and then with $CHCl_3$–MeOH–HCOOH (9:1:0.05). The fractions with the targeted compound were combined and evaporated to a small volume. Addition of petroleum ether gave a precipitate, which was filtered off, washed with petroleum ether and dried in vacuum to yield **5** as an amorphous powder (30 mg, 55%). Rf 0.27 (system A), HPLC at Rt 19.46 min, m.p. 155–157 °C (decomposition). MALDI-MS: calculated MW for $C_{70}H_{102}N_2O_{27}$ 1402.67, observed MW 1425.47 $(M+Na)^+$.

The ^{1}H-NMR spectrum (DMSO-d_6) of **5** corresponded to the ^{1}H-NMR spectrum of olivomycin I,[13] except for the presence of signals of adamantyl residue at 1.0–2.43 p.p.m. (15H), 4.49 p.p.m. (m, 1H) and 4.63 p.p.m. (s, 2H, $-OCH_2$).

Amide of 2′-(carboxymethoxime)-olivomycin I (3)

Compound **3** was obtained in the same manner as **5**, starting from **2** (50 mg, 0.039 mmol), NH_4Cl (6.3 mg, 0.12 mmol) and PyBOP (30 mg, 0.058 mmol). Yield 19 mg (38%). Rf 0.44 (system A), HPLC at Rt 10.15 min, m.p. 162–167 °C (decomposition). ESI-MS: calculated MW for $C_{60}H_{88}N_2O_{27}$ 1268.56, observed MW 1291.34 $(M+Na)^+$.

The ^{1}H-NMR spectrum (DMSO-d_6) of **3** corresponded to the ^{1}H-NMR spectrum of olivomycin I,[13] except for the presence of the signal at 4.63 p.p.m. (s, 2H, $-OCH_2$).

Ethanolamide of 2′-(carboxymethoxime)-olivomycin I (4)

Compound **4** was obtained in the same manner as **5**, starting from **2** (30 mg, 0.024 mmol), ethanolamine hydrochloride (6.9 mg, 0.071 mmol) and PyBOP (18.4 mg, 0.035 mmol). Yield 16 mg (51%). Rf 0.51 (system A), HPLC at Rt 9.71 min, m.p. 140–142 °C (decomposition). ESI-MS: calculated MW for $C_{62}H_{92}N_2O_{28}$ 1312.58, observed MW 1313.39 $(M+Na)^+$.

The ^{1}H-NMR spectrum (DMSO-d_6) of **4** corresponded to the ^{1}H-NMR spectrum of olivomycin I,[13] except for the presence of signals at 3.40–3.80 p.p.m. (4H, $-OCH_2CH_2N$) and 4.63 p.p.m. (s, 2H, $-OCH_2$).

Tert-butylamide of 2′-(carboxymethoxime)-olivomycin I (6)

Compound **6** was obtained in the same manner as **5**, starting from **2** (60 mg, 0.047 mmol), *tert*-butylamine hydrochloride (15.2 mg, 0.142 mmol) and PyBOP (36.8 mg, 0.071 mmol). Yield 26 mg (42%). Rf 0.66 (system A), HPLC at Rt 15.26 min, m.p. 145–147 °C (decomposition). ESI-MS: calculated MW for $C_{62}H_{92}N_2O_{28}$ 1312.58, observed MW 1313.39 $(M+Na)^+$.

^{1}H-NMR spectrum (DMSO-d_6) of **6** corresponded to the ^{1}H-NMR spectrum of olivomycin I,[13] except for the presence of signals at 1.30 p.p.m. (s, 9H, $-C(CH_3)_3$) and 4.63 p.p.m. (s, 2H, $-OCH_2$).

2′-(Carboxymethoxime)-olivomycin I amide of 2-amino-2-methylpropane-1,3-diol (7)

Compound 7 was obtained in the same manner as **5**, starting from **2** (30 mg, 0.024 mmol), 2-amino-2-methylpropane-1,3-diol hydrochloride (10 mg, 0.071 mmol) and PyBOP (18.4 mg, 0.071 mmol). Yield 10 mg (30%). Rf 0.56 (system A), HPLC at Rt 8.11 min, m.p. 141–143 °C (decomposition). MALDI-MS: calculated MW for $C_{64}H_{96}N_2O_{29}$ 1356.61, observed MW 1379.97 $(M+Na)^+$.

The ^{1}H-NMR spectrum (DMSO-d_6) of **7** corresponded to the ^{1}H-NMR spectrum of olivomycin I,[13] except for the presence of signals at 1.30 p.p.m. (s, 3H, $-C(CH_2OH)_2\underline{CH_3}$), 4.5–4.78 p.p.m. (4H, $-C(\underline{CH_2}OH)_2CH_3$) and 4.63 p.p.m. (s, 2H, $-OCH_2$).

Cell lines and viability assays

All antiproliferation assays were performed in 96-well microliter plates (Falcon, Grenoble, France). In all, 5–7.5×10^4 tumor cells and a given amount of test compound were added to each well. The cells were allowed to proliferate for 48 h (L1210), or for 72 h (Molt4/C8 or CEM) at 37 °C in a humidified CO_2-controlled atmosphere.

The K562 human leukemia cells were cultured for 72 h in RPMI-1640 (PanEco, Moscow, Russia) supplemented with 5% fetal calf serum (BioWhittaker, Verviers, Belgium), 2 mmol L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin at 37 °C and 5% CO_2 in humidified atmosphere. At the end of the incubation period, the cells were counted in a Coulter Counter (Counter Electronics LTD, Luton, UK). IC_{50} (50% inhibition concentration) was defined as the concentration of compound that reduced the number of viable cells by 50%.

Topo-I-poisoning activity

The reaction mixture (0.25 μg of plasmid pUC19, 1 U of Topo-I and the antibiotics examined) was incubated in buffer (35 mM Tris-HCl, pH 8.0; 0.72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine, 0.01% bovine serum albumin) for 30 min at 37 °C. The reaction was terminated by adding sodium dodecyl sulfate to a final concentration of 1%. After the addition of protease K (37 °C, 40 min), the reaction products were separated in 1% agarose gel (3 A cm^{-1}) for 4–5 h (buffer composition: 40 mM Tris base, 1 mM EDTA and 30 mM glacial acetic acid). Reaction products were analyzed after staining the gels with an ethidium bromide solution (0.5 μg ml^{-1}) and photographed in ultraviolet light.

Antitumor activity

The antitumor activity of **5** was studied in mice bearing lymphocyte leukemia P-388 using both single and multiple i.p. injection regimens. B6D2F1 male mice were injected with 10^6 ascitic cells of lymphoma P-388 cells on day 0. The sample of **5** was dissolved in 0.1% DMSO, diluted with physiological solution and injected i.p. on day 3 (single injection regimen), or from day 3 to day 7 (multiple injection regimen).

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ORIGINAL ARTICLE

Genetic analysis of *Helicobacter pylori* clinical isolates suggests resistance to metronidazole can occur without the loss of functional *rdxA*

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Resistance to metronidazole (MTZ) in *Helicobacter pylori* is associated with mutations in *rdxA*, encoding an oxygen-insensitive NADPH nitroreductase, and mutations in *frxA*, encoding a NAD(P)H-flavin oxidoreductase. Despite this association, the strict correlation of MTZ resistance with mutations in *rdxA* or *frxA* is still controversial. In this study, *rdxA* allelic replacement was used to distinguish resistance-associated nucleotide mutations from the natural genetic diversity of *H. pylori*. Replacement with truncated *rdxA* resulted in MTZ resistance, whereas replacement with missense-mutated *rdxA* from resistant clinical isolates failed to yield MTZ resistance. Thus, although truncation of *rdxA* confers MTZ resistance in G27 *H. pylori*, MTZ resistance found in other clinical isolates is not due to the identified amino-acid substitutions. Three of our MTZ-resistant clinical isolates expressed functional *rdxA* and two of these also encoded full-length *frxA*. Therefore, MTZ resistance can arise in *H. pylori* possessing functional *rdxA*, suggesting that other factors are involved in MTZ resistance.
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INTRODUCTION

Since *Helicobacter pylori* was discovered in human gastric biopsy specimens in 1982,[1] it has been recognized as a significant contributing factor to the development of a number of gastric disorders. These include gastritis, ulcer disease and two distinct forms of gastric cancer, gastric adenocarcinoma and B-cell-mucosa-associated lymphoma.[2–6] *H. pylori* chronically infects more than 50% of the world's population, making it one of the most common bacterial infections worldwide. Given the large number of infected individuals and the strong association of *H. pylori* infection with gastric disease, *H. pylori* exerts a tremendous medical burden. This fact makes effective treatment regimens extremely important.

