Review

Current perspectives in drug discovery against tuberculosis from natural products

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ABSTRACT

Currently, one third of the world's population is latently infected with *Mycobacterium tuberculosis* (MTB), while 8.9–9.9 million new and relapse cases of tuberculosis (TB) are reported yearly. The renewed research interests in natural products in the hope of discovering new and novel antitubercular leads have been driven partly by the increased incidence of multidrug-resistant strains of MTB and the adverse effects associated with the first- and second-line antitubercular drugs. Natural products have been, and will continue to be a rich source of new drugs against many diseases. The depth and breadth of therapeutic agents that have their origins in the secondary metabolites produced by living organisms cannot be compared with any other source of therapeutic agents. Discovery of new chemical molecules against active and latent TB from natural products requires an interdisciplinary approach, which is a major challenge facing scientists in this field. In order to overcome this challenge, cutting edge techniques in mycobacteriology and innovative natural product chemistry tools need to be developed and used in tandem. The present review provides a cross-linkage to the most recent literature in both fields and their potential to impact the early phase of drug discovery against TB if seamlessly combined.

Contents

Introduction

Tuberculosis (TB), an old, highly infectious disease, declared a global health emergency by the World Health Organization (WHO) in 1993, is still the second leading killer in the world, with an approximate 2 billion people being latently infected. These latently infected individuals with *Mycobacterium tuberculosis* (MTB) represent one third of the world's population. It still remains one of the world's deadliest infectious diseases. WHO estimates that there were approximately 9.0 million new cases and 1.5 million cases of mortality in 2013–360,000 of whom were positive for HIV [1]. TB treatment is generally comprised of 2 months with isoniazid, rifampicin, ethambutol and pyrazinamide (the intensive phase), followed by four additional months of isoniazid and rifampicin therapy (the continuation phase) [1]. Unfortunately, lack of adherence to prescribed treatment procedures and inefficient healthcare structures have contributed to the development of multidrug-resistant TB (MDR-TB, defined as resistance to at least isoniazid and rifampicin, two front-line drugs used for the treatment of TB) that requires at least 20 months of treatment with second-line drugs comprised of capreomycin, kanamycin, amikacin and fluoroquinolones; these are more toxic and less efficient, with cure rates in the range of 60–75% [2].

Riccardi et al. [3] notes that in 2012, 450,000 people developed MDR-TB in the world. It is estimated that about 9.6% of these cases were extensively drug resistant (XDR-TB), showing additional resistance to at least one fluoroquinolone and one injectable second-line drug [1,4]. In patients affected by XDR-TB, the chances of successful treatment are quite low [3], underpinning the need for urgent discovery of novel compounds with activity against MTB strains resistant to second-line drugs. Recently, a few reports have claimed the emergence of a 'totally drug-resistant TB' strain with a limited chance of successful therapy [3,5–8]. Moreover, there is an urgent need to come to an agreement on the definition of these strains of MTB, mainly in terms of their severity [9]. Hence, the search for new antitubercular drugs is a priority so as to overcome the problem of drug resistance and to finally eradicate TB.