Metronidazole (MTZ), a synthetic nitroimidazole, was a critical ingredient of the first successful *H. pylori* therapy and remains a major component of several multidrug therapies that contain a proton pump inhibitor and a combination of two or more antibiotics (MTZ, clarithromycin, amoxicillin or tetracycline).[7,8] A major obstruction to successful *H. pylori* treatment is the presence of antibiotic-resistant strains. In fact, resistance to MTZ is common among clinically isolated *H. pylori*, with frequencies ranging from 10% to more than 90%, depending on the geographic region and patient group.[9,10] MTZ resistance is of clinical significance because it decreases the effectiveness of popular and affordable MTZ-containing anti-*H. pylori* therapies.[11,12] In addition, as MTZ is also used against a wide variety of prokaryotic and eukaryotic pathogens,[13–15] understanding the whole range of MTZ resistance mechanisms utilized by *H. pylori* may shed light on similar pathways in other clinically significant microbes.

The mechanism of antimicrobial action of MTZ has been investigated in anaerobic microbes.[13,16,17] The cytotoxicity of MTZ is due to unstable intermediates that damage the DNA, resulting in strand breakage, helix destabilization, unwinding and ultimately cell death.[18,19] Moreover, the anti-microbial action of MTZ is dependent on its reductive activation by the redox system of the target cell.[20]

This study focuses on the mechanisms of susceptibility and resistance of *H. pylori* to MTZ. In this bacterium, MTZ resistance has been shown to be strongly associated with mutations in *rdxA*,

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a chromosomal gene encoding an oxygen-insensitive NADPH nitroreductase, and mutations in *frxA*, a chromosomal gene encoding a NAD(P)H-flavin oxidoreductase. However, the absolute association of *H. pylori* MTZ resistance with mutations of *rdxA* or *frxA* is still a debated topic.[21–23] To conclusively prove this association, the contribution of specific *rdxA* or *frxA* mutations to MTZ resistance needs to be confirmed using classical genetic techniques. However, genetic tools and strategies for manipulation of *H. pylori* still lag behind those available for other model organisms. In this study, both *rdxA* and *frxA* genes from clinically isolated *H. pylori* strains were analyzed for sequence variation. To determine whether specific identified mutations in *rdxA* were responsible for the resistance patterns associated with the clinical isolates, allelic exchange was used to replace the *rdxA* gene of an MTZ-sensitive wild-type *H. pylori* strain with the *rdxA* gene from most of the clinical isolates. Minimum inhibitory concentrations (MICs) of MTZ of these *rdxA*-replaced transformants were then determined to evaluate the contribution of the *rdxA* mutations to MTZ resistance.

MATERIALS AND METHODS

H. pylori strains and culture condition

A total of 10 clinical *H. pylori* isolates, five MTZ-sensitive (S1–S5) and five MTZ-resistant (R1–R5), were obtained from patients at the Division of Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea. A written informed consent was received from each patient. The Institutional Review Board of Human Research at the Catholic University of Korea approved the protocol. The 3 previously characterized strains, ATCC43504, 26695 and G27,[24] and the 10 *H. pylori* clinical isolates were cultured on Columbia blood agar plates (Difco, Detroit, MI, USA) containing 5% horse blood (Oxoid, Basingstoke, UK) in 10% CO_2 at 37 °C.

Determination of MIC

MIC was defined as the lowest concentration of MTZ that completely inhibited the growth of the inoculum. MICs were determined by the *E*-test method (AB Biodisk, Solna, Sweden) and agar dilution method.[25] The *E*-test was performed on Columbia blood agar plates containing 5% horse blood according to the manufacturer's instructions. The agar dilution method was performed on Columbia blood agar plates containing 5% horse blood as described earlier.[25] Frozen bacterial stocks were streaked on Columbia blood agar and incubated for 3 days. Cells from a few colonies from these initial plates were then restreaked on fresh Columbia blood agar plates and incubated for one more day. The resulting exponentially growing cells were suspended in phosphate-buffered saline; series of 10-fold dilutions of these cell suspensions were prepared, and 10 µl of each dilution was spotted on freshly prepared Columbia blood agar containing various concentrations (0, 0.2, 0.5, 1.5, 3, 5, 8, 16, 32, 64 or 256 µg ml^{-1}) of MTZ (Sigma Chemical Co., St Louis, MO, USA). The plates were incubated for 3 days. MTZ-resistant *H. pylori* ATCC43504 and MTZ-sensitive 26695 were used as control strains. MTZ resistance was considered to be attained at an MIC of >8 µg ml^{-1}.[21]

Cloning of *rdxA* and *frxA genes* of clinical *H. pylori* isolates

Chromosomal bacterial DNA was extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). PCR amplifications of *rdxA* and *frxA* were carried out in a DNA thermal cycler (Biometra, Goettingen, Germany) using the Expand High Fidelity PCR System (Roche Applied Science, Mannheim, Germany) as follows: 1 cycle at 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final elongation step at 72 °C for 10 min. The oligonucleotide PCR primers used to amplify an 851-bp fragment containing the entire *rdxA* open reading frame were a forward primer (5′-aatttgagcatggggcaga-3′) and a reverse primer (5′-gaaacgcttgaaaacacccct-3′). Similarly, a forward primer (5′-ccatcgatatggacagagaacaagtgg-3′) and a reverse primer (5′-gctaacagcgtttttattcaatc-3′) were used to amplify the 675-bp fragment containing the entire *frxA* open reading frame. The amplified PCR fragments were extracted from 1% agarose gels using the QiaQuick Gel Extraction kit (Qiagen, Hilden, Germany), ligated into the pGEM-T-easy vector (Promega) and transformed into *Escherichia coli* DH5α, generating plasmid pRDXA-G27, -43504, -S1 to -S5 and -R1 to -R5, and pFRXA-G27, -43504, -S1 to -S5 and -R1 to -R5.

Nucleotide sequence analysis of *rdxA* and *frxA* of G27, ATCC43504 and clinically isolated *H. pylori*

DNA sequences of *rdxA* and *frxA* of G27, ATCC43504 and the clinical isolates were determined for both strands of the inserts of pRDXA and pFRXA series (Cosmogenetech, Seoul, Republic of Korea) using T7 and SP6 primers. The resulting DNA sequences were analyzed by the vector NTI v9.1 (Invitrogen, Carlsbad, CA, USA) and Sequencher v4.5 (Gene Code, Ann Arbor, MI, USA) programs. The GenBank accession numbers for the sequences reported in this paper are EF444879–EF444880, EF471983–EF471992 and EF521388–EF521397.

Generation of *rdxA* constructs for natural transformation

A 1.4-kb kanamycin (Km) resistance cassette (*aphA-3*) was PCR amplified from pILL600[26] using a forward primer (5′-ccaagcttggcgtatcacgaggccctttcg-3′) and a reverse primer (5′-ccaagcttctaaaacaattcatccag-3′), each containing a *Hin*dIII restriction enzyme site and two additional nucleotides. A unique *Hin*dIII restriction enzyme site in the *rdxA* fragment in pRDXA-S1 to -S5 and -R1 to -R5 was used to insert the Km resistance cassette. To avoid the leaky expression of *rdxA* due to readthrough from the expression of the Km resistance cassette, the Km resistance cassette was subcloned in the reverse direction to the *rdxA* gene. The direction was confirmed by sequencing the pRDXA series with the *rdxA* forward primer. The resulting plasmids carrying a Km resistance cassette were named pRDXA(Km) -S1 to -S5 and -R1 to -R5 and used for the *rdxA* replacement experiments.

Replacement of *rdxA* by natural transformation

The G27 *H. pylori* strain was used for replacement of the endogenous *rdxA* allele with the *rdxA* genes from the clinical isolates. Natural transformation with the pRDXA(Km) series was performed as described earlier[27] with the following modification: the G27 strain was struck and incubated for 3 days. Sweeps of G27 colonies from the initial plate were then restruck on a fresh Columbia blood agar plate and incubated. After 24 h, these cells were inoculated as a circle on a fresh Columbia blood agar plate with four 1-inch scrapes. After 6 h, 2 µg DNA of the pRDXA(Km) series was added to the G27 circle. After 20 h, the whole circle was restreaked onto Columbia blood agar containing Km (10 µg ml^{-1}) and incubated for 3–5 days until transformants appeared.

PCR screening for the *rdxA* replacement of transformants

Integration of the pRDXA(Km) series in the *rdxA* locus of G27 *H. pylori* by single crossover homologous recombination was selected for by Km resistance (Figure 1). The *rdxA* replacement of the Km-resistant transformants was confirmed by PCR screening with the *rdxA* primers as described above and illustrated in Figure 1. If the pRDXA(Km) series were integrated successfully into the wild-type *rdxA* locus, two bands of 851 bp (*rdxA* gene expressed by the endogenous *rdxA* promoter) and 2275 bp (promoterless *rdxA* gene containing the Km resistance cassette) should be observed. The small PCR band (851 bp) and large band (2275 bp) were extracted and sequenced (Cosmogene Tec.) with the *rdxA* primers and Km(seq) primer (5′-cgaaagggcctcgtgatacg-3′) to confirm the single crossover site of homologous recombination, the integration of *rdxA* of clinically isolated *H. pylori* and the interruption of G27 *rdxA* by the Km resistance cassette (Figure 1).

Reverse transcription-PCR

To confirm *rdxA* expression in the R3–R5 clinical *H. pylori* isolates, as well as expression of the integrated *rdxA* gene and lack of expression of the original G27 endogenous *rdxA* locus in *H. pylori* transformants, reverse transcription (RT)-PCR was performed on RNA from the R3–R5 clinical *H. pylori* isolates and *H. pylori* transformants, respectively. Total RNA was isolated using the RNeasy Protect Bacteria Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with 1 U of RNase-free DNase I (Sigma Chemical Co.) for 15 min, which was then inactivated by adding 50 mM EDTA

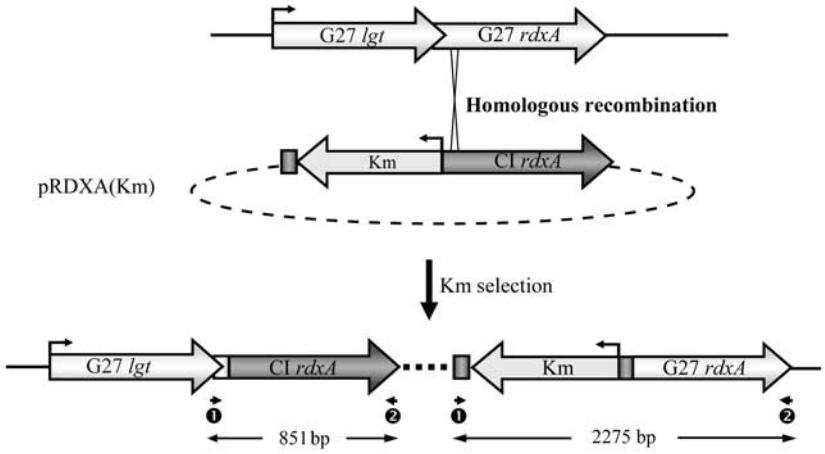

Figure 1 Schematic representation of the integration of pRDXA(Km) in the *rdxA* locus of G27 chromosomal DNA by single crossover homologous recombination. In *H. pylori*, *rdxA* is transcribed as part of an operon with *lgt*.[25] The promoterless *rdxA* gene from individual clinical isolates (CIs) was amplified by PCR and a kanamycin resistance cassette was inserted into a unique *Hin*dIII restriction site within the context of the pRDXA(Km) integration vectors as described in Materials and methods. After transformation of G27 and selection for kanamycin resistance, transformants were screened by PCR to identify those where the pRDXA(Km) plasmid had integrated into the endogenous *rdxA* locus by homologous recombination. As illustrated in the bottom portion of the figure, two structural *rdxA* genes exist in the transformants, but only one of the genes can be expressed by the endogenous *rdxA* promoter and the other one is silent. Promoter elements are indicated by small arrows on the respective open reading frames. The relative position of the primers used to verify proper integration is indicated by the numbers 1 and 2 and the sizes of the indicated PCR products are indicated in base pairs. Items in the figure are not drawn to scale.

Table 1 Deduced RdxA amino-acid changes in MTZ-sensitive and -resistant *H. pylori* isolates

Strain	*MIC* ($\mu g\,ml^{-1}$)	*Amino-acid position and change in RdxA*																	
		21	*26*	*37*	*40*	*50*	*52*	*62*	*64*	*88*	*90*	*98*	*106*	*108*	*117*	*131*	*153*	*172*	*206*
G27	0.75	M	Y	A	A	Q	H	L	K	S	K	G	P	S	A	K	L	V	A
S1	0.38	.[a]	.	.	.	.	.	V[b]	.	P	R	S	.	.	.	R	.	.	.
S2	0.25	.	.	.	.	.	R	V	.	P	R	S	.	A	S	.	.	I	.
S3	0.13	.	.	.	.	.	R	.	.	P	R	S	S	.	S	.	.	.	.
S4	0.19	.	.	.	.	.	R	V	.	P	R	S	.	.	.	.	.	I	.
S5	0.25	A	.	.	.	.	R	V	.	P	R	S	.	.	.	.	.	I	T
R1	64	.	.	.	.	~[c]	~	~	~	~	~	~	~	~	~	~	~	~	~
R2	128	A	.	V	.	.	.	V	.	P	R	S	.	.	.	.	~[d]	~	~
R3	256	.	.	.	.	.	.	V	N	P	R	N	.	.	.	.	.	I	.
R4	256	.	.	.	.	.	R	V	.	P	R	S	.	.	.	.	.	I	T
R5	256	.	V	.	P	.	R	V	.	P	R	S	.	A	S	.	.	.	.

Abbreviations: MIC, minimum inhibitory concentration; MTZ, metronidazole.
[a]The amino acid is the same as that of the G27 strain.
[b]A missense mutation resulted in the amino-acid substitution.
[c]A nonsense mutation resulted in the stop codon at residue 50.
[d]A frame-shift mutation resulted in the stop codon at residue 153.

and heating to 70 °C for 10 min. Here, 1 μg of total RNA as a template and 10 pM of the *rdx*A(RT) reverse primer (5′-gcattgctctaaaatatagc-3′) were then used for cDNA synthesis using an RT kit (Bioneer, Daejeon, Republic of Korea) according to the manufacturer's instructions. PCR amplification of the *rdx*A cDNA was then carried out with the *rdx*A(RT) forward primer (5′-ctatcgc-caagctcttacaa-3′) and reverse primer in a DNA thermal cycler (Biometra) using Maxime PCR PreMix (i-StarTaq) (iNtRON Biotechnology, Seongnam-Si, Republic of Korea) as follows: 1 cycle at 95 °C for 2 min; 35 cycles at 95 °C for 30 s, 48 °C for 30 s and 72 °C for 30 s and a final elongation step at 72 °C for 10 min. The final RT-PCR products (297 bp) were then run on an agarose gel to examine *rdxA* expression. For the negative control, total RNA of each sample was amplified by PCR without conducting a RT reaction. For these controls, no PCR products were observed, indicating no chromosomal DNA contamination. To confirm the expression of only the integrated *rdxA* gene in the *H. pylori* transformants, the final RT-PCR products from the transformants were extracted and sequenced as described above.

RESULTS

Identification of MTZ-sensitive or -resistant clinical *H. pylori* isolates

As we wished to elucidate the specific contribution of *rdxA* and *frxA* to MTZ resistance in current clinical *H. pylori* isolates, the MICs of MTZ of several isolates from patients undergoing gastroscopy were first determined by both the *E*-test and the agar dilution method. For reference, the MTZ MICs were also determined for the well-studied

Table 2 Deduced FrxA amino-acid changes in MTZ-sensitive and -resistant *H. pylori* isolates

Strain	MIC ($\mu g\,ml^{-1}$)	Amino-acid position and change in FrxA																							
		2	16	18	19	20	21	32	37	43	44	68	72	73	81	103	111	117	124	149	152	153	155	176	193	208
26695	0.75	D	A	K	Y	D	P	A	G	S	I	W	F	G	V	V	N	I	N	M	A	A	M	E	C	K
G27	0.75	.[a]	.	I[b]	R	S	~[c]	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~	~
S1	0.38	.	T	.	.	.	.	V	E	.	V	.	S	S	I	.	.	.	S	.	.	.	T	.	S	N
S2	0.25	.	T	.	.	.	.	.	.	.	.	.	S	S	.	.	.	.	.	.	.	.	.	K	S	.
S3	0.13	V	T	.	.	.	.	.	.	A	F	.	S	S	.	I	D	M	S	.	.	.	.	K	S	.
S4	0.19	V	T	.	.	.	.	.	.	A	F	.	S	S	.	.	D	M	S	K	.	.	.	K	S	.
S5	0.25	V	T	.	.	.	.	.	.	A	F	.	S	S	.	.	D	M	S	.	.	.	.	K	S	.
R1	64	.	T	.	.	.	.	V	.	A	F	.	S	S	.	.	D	M	S	.	.	.	.	K	S	.
R2	128	V	T	.	.	.	.	.	.	A	F	.	S	S	.	.	D	M	S	.	V	.	.	K	S	.
R3	256	.	T	.	.	.	.	.	.	A	F	.	S	S	.	.	D	M	S	.	.	.	.	.	S	N
R4	256	V	T	.	.	.	.	.	.	A	F	~[d]	~	~	~	~	~	~	~	~	~	~	~	~	~	~
R5	256	.	T	.	.	.	.	V	.	A	F	.	S	S	.	.	D	M	S	.	.	V	.	K	S	.