Trends in discovery of TB drugs

The four pioneer first-line drugs

Pyridine-4-carboxy hydrazide, isoniazid (INH; isonicotinyl hydrazide, Fig. 1a) was discovered at the same time in 1952 by three different pharmaceutical companies: BAYER
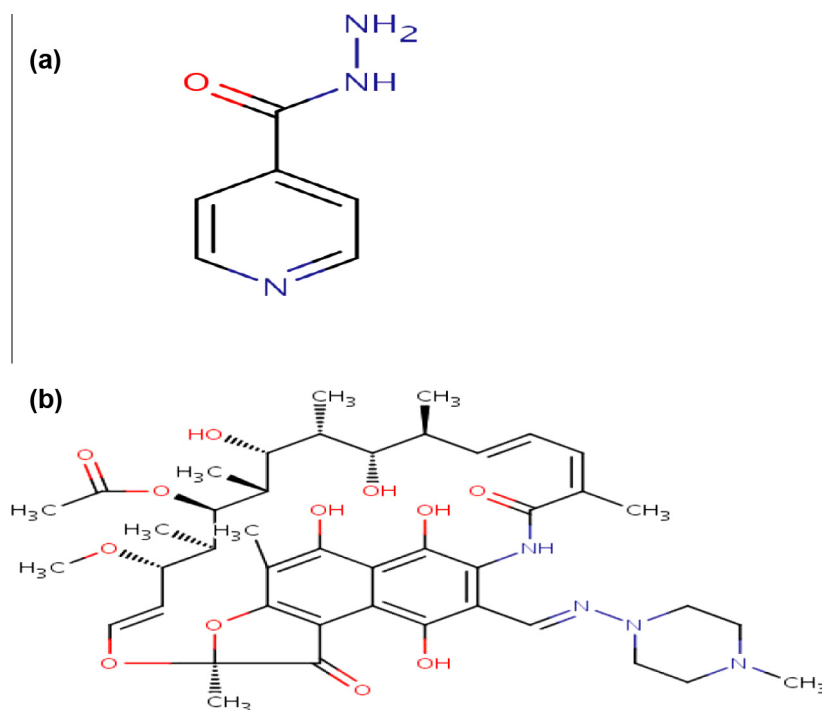
Fig. 1 – Chemical structure of isoniazid (a) and rifampicin (b).

(Leverkusen, Germany); Hoffmann LaRoche (Nutley, NJ, USA); and ER Squibb & Sons (Princeton, NJ, USA). INH cured many patients and was defined as a 'magic drug' [3]. Since 1952, INH continues to be an essential drug in the fight against TB [10].

Isoniazid is a prodrug that requires activation by MTB catalase-peroxidase katG enzyme to form an INH-NAD complex which inhibits the nicotinamide adenine dinucleotide (NADH)-dependent enoyl-ACP reductase (encoded by inhA gene) of the fatty acid synthase type II system, a key player in the mycolic acid biosynthetic pathway of MTB [3]. The inhibition of enoyl-ACP reductase (encoded by inhA gene) causes an accumulation of long-chain fatty acids and cell death [11].

Fig. 2 – Chemical structure of pyrazinamide (a) and ethambutol (b).

Mutations in the katG and inhA genes have been shown to contribute approximately 70% and 80% respectively to isoniazid resistance in MTB clinical isolates [3,10]. Since isoniazid is a prodrug, its activity is greatly influenced by mutations in the katG enzyme, and, as such, a reasonable way to bypass this mechanism of resistance is designing drugs that do not require the katG enzyme activation, but mainly target the inhA enzyme. Triclosan inhibits the inhA enzyme [3], but its usefulness as an antitubercular drug has not been successful because of its sub-optimal bioavailability [12]. A series of triclosan derivatives have been synthesized using a structure-based drug design approach [13]. It is interesting to note that these derivatives have been shown to be effective against MTB isoniazid resistant clinical and laboratory strains. The best triclosan derivative inhibitor had a minimum inhibitory concentration (MIC) value of 4.7 μg/ml, which represents a tenfold improvement compared with the activity of the parent compound, triclosan, but less potent than isoniazid with an MIC value of 50 ng/ml [3,13]. Investigators are searching for new anti-TB drugs targeting the inhA gene which do not require activation by the katG enzyme with a susceptibility pattern similar to that of isoniazid. This will be a hard task because isoniazid is a very potent antitubercular drug [3]. New inhibitors of the inhA enzyme have been synthesized of late, but their effectiveness is not as good as isoniazid [14].