Abbreviations: MIC, minimum inhibitory concentration; MTZ, metronidazole.
[a]The amino acid is the same as that of the 26695 strain.
[b]A missense mutation resulted in the amino-acid substitution.
[c]A frame-shift mutation resulted in the stop codon at residue 21.
[d]A nonsense mutation resulted in the stop codon at residue 68.

H. pylori strains G27, ATCC43504 and 26695 (Tables 1 and 2). As expected, G27 and 26695 were sensitive to MTZ (MIC, 0.75 $\mu g\,ml^{-1}$), whereas ATCC43504 was resistant (MIC, $\geqslant 256\,\mu g\,ml^{-1}$). Five isolates (S1–S5) that were sensitive to MTZ (MIC, 0.38, 0.25, 0.13, 0.19 and 0.25 $\mu g\,ml^{-1}$, respectively) and five isolates (R1–R5) that were resistant to MTZ (MIC, 64, 128 and R3–R5 $\geqslant 256\,\mu g\,ml^{-1}$, respectively) were selected for further in-depth analysis (Table 1).

Amino-acid sequences of RdxA and FrxA from MTZ-sensitive and -resistant *H. pylori* isolates

To identify putative MTZ resistance-associated mutations in *rdxA* and *frxA*, both genes were sequenced from the 10 *H. pylori* clinical isolates, as well as from the G27 and ATCC43504 reference strains. The DNA sequences obtained for *rdxA* and *frxA* from ATCC43504 were identical to the sequences reported earlier.[4,28] Moreover, the deduced amino-acid sequence of RdxA and FrxA from the DNA sequence of ATCC43504 indicates that this strain produces truncated non-functional forms of both proteins.

The deduced RdxA amino-acid sequences of G27 and the 10 clinical isolates were compared to identify any potential resistance-associated nucleotide mutations (Table 1). All of the six MTZ-sensitive strains (G27 and the five clinical isolates) encode full-length RdxA, but each clinical isolate contains 5–8 amino-acid substitutions arising as a result of missense mutations (Table 1). As these strains are all MTZ-sensitive, these amino-acid changes are probably not important for nitroreductase function and hence MTZ resistance. Instead, these variations are likely a by-product of the natural genetic diversity of *H. pylori*, which, in the case of *rdxA*, is estimated to be 5–8%.[29,30]

Two of five MTZ-resistant *H. pylori* isolates (R1 and R2) contained truncated RdxAs due to a nonsense mutation at amino acid 50 and a frameshift resulting in termination at residue 153, respectively. The amino-acid sequence of three (R3–R5) of the MTZ-resistant isolates identified full-length RdxA containing 6–9 amino-acid substitutions. Comparison of the amino-acid sequence between the MTZ-sensitive and -resistant *H. pylori* isolates suggests that substitutions of asparagine at residue 64 and asparagine at residue 98 in the R3 strain, and substitutions of valine at residue 26 and proline at residue 40 in the R5 strain might be important for MTZ resistance. The amino-acid sequence of R4 was virtually identical to that of S5 except for a predicted neutral mutation (Met to Ala) at residue 21 in S5, suggesting that the R4 RdxA is functional similar to the S5 RdxA.

As FrxA has been suggested to play a role in MTZ resistance, we next analyzed the deduced FrxA amino-acid sequences to identify any amino-acid changes. As FrxA is truncated in G27, the FrxA amino-acid sequence from 26695 was used to compare the FrxA sequence of the 10 clinical isolates (Table 2). All five of the MTZ-sensitive *H. pylori* strains encoded full-length FrxA containing 5–12 amino-acid substitutions by missense mutations. Among the five MTZ-resistant *H. pylori* isolates, R4 encodes a truncated FrxA due to a nonsense mutation at residue 68. The amino-acid sequences of the four remaining MTZ-resistant isolates (R1, R2, R3 and R5) showed full-length FrxA containing 10–12 amino-acid substitutions by missense mutations. Surprisingly, no putative MTZ resistance-associated missense mutations of *frxA* were identified as no unique missense mutation in the MTZ-resistant strains was identified, as compared with the MTZ-sensitive strains. The one exception was a neutral mutation (Ala to Val) at residue 152 or 153. It is noted that the FrxA sequence from G27 showed that this MTZ-sensitive strain, which encodes a full-length RdxA protein, contained a truncated FrxA due to a frameshift mutation at residue 21. This strongly suggests that truncation of *frxA* alone does not result in MTZ resistance. Given that mutation of *frxA* was not sufficient to impart MTZ resistance, we focused the remainder of our studies on the role of *rdxA* mutations on MTZ resistance.

Analysis of clinical *rdxA* alleles in an isogenic *H. pylori* background

To directly evaluate the contribution of the truncated *rdxA* and substituted *rdxA* alleles to MTZ resistance in an isogenic strain background, the *rdxA* locus of MTZ-sensitive wild-type *H. pylori* was genetically replaced with the *rdxA* gene of several of the clinical isolates (Figure 1). G27 was chosen as the recipient for the *rdxA* replacement because G27 is MTZ sensitive (MIC, 0.75 $\mu g\,ml^{-1}$), is capable of natural transformation and encodes a truncated FrxA

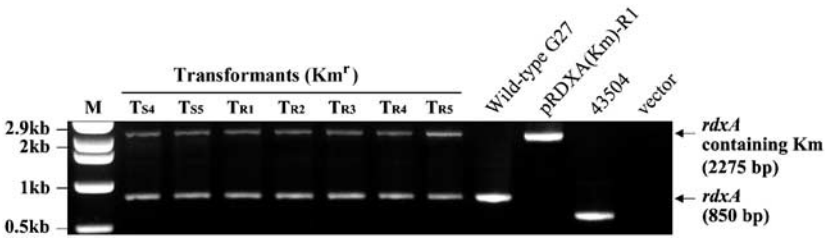

Figure 2 PCR screening for the *rdxA* replacement of transformants. The *rdxA* replacement of Km-resistant *H. pylori* transformant was confirmed by PCR screening with an *rdxA* primer set. Transformants showed two bands—an 851 bp (*rdxA* gene) and a 2275 bp (*rdxA* containing the Km resistance cassette).

protein. We considered the truncation of FrxA to be important as an earlier study suggested that combined inactivation of FrxA and RdxA enhances MTZ resistance.[21] The *rdxA* gene from S4, S5 and R1–R5 were introduced into G27 by natural transformation with pRDXA(km)-S4, -S5 and -R1–R5 (Figure 1). The S4 and S5 *rdxA* alleles were selected as representative forms of *rdxA* from the MTZ-sensitive strains. The pRDXA(km) plasmid series does not replicate in *H. pylori*, and thus to obtain the Km resistance phenotype, the plasmid must integrate into the *rdxA* chromosomal locus by single crossover homologous recombination (Figure 1). Thus, after transformation a merodiploid is produced where only the introduced *rdxA* is expressed by the endogenous *rdxA* promoter. To avoid the leaky expression of *rdxA* from the Km resistance cassette promoter, the Km resistance cassette was subcloned in the reverse orientation to the *rdxA* gene (Figure 1).

The proper *rdxA* replacement in the Km-resistant transformants was confirmed by PCR with the *rdxA* primer set that showed the following two bands; an 851-bp band (*rdxA* gene expressed from the endogenous promoter) and a 2275-bp band (a promoterless *rdxA* containing the Km resistance cassette) (Figures 1 and 2). In addition to the PCR analysis, the expressing *rdxA* gene (small PCR product) was sequenced to confirm the single crossover site and the complete replacement with the clinical isolates *rdxA* locus (data not shown). Finally, interruption of the native G27 *rdx*A gene by the Km resistance cassette was confirmed by sequencing of the large PCR product (2275 bp) (data not shown). For all natural transformations, we isolated transformants in which the single crossover occurred upstream from the first identified amino-acid change. Therefore, the clinical isolates *rdxA* allele completely replaced the G27 *rdxA* gene. At least three individual transformants from each complete replacement were obtained and analyzed further.

Verification of expression of the introduced *rdx*A gene but not the G27 endogenous *rdx*A

To confirm that the *rdxA* expressed by each of our merodiploid strains was indeed from the integrated copy of *rdxA* from our clinical isolates, we performed RT-PCR for the *rdxA* locus using total RNA harvested from the *H. pylori* transformants. On the basis of the assumption that the sequence of the cDNA produced from these RT reactions would show whether the integrated *rdxA* or endogenous *rdxA* was being expressed, we compared these cDNA sequences to the sequences we obtained earlier (Table 1). Total RNA from each sample was also amplified by PCR without first conducting a RT reaction and no PCR products were observed, indicating that there was no chromosomal DNA contamination (data not shown).

The RT-PCR products of R1–R5 were each extracted from an agarose gel and sequenced. The sequence analysis indicated that each *H. pylori* transformant indeed expressed only the integrated *rdxA* from the clinical *H. pylori* isolates as the expression of the disrupted original G27 *rdx*A could not be detected.

Table 3 MTZ MICs of transformants

Clinical isolates (MICs)	*Transformants (MICs)*
S4 (0.19 μg ml^{-1})	T_{S4} (0.25–0.75 μg ml^{-1})
S5 (0.25 μg ml^{-1})	T_{S5} (0.25–0.35 μg ml^{-1})
R1 (64 μg ml^{-1})	T_{R1} (⩾256 μg ml^{-1})
R2 (128 μg ml^{-1})	T_{R2} (⩾256 μg ml^{-1})
R3 (⩾256 μg ml^{-1})	T_{R3} (0.25–0.75 μg ml^{-1})
R4 (⩾256 μg ml^{-1})	T_{R4} (0.25–3 μg ml^{-1})
R5 (⩾256 μg ml^{-1})	T_{R5} (0.25–3 μg ml^{-1})

Abbreviations: MIC, minimum inhibitory concentration; MTZ, metronidazole.
The *rdxA* replacement of MTZ-sensitive G27 (MIC, 0.75 μg ml^{-1}) with the *rdxA*s of the clinical isolates resulted in the following MTZ MICs.

MIC determination of *rdxA*-replaced *H. pylori* transformants

To determine whether the MTZ resistance/sensitivity profile of the clinical isolates was transferred by the expression of the clinical *rdxA* locus in the isogenic strain background, the MTZ MICs of the *rdxA*-replaced *H. pylori* transformants were determined by both the *E*-test and agar dilution method. As shown in Table 3, replacement with the *rdxA* genes from the MTZ-sensitive strains (S4 and S5) resulted in *H. pylori* transformants that remained sensitive to MTZ (MIC, 0.75 and 0.35 μg ml^{-1}, respectively). This was expected as the RdxAs of the MTZ-sensitive strains should be functional. The MICs were similar to that of the recipient strain, G27 (MIC, 0.75 μg ml^{-1}). Replacement with the *rdxA* genes from the MTZ-resistant strains (R1–R5) resulted in a wide range of MICs (0.25–⩾256 μg ml^{-1}). As expected, replacement with *rdxA* from R1 and R2 resulted in high resistance to MTZ (MIC, ⩾256 μg ml^{-1}). These results indicate that truncated, and hence non-functional, RdxA causes MTZ resistance in *H. pylori*. In addition, the MICs were higher than those of the original clinical isolated *H. pylori* strains (R1 MIC, 64 μg ml^{-1} and R2 MIC, 128 μg ml^{-1}). This is in keeping with the idea that the truncated FrxA already found in G27 can enhance MTZ resistance in the presence of a non-functional *rdxA* mutation. Unexpectedly, replacement with the *rdxA* genes from the MTZ-resistant strains R3, R4 and R5 still resulted in sensitivity to MTZ (MIC, 0.25–0.75, 0.25–3 and 0.25–3 μg ml^{-1}, respectively). We originally predicted that the asparagine substitution at residue 64 and asparagine at residue 98 in the R3 strain, and the valine at residue 26 and proline at residue 40 in the R5 might be important for MTZ resistance (Table 1). However, the fact that the *rdxA* replacement containing these amino acids still resulted

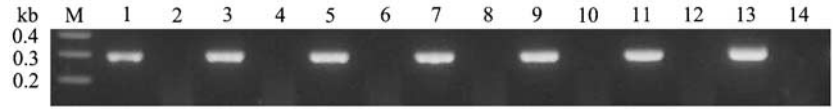

Figure 3 Reverse transcription (RT)-PCR to confirm *rdxA* expression in metronidazole (MTZ)-sensitive and -resistant *H. pylori* strains. Total RNA was isolated from the indicated strains, treated with DNase and then used to examine *rdxA* expression by RT-PCR as detailed in Materials and methods. Each of the strains expressed *rdxA* as they yielded the expected 297-bp product. For the negative control, total RNA from each sample was amplified by PCR without first conducting a reverse transcription reaction. No PCR products were observed, indicating no chromosomal DNA contamination. Lanes M=Marker, 1=G27, 2=G27 no RT, 3=S3, 4=S3 no RT, 5=S4, 6=S4 no RT, 7=S5, 8=S5 no RT, 9=R3, 10=R3 no RT, 11=R4, 12=R4 no RT, 13=R5 and 14=R5 no RT.

Table 4 Sequence variation in the upstream region of *rdxA* in *H. pylori* clinical isolates

Strain	*Sequence of rdxA upstream region*	
	SD[a] sequence	*Start codon*
HP2600	GCTACGAAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
G27	GCTAC**A**A**GGC**ATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
S1	GCTACGAAAAATTCTAAAAAAAATA**AAGG****G**AATCA	***ATG***
S2	GCTACGAAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
S3	GCTACGAAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
S4	GCTACGAAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
S5	GCTACGAAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
R1	GCTAC**–**[b]AAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
R2	GCTACGAAAAATTCTAAAAAAAATA**AAGG****G**AATCA	***ATG***
R3	GCTACGAAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
R4	GCTACGAAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***
R5	GCTAC**–**AAAAATTCTAAAAAAAATA**AAGGAA**AATCA	***ATG***

Gray blocks indicate differences from the sequence of HP2600.
RdxA start codon (ATG) is indicated in italic bold.
[a]SD (Shine–Dalgarno) sequence (AAGGAA) is indicated in bold.
[b]– indicates a nucleotide deletion.

in MTZ sensitivity suggests that these substitutions are not significant for MTZ resistance and thus reductase function.

Verification of *rdxA* expression in the clinical isolates

As we examined expression of the clinical *rdxA* allele in G27 using the G27 promoter to drive expression of the transferred *rdxA* gene, we sought to ensure that the R3–R5 clinical isolates did not contain any promoter mutations that prevented *rdxA* expression in the context of the clinical strain background. Therefore, *rdxA* expression was examined by RT-PCR in the R3–R5 clinical isolates as well as in G27. This analysis showed that each of the strains expressed *rdxA* (Figure 3). Therefore, any variations in the upstream sequences in R3–R5 do not prevent *rdxA* expression and thus, do not explain the obtained MTZ resistance profiles of the clinical isolates.

Finally, as R3–R5 apparently encode functional RdxA and as mutations upstream of the RdxA start codon have been identified that affect MTZ resistance,[21] we investigated whether there were any nucleotide mutations in this region that might affect RdxA translation in the original R3–R5 clinical isolates. To examine sequence polymorphisms, the region upstream of the ATG start codon, which includes the Shine–Dalgarno sequence of *rdxA*, were sequenced in all 10 clinical isolates (Table 4). The sequences were then compared with that of HP2600[28] and G27, which are both sensitive to MTZ. As all the clinical isolates showed sequences that were most similar to HP2600, we focused our comparison to this MTZ-sensitive strain. Because the sequences of S2–S5 and R3 and R4 were the same as that of HP2600 there are no changes that can account for the MTZ resistance seen in R3 and R4. In addition, the sequences of S1 and R2 were identical, suggesting that the single nucleotide difference from the HP2600 sequence is not important for MTZ resistance. The sequence of R1 and R5 showed a one nucleotide (G) deletion, which might affect *rdxA* expression; however, the sequence of G27 has four nucleotide variations in this same region. As G27 is sensitive to MTZ, it seems unlikely that these variations should affect *rdxA* expression in R1 and R5.

DISCUSSION

This study examined the resistance of *H. pylori* to the anti-microbial agent MTZ and specifically focused on the contribution of the *rdxA* and *frxA* genes to MTZ resistance. Five MTZ-sensitive and five MTZ-resistant *H. pylori* strains were obtained from the clinical isolates. Analysis of the deduced RdxA and FrxA amino-acid sequences of these isolates revealed various mutations. To directly evaluate the contribution of these specific *rdxA* mutations to MTZ resistance, we introduced these *rdxA* variants into G27 while inactivating its own *rdxA* gene. Our results show that (i) premature truncation of *frxA* alone is not sufficient to cause MTZ resistance in *H. pylori*, but has the capacity to enhance MTZ resistance in *H. pylori* containing a deficient *rdxA*; (ii) introduction of an inactivated *rdxA* (non-functional premature truncation) is sufficient to confer MTZ resistance to G27 (normally MTZ sensitive); (iii) introduction of the missense-mutated *rdxA* alleles (6–9 amino-acid substitutions each) does not result in MTZ resistance; (iv) three high MTZ-resistant isolates (MIC, $\geqslant 256\,\mu g\,ml^{-1}$) have functional *rdxA*, suggesting that an MTZ resistance phenotype can arise in *H. pylori* in the absence of inactivating mutations in *rdxA*.