Pyridomycin, a natural compound produced by *Dactylosporangium fulvum* with specific 'cidal' activity against mycobacteria, has been recently demonstrated to target the inhA enzyme [15]. Moreover, biochemical and structural approaches have showed that pyridomycin inhibits the inhA enzyme directly via the competitive inhibition of the NADH binding site, without activation by the katG enzyme [3].
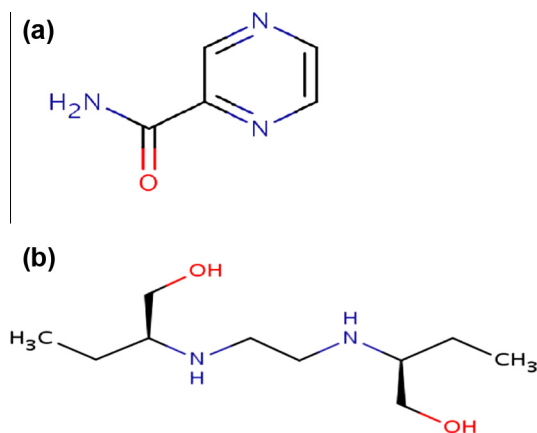
Interestingly, the majority of the MTB isoniazid-resistant clinical isolates are sensitive to pyridomycin, underpinning the potency of this drug [15].

Pyrazinamide (PZA), an analog of nicotinamide (Fig. 2a), is a prodrug that requires conversion by MTB pyrazinamidase (coded by the pncA gene) to pyrazinoic acid [16]. RpsA has recently been shown to be a pyrazinamide cellular target [17], and its over-expression (wild-type RpsA) has been implicated in PZA resistance in MTB. Binding of the activated pyrazinoic acid to RpsA interferes with its binding to the messenger RNA. In addition, some PZA-resistant MTB strains without mutations in pncA have shown mutations in RpsA [3]. This enzyme (RpsA) is essential for translation, and its C terminus is also involved in trans–translation [18] in MTB. Trans–translation in MTB is dispensable during active growth conditions, but it is also required by some bacterial strains for survival under stress conditions and also for disease progression. The inhibition of the trans–translation step in MTB may interfere with its survival under the dormancy state, and this could explain how diverse stress conditions, such as starvation, acidic pH and hypoxia, can potentiate the activity of pyrazinamide [3,17,19–21].

Ethambutol (EMB) (Fig. 2b), interferes with mycobacterial cell wall synthesis in MTB by inhibiting polymerization of arabinogalactan, an important cell wall component in MTB [22]. Moreover, it also interrupts the utilization of the arabinose donor by inhibiting either arabinosyltransferase enzymatic activity or the formation of an arabinose acceptor in mycobacteria [22]. The embCAB operon has been shown to be responsible for ethambutol resistance in MTB [23]. It is worth noting that ethambutol acts at the same pathway that is blocked by benzothiazinones, but not at the same step of metabolism.

Rifampicins comprise a group of antibacterial drugs and include the following derivatives: rifampicin, rifapentine, rifabutin and rifalazil [3]. They bind to the bacterial beta RNA polymerase subunit, thus interfering with transcription [3]. Resistance to rifampicins in MTB is conferred by mutations in the 81-bp region of the rpoB gene (encodes beta RNA polymerase) [24]. Both rifampicin (Fig. 1b) and isoniazid are essential and commonly used first-line drugs for TB therapy in combination with other molecules [3].

Following the introduction of rifampicin into clinical use, the treatment of active TB was reduced from 9–12 months to 6 months, while the duration for treatment of latent TB was reduced from 9 months to 3 months [10]. It is important to note that rifampicins are among the few drugs that can kill the dormant (non-replicating) strains of MTB. Rifampicin (RIF) was developed by blind whole-cell screening in an extensive program of chemical modification of the rifamycins, the natural metabolites of *Amycolatopsis mediterranei* under the supervision of Professor Piero Sensi [25]. Since rpoB is an essential gene in MTB, and RNA polymerase is a proven target for antibacterial and anti-TB therapy, it would be reasonable to search for new RNA polymerase inhibitors binding at sites different from that utilized by rifampicin [26]. In 1989 Professor Piero Sensi wrote: "In the last two decades, no new major anti-TB drug has been developed. Although dramatic improvements in chemotherapy for TB have been achieved through careful studies of drug regimens, there is still a need for new agents that are highly active. The antimycobacterial drugs used at present in therapy for TB were obtained by either blind screening or chemical modification of active compounds. Other approaches based on the knowledge of the biochemistry of the mycobacterial cell should be tried. Certain constituents of the cell, such as mycolic acids, arabinogalactan, peptidoglycan and mycobactin, may represent specific targets for new anti-TB drugs [27]." As an outstanding scientist, Professor P. Sensi understood what research scientists in the field of TB drug discovery would realize many years later. Afterwards, a lot of compounds have been discovered that inhibit specific steps involved in either arabinogalactan or mycolic acid biosynthesis [28]. Novel efficacious and safe anti-TB drugs are currently needed so as: (1) to shorten the duration of TB therapy; (2) to be able to treat MDR, XDR and totally drug resistant (TDR) TB strains; (3) to be able to treat latent TB; (4) to be able to act in a synergistic manner with other co-administered anti-TB drugs; and, finally, (5) to be able to be safely co-administered with anti-HIV agents.