MTZ resistance associated with mutation of *frxA* is still one of the most controversial topics. Kwon *et al*.[22] reported that *frxA* inactivation resulted in MICs (32 or $128\,\mu g\,ml^{-1}$) similar to those seen with *rdxA* inactivation, whereas a study by Jeong *et al*.[21] showed that *frxA* inactivation enhanced MTZ resistance in *rdxA*-deficient *H. pylori* but had little effect on the MTZ susceptibility of strains carrying a functional *rdxA* allele. In addition, Yang *et al*.[23] recently identified the truncation of FrxA in an MTZ-sensitive *H. pylori* isolate. Our results agree with the studies of the Jeong and Yang groups. MTZ-sensitive G27 (MIC, $0.75\,\mu g\,ml^{-1}$) contains a truncated FrxA due to a frameshift-causing termination at residue 21. As full-length FrxA is 217 amino acids, the truncated FrxA is most likely non-functional. Therefore, the functional inactivation of FrxA alone cannot induce MTZ resistance in this strain. In addition, the comparison of FrxA amino-acid sequences (Table 2) shows that none of the putative missense mutations is likely to be responsible for MTZ resistance as no unique missense mutations in the MTZ-resistant strains were identified with the exception of a neutral mutation (Ala to Val) at residue 152 or 153. It is worth noting that the replacement of G27 *rdxA* with the nonsense-mutated *rdxA* (premature truncation) from R1 (MIC, $64\,\mu g\,ml^{-1}$) and the frameshift-mutated *rdxA* (premature truncation)

from R2 (MIC, 128 μg ml^{-1}) resulted in higher MTZ resistance (MIC, ⩾256 μg ml^{-1}) than in the original isolates. Although this comparison is between *H. pylori* strains of different genetic backgrounds, this result is probably due to double functional inactivation of RdxA and FrxA in the transformants as G27 contains a non-functional FrxA and both R1 and R2 isolates contain full-length and presumably functional FrxA (Table 2). This fact suggests that FrxA inactivation does indeed enhance MTZ resistance in *rdxA*-deficient *H. pylori* and is in agreement with the study of Jeong *et al.*[21]

Several *rdxA* replacements were performed to evaluate the role of *rdxA* mutations in MTZ resistance. We designed our study such that the replaced *rdxA*s of all transformants were expressed by the same G27 endogenous *rdxA* promoter. This design enabled us to avoid the potential problem of differential promoter regulation and as a result, all transformants are in the same genetic background. Earlier, presumably important mutations of *rdxA* for MTZ resistance were simply identified by comparison of the RdxA sequence between MTZ-sensitive and -resistant *H. pylori* strains.[2,31,32] However, these mutations were not evaluated directly for their contribution to MTZ resistance. Comparative analysis of the RdxA sequences in our study predicted that the changes of Lys64→Asn and Gly98→Asn in the R3 strain, and Tyr26→Val and Ala40→Pro in the R5 strain might be responsible for MTZ resistance. *rdxA* replacement proved that these substitutions are not critical changes in MTZ resistance as the G27 strain carrying them remained MTZ sensitive. These findings suggest that the amino-acid changes should be attributed to natural genetic diversity and are not associated with MTZ resistance. Recent studies revealed the RdxA amino-acid changes of Arg10→Lys, Arg16→His, Met21→Ala, His53→Arg, Met56→Ile, Leu62→Val, Ala68→Val, Gly98→Ser, Gly163→Asp and Ala206→Thr in MTZ-resistant strains,[2,31,32] suggesting that those substitutions may be important in MTZ resistance. However, in our study the replacement with the missense-mutated *rdxA* containing amino-acid substitutions (Met21→Ala, Leu62→Val, Gly98→Ser and Ala206→Thr) failed to transfer the MTZ-resistant phenotype, suggesting that the substitutions are not important for MTZ resistance. In addition, only Paul *et al.*[32] confirmed experimentally that RdxA amino-acid substitutions (Cys19→Tyr and Thr49→Lys) were causative for MTZ resistance. In that study, MTZ-sensitive strains were transformed with PCR products of MTZ-resistant *rdxA* and the appearance of MTZ-resistant isolates was selected for studies on MTZ. MTZ-resistant transformants were then analyzed for their *rdxA* sequences. It should be noted that it is formally possible that the selective pressure of MTZ applied in that study forced other mutations to arise elsewhere that result in MTZ resistance. The PCR-transformed strains were not screened for putative resistance-associated mutations in other genes. To prevent MTZ-selective pressure in our study, the transformants were selected by Km resistance and MTZ MICs were then measured. In the future, it will be interesting to directly assess the role of the RdxA substitutions identified by Paul *et al.*[32] for their significance in MTZ resistance using the *rdxA* replacement strategy.

Three clinical isolates (R3–R5) showed high resistance to MTZ (MIC, ⩾256 μg ml^{-1}), even though our data indicate that they have functional *rdxA*. This result is in agreement with Marais *et al.*[33] who suggested that an MTZ resistance phenotype may arise in *H. pylori* without mutation in *rdxA* or *frxA*. Interestingly, several groups[22,34–37] and genome sequence annotation[38–40] suggest other putative redox systems that may play a role in MTZ resistance. These include *fdxB* (encoding a ferredoxin-like protein), *fdxA* (ferredoxin), *fldA* (flavodoxin), *oorD* (the γ-subunit of 2-oxoglutarate oxidoreductase and *porD* (the γ-subunit of pyruvate ferredoxin oxidoreductase). However, with the exception of *fdxB*, research to identify other MTZ important redox systems has been hampered because the deletion of the genes seems to be lethal for *H. pylori*.[22] Recently, van Amsterdam *et al.*[41] reported that double mutation of HP0605 and HP0971 (TolC-like proteins) results in decreased MTZ resistance (from wild-type MIC, >256 μg ml^{-1} to 8 μg ml^{-1}), indicating that the TolC efflux pump may confer resistance to MTZ. Therefore, *H. pylori* possessing TolC efflux pumps may be resistant to MTZ independent of *rdxA* or *frxA* mutations.

In summary, the findings of this study clarify some of the debate surrounding the questions of whether deletion of *frxA* alone can induce MTZ resistance or simply enhance the resistance in an *rdxA*-deficient *H. pylori* strain. Additionally, our *rdxA* replacement approach provides some of the first analysis of the role of specific mutations in MTZ resistance. Our results show that deletion of *rdxA* induces MTZ resistance, but that none of the 15 amino-acid substitutions found in *rdxA* of the MTZ-resistant strains is able to cause MTZ resistance. This strongly suggests that the substitution mutations identified previously by sequence comparison need to be directly analyzed for their contribution to MTZ resistance. Finally, MTZ resistance in *H. pylori* can arise without mutations in *rdxA* or *frxA*, clearly suggesting that other genetic elements are involved in MTZ resistance. Future work from our group will focus on the identification of the genes responsible for this MTZ resistance phenotype.

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ORIGINAL ARTICLE

Expression of two human acyl-CoA:diacylglycerol acyltransferase isozymes in yeast and selectivity of microbial inhibitors toward the isozymes

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Two isozymes for human acyl-coenzyme A:diacylglycerol acyltransferase (DGAT), DGAT1 and DGAT2, were independently expressed in DGAT-deficient *Saccharomyces cerevisiae* to establish DGAT1- and DGAT2-*S. cerevisiae*. The selectivity of DGAT inhibitors of natural origin towards the isozymes was assessed in enzyme assays using the microsomal fractions prepared from DGAT1- and DGAT2-*S. cerevisiae*. Amidepsines and xanthohumol inhibited DGAT1 and DGAT2 with similar potency, whereas roselipins were found to inhibit DGAT2 selectively.
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Keywords: acyl-CoA:diacylglycerol acyltransferase; amidepsine; DGAT; isozyme; roselipin; triacylglycerol; xanthohumol

INTRODUCTION

Triacylglycerol (TG) is the major energy-storage form of long-chain fatty acids in animals.[1,2] TG synthesis is important in many biological processes, including intestinal fat absorption, fat storage in adipocytes and energy metabolism in muscle, but excessive accumulation of TG in adipocytes as a result of a fat-rich diet or sedentary lifestyle causes obesity.

Acyl-coenzyme A (CoA):diacylglycerol acyltransferase (DGAT, EC2.3.1.20) is a membrane-bound enzyme that catalyzes TG formation by acyl esterifications of diacylglycerol. Two biological pathways for TG synthesis, the glycerol phosphate pathway and the monoacylglycerol pathway, have been reported. These pathways form diacylglycerol (DG), which in turn is acylated by DGAT to form TG.[3] Recent molecular biological studies have revealed the existence of two different DGAT isozymes, DGAT1 and DGAT2,[4–6] in mammals, and extensive studies including biological experiments and knockout mice have shown that these isozymes have different functions in mammals.[7–13] Increased DGAT2 activity in the liver causes hepatic steatosis, whereas DGAT1 plays a role in very low-density lipoprotein (VLDL) synthesis in the liver and increases plasma VLDL concentration. Furthermore, newborn DGAT2-deficient mice die within hours of birth, whereas DGAT1-deficient mice are viable and have a modest reduction in tissue TG. Therefore, it is important to determine the selectivity of inhibitors towards the two DGAT isozymes for developing them as pharmaceutical drugs.

Our research group conducted an enzyme assay involving rat liver microsomes to discover several DGAT inhibitors from natural sources, including fungal amidepsins,[14–16] plant xanthohumols[17] and fungal roselipins[18–20] (Figure 1). The selectivity of these inhibitors towards the two DGAT isozymes could not be assessed in the rat liver system. In this study, enzyme-based assay systems for DGAT1 and DGAT2 isozymes were established by transforming DGAT-deficient *Saccharomyces cerevisiae* using complementary DNA (cDNA) of human DGAT isozymes, which allowed us to investigate the selectivity toward the isozymes of natural DGAT inhibitors discovered earlier.

MATERIALS AND METHODS

Materials

Amidepsines A–D and roselipins 1A, 1B, 2A and 2B were purified from the culture broth of the respective microorganism according to established methods.[14,18] Xanthohumol was purified from the methanol extracts of *Humulus lupulus* according to an established method.[17] [1-^{14}C]Palmitoyl-CoA was purchased from GE Healthcare UK Ltd (Amersham Place, Little Chalfont, UK).

General

Standard molecular biological techniques were applied[21]. For polyethylene glycol-mediated transformation of yeast cells, the lithium acetate method was used.[22]

Strains, media and chemicals

S. cerevisiae BY4742-DGA1, a DGAT-deficient mutant of *S. cerevisiae* BY4742 (MATα *his3 leu2 lys2 ura3*), was purchased from Open Biosystems (Huntsville, AL, USA) (www.openbiosystems.com). *S. cerevisiae* OP3-C (MATα *leu2 ura3*) was used for the reference strain of DGAT activity. *Escherichia coli* strain DH5α (F$^-$ Φ80d*lacZ* ΔM15 Δ (*lacZYA-argF*) U169 *deoR recA*1 *endA*1 *hsdR*17 (rk$^-$, mk+) *phoA supE*44 λ$^-$ *thi*-1 *gyrA*96 *relA*1) was used in DNA manipulations.

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YPD medium (1.0% bacto-yeast extracts, 2.0% bacto-peptone, 2.0% glucose) was used to grow non-transformed yeast strains. Selection medium (SM-Glc; yeast nitrogen base w/o amino acids 0.67%, cassamino acid 0.5%, glucose 2.0%) was used for the maintenance of yeast strains transformed with pYES, harboring either no insert or DGAT1 or DGAT2 cDNA. Production medium (PM-Gal; yeast nitrogen base w/o amino acid 0.67%, cassamino acid 0.5%, galactose 2.0%) was used for production of the recombinant human DGAT isozymes by the transformed yeasts.

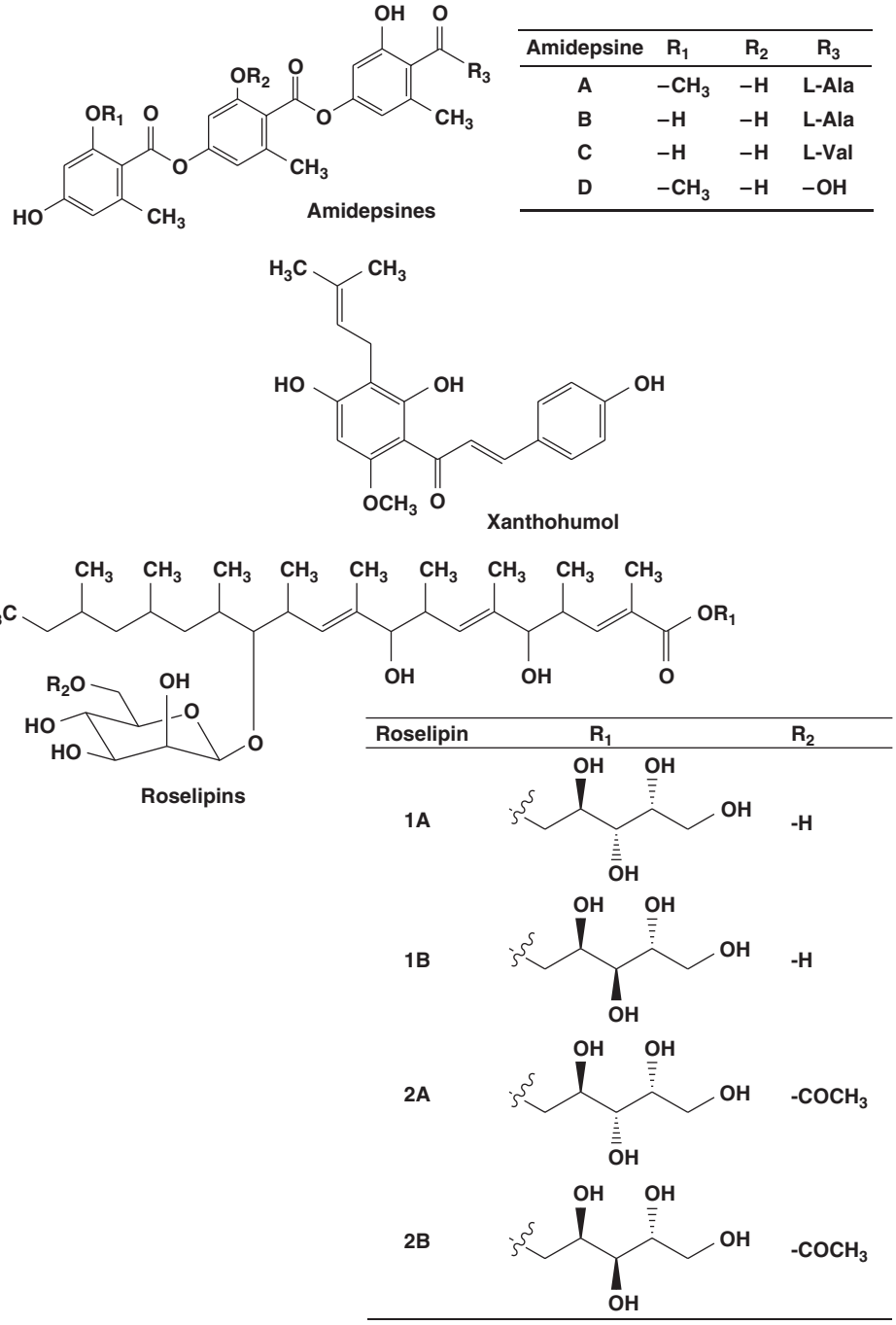

Figure 1 Structures of acyl-CoA:diacylglycerol acyltransferase (DGAT) inhibitors discovered using rat liver microsomes in an enzyme-based assay.

Plasmids

The coding regions of human DGAT1 (accession no. AF059202) and DGAT2 (accession no. AY358532) genes were amplified using cDNA prepared from HeLa cells and a human liver cDNA library (Takara Bio Inc., Shiga, Japan), respectively, by polymerase chain reaction (PCR) (30 cycles, 1 min at 94 °C, 1 min at 55 °C and 6 min at 68 °C) using the following oligonucleotide sets: N-terminal primer 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GGG CGA CCG CGG CAG C-3′ and C-terminal primer 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA GCT CAG GCC TCT GCC GCT G-3′ for DGAT1, and N-terminal primer 5′-GGG GAC AAG TTT GAT CAA AAA AGC AGG CTC CAT GAA GAC CCT CAT AG-3′ and C-terminal primer 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CAG CTG GTT CCT CCA GG-3′ for DGAT2. The DGAT1 and DGAT2 cDNAs were transferred to pDONR201 (Invitrogen, Carlsbad, CA, USA) between the att1 and att2 sites by site-specific recombination according to the protocols, and the nucleotide

sequences of the cDNA regions were determined by a PRISM 3100 Genetic Analyzer (Applied Biosystems) using M13 Primer M4 and M13 Primer RV (Takara Bio). The cDNAs were then transferred to pYES-DEST52 (Invitrogen) according to the manufacturer's protocols. The vector carried the URA3 marker for auxotrophic selection and a GAL1 promoter for protein expression.