New TB drugs in the pipeline

After a long period of inactivity, the last few years have seen an increase in the number of new anti-TB drugs in the pipeline. As it can be seen in Fig. 3, there are currently adequate numbers of drug candidates in the lead optimization stage, preclinical development, phase II and phase III clinical trials. However, there is a worrying gap within phase I that needs to be filled to have a constant delivery of molecules in case of failure of advanced drug candidates. Many molecules under clinical evaluation, such as fluoroquinolones, were developed to treat other infectious diseases and have now been repurposed for TB treatment and this call for increased efforts towards discovery of new compounds against different physiological states of tubercle bacilli.

Bedaquiline (TMC-207), a diarylquinoline, was approved by the FDA (Food and Drug Administration) in December 2012 as part of the combination therapy for the treatment of adult patients affected by MDR-TB, and it is now in phase III of clinical development (Fig. 3). Bedaquiline can be considered to be the first major drug approved by the FDA for TB therapy in the last four decades (40 years). It came out following a phenotypic screening of compounds against MTB, while the corresponding target was identified through the whole-genome sequencing of MTB and *Mycobacterium smegmatis* spontaneous mutants that were resistant to chemical molecules. Interestingly, the resistant mutants showed missense mutations in the atpE gene (encoding the c subunit of ATP synthase) [29]. Bedaquiline acts by inhibiting ATP synthase and has activity against active and dormant MTB strains. Currently, it is well known that TB patients with pulmonary TB can have both active and dormant tubercle bacilli, the latter being difficult to eliminate with the currently used anti-TB drugs, hence favoring the development of resistant strains and latent infection [30]. It is well known that human mitochondrial ATP synthase is 20,000-fold less sensitive to diarylquinoline than the mycobacterial one, thus validating the enzyme as an important drug target against MTB [31]. Bedaquiline has been associated with an increased risk of inexplicable mortality and QT prolongation, but it still
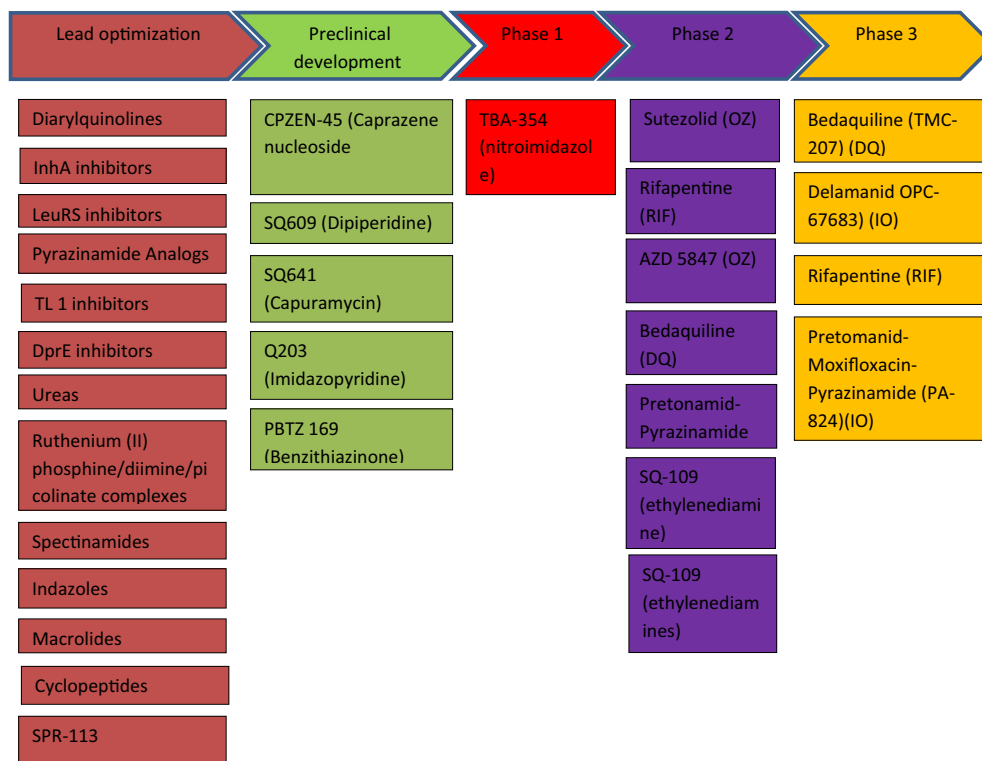
Fig. 3 – **Current global drug pipeline (modified by** www.newtbdrugs.org**). OX, oxazolidinone; RIF, rifamycin; DQ, diarylquinoline; IO, imidazooxazole. Source: @2015 Working group on New TB Drugs. Stop TB Partnership. (**http://www.newtbdrugs.org/pipeline.php**).**