Expression of the recombinant DGAT1 and DGAT2 in yeasts and preparation of microsomes

S. cerevisiae BY4742-ΔDGA1 transformed with pYES, harboring either no insert or DGAT1 or DGAT2 cDNA (designated as mock *S. cerevisiae*, DGAT1-*S. cerevisiae* and DGAT2-*S. cerevisiae,* respectively), was grown for 24 h in SM-Glc medium. The cells were washed twice with PM-Gal, resuspended in the same medium at $OD_{590\,nm}$=0.4 and grown for another 24 h. The cells were washed once with ice-cold PBS(−) and resuspended in DGAT buffer containing 50 mM Tris–HCl, 140 mM KCl, 0.1 mM EDTA (pH 7.5) and complete Mini (F. Hoffmann-La Roche AG, Basel, Switzerland). Cell lysates obtained by disintegration using a French Press (GLEN MILLS INC., Clifton, NJ, USA) were centrifuged at 15 000 *g* for 30 min. The supernatant was collected and centrifuged at 105 000 *g* for 1 h to precipitate the microsomes. The pellet was resuspended in DGAT buffer and the protein concentration was determined by a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). The microsomes were aliquoted and frozen at −80 °C.

DGAT assay

Microsomal fractions from mock, DGAT1- and DGAT2-*S. cerevisiae* were used to measure DGAT activity. Assays were performed in duplicate as described previously.[14] An assay mixture contained 175 mM Tris–HCl (pH 8.0), 50–75 μg yeast microsomal protein, 14.5 μM bovine serum albumin, [1-^{14}C]palmitoyl-CoA (30 μM, 7.4 kBq), 8 mM $MgCl_2$, 2.5 mM diisopropylfluorophosphate and 150 μM 1,2-dioleoyl-*sn*-glycerol; experimental inhibitors were dissolved in 2.5 μl of methanol and included in a total assay reaction volume of 0.2 ml. The assay was initiated by addition of the microsomal fraction. After incubation at 23 °C for 15 min, the reaction was stopped by addition of 1.2 ml of chloroform: methanol (1:2) and lipids were extracted according to the method of Folch *et al.*[23] TG was separated by TLC on Silica gel 60 plates (Merck Co.) using a petroleum ether:diethylether:acetic acid (80:20:1) solvent system. The TLC plates were exposed to a Fujifilm imaging plate to assess the formation of [^{14}C]TG. Imaging signals were visualized and quantified with a BAS 2000 imaging analyzer (FUJIFILM Corporation, Tokyo, Japan).

RESULTS

Construction of DGAT1- and DGAT2-*S. cerevisiae*

To obtain active human DGAT isozymes for the construction of the enzyme-based assay system, DGAT1 and DAGT2 cDNAs were originally isolated from HeLa cells and a cDNA library of human liver using PCR. The deduced amino acid sequence of these clones revealed perfect matches with previously reported sequences (accession nos. AF059202 and AF384161). The coding regions of DGAT1 and DGAT2 were introduced to the yeast expression vector pYES-DES52 by *in vitro* recombination, and the constructed plasmids were transformed into DGAT-deficient *S. cerevisiae* to establish DGAT1- and DGAT2-*S. cerevisiae*.

DGAT1 and DGAT2 activity

TG synthesis catalyzed by DGAT in the microsomes prepared from DGAT-deficient *S. cerevisiae* and mock *S. cerevisiae* was measured. As shown in Figure 2, [^{14}C]TG was not detected (lanes 2 and 3), indicating that these mutant yeasts completely lack DGAT activity.

On the other hand, TG synthetic activity was recovered in the microsomes prepared from DGAT1- and DGAT2-*S. cerevisiae* (lanes 4 and 5). Thus, it is possible to measure human DGAT1 and DGAT2 activities by using these transformed yeasts. The activities of both DGAT isozymes were more potent than that in rat liver microsomes or in the reference yeast strain OP3-C with native yeast DGAT (Figure 2).

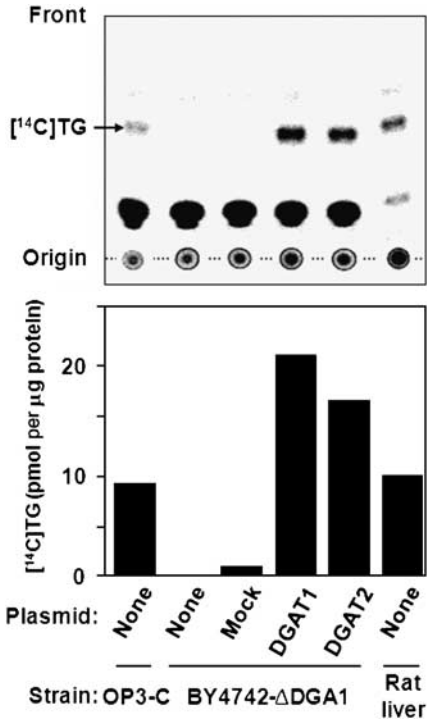

Figure 2 *In vitro* diacylglycerol esterification by recombinant human DGATs produced in yeast. DGAT activities of membrane fractions from *S. cerevisiae* OP3-C (lane 1), *S. cerevisiae* BY4742-ΔDGA1 (lane 2), *S. cerevisiae* BY4742 transformed with pYES harboring either no insert (lane 3) or DGAT1 (lane 4) or DGAT2 DNA (lane 5) and rat liver (lane 6) were measured. The enzyme reactions and the analysis of the reaction products were performed as described in text.

Table 1 Selectivity of DGAT inhibitors of natural origin toward acyl-CoA:diacylglycerol acyltransferase isozymes

Compound	*IC_{50} (μM)*		
	DGAT1	*DGAT2*	*Rat liver*[a]
Amidepsine A	40	70	10
Amidepsine B	30	60	19
Amidepsine C	200	>200	52
Amidepsine D	20	30	18
Xanthohumol	40	40	58
Roselipin 1A	>200	30	17
Roselipin 1B	>200	40	15
Roselipin 2A	>200	45	22
Roselipin 2B	>200	50	18

Abbreviation: DGAT, diacylglycerol acyltransferase.
[a]Data cited from Matsuda & Tomoda.[8]

Selectivity of DGAT inhibitors toward the isozymes

The selectivity of the three types of DGAT inhibitors, namely amidepsines, xanthohumol and roselipins (Figure 1), toward the isozymes was tested in the DGAT1 and DGAT2 assays. The IC_{50} values are summarized in Table 1. IC_{50} values previously determined in rat liver microsomes are also shown for comparison.

Amidepsines and xanthohumol inhibited both DGAT1 and DGAT2 isozymes with similar potency. The order of potency is similar to that seen with rat liver microsomes. Amidepsine C, which contains a Val

residue, is less potent than those inhibitors that have an Ala residue (amidepsines A and B) or no amino acid (amidepsine D). On the other hand, roselipins inhibited DGAT2 isozymes with IC_{50} values of 15–40 μM, but showed almost no inhibition against DGAT1 even at 200 μM. Thus, roselipins are found to be selective inhibitors of DGAT2.

DISCUSSION

In this study, human DGAT1 and DGAT2 genes were transformed into DGAT-deficient *S. cerevisiae* to establish DGAT1- and DGAT2-expressing yeasts.

Biosynthesis of TG in *S. cerevisiae* is mainly catalyzed by the two acyltransferases, *DGA1* and *LRO1* proteins. DGA1 encodes an acyl CoA:DGAT, which is similar to the MrDGAT2A and MrDGAT2B proteins from *Mortierella ramanniana* with ~36% identity, and thus *DGA1* protein exhibits acyl-CoA-mediated DG esterification activity.[24] On the other hand, LRO1 shows a significant sequence similarity to the human lecitin:cholesterol acyltransferase: *LRO1* protein mediates esterification of DG using the *sn*-2 acyl group of phospholipids as an acyl donor.[25]

Whether these enzymes would interfere with our assay was tested using assays with microsomes prepared from yeast lacking DGA-1 and from the same strain transformed with vector lacking the cDNA insert. Our results showed that DGAT activity was not detected in DGA1-deficient yeast (Figure 2). In contrast, the microsomal fractions prepared from yeasts transformed with cDNA for DGAT1 or DGAT2 showed strong enzyme activity. Using these enzymes we found that amidepsines and xanthohumol inhibit both DGAT1 and DGAT2, but roselipins are DGAT2-selective inhibitors (Table 1). Thus, the assay system is useful for the study of DGAT inhibitors. Furthermore, it is plausible that rat liver microsomes contain DGAT1 and DGAT2 isozymes.

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