represents a great addition for the treatment of MDR- and XDR-TB strains, especially in TB-endemic regions of the world [32].

Delamanid (OPC67683) and pretomanid–moxifloxacin–pyrazinamide combination (PA-824) are two new imidazooxazoles in phase III clinical development (Fig. 3). Both molecules are pro-drugs whose activation depends on a F420-deazaflavin-dependent nitroreductase (Ddn) which is present in MTB. The active form of PA-824 is the corresponding des-nitroimidazole molecule, which releases reactive nitrogen species, such as nitric oxide [33], causing respiratory poisoning which appears to be crucial for its anaerobic activity [34]. PA-824 has activity against both active and latent TB infection, which could shorten the duration of TB therapy [35]. Delamanid inhibits mycolic acid biosynthesis and has been associated with increased sputum-culture conversion in MDR-TB patients [36]. In addition, Delamanid has been shown to be effective with acceptable toxicity when combined with other anti-TB drugs in an MDR-TB regimen [37].

Rifapentine, a semi-synthetic cyclopentyl rifamycin derivative, acts by binding the b-subunit of the RNA polymerase in MTB, a mechanism of action that is also utilized by rifampicin [38]. It is more effective than rifampicin against MTB, both *in vitro* and *in vivo* with an MIC value in the range of 0.02–0.06 µg/ml [39]. Both rifamycin and rifapentine exhibit cross resistance. The United States Food and Drug Administration (US FDA) in 1998 approved rifapentine at a dosage rate of 10 mg/kg (oral administration) once or twice weekly for the therapy of active and latent TB. There is good

clinical evidence that supports the use of rifapentine plus isoniazid for 3 months (once-weekly regimen) against latent TB [40], but quite different for the treatment of active TB, where it is approved by the FDA at a dose of 600 mg orally, twice weekly during the intensive phase of TB treatment (2 months), and then once weekly during the continuation phase (4 months) [41]. Recently, animal studies have suggested that more frequent administration of rifapentine might cure both active and latent TB in 3 months or less, however, the observed findings could not be reproduced in clinical trials involving human subjects. Moreover, in animal studies, the administration of the drug via inhalation appeared to improve tubercle clearance in the lungs, but clinical data has not yet been generated [39].

SQ109, a 1,2-ethylenediamine {N0-(2-adamantyl)-N-[(2E)-3, 7-dimethylocta-2,6-dienyl]ethane-1,2-diamine} is in phase II clinical trials (Fig. 3). SQ109 is active against sensitive MTB, MDR-TB and XDR-TB strains [42,43]. This compound was found while screening a 63,238 chemical library, designed around the active 1,2-ethylenediamine pharmacophore of ethambutol, an essential first-line antitubercular drug, with the hope of identifying an ethambutol-like chemical molecule, probably more effective and safer than ethambutol. Interestingly, both ethambutol (EMB) and SQ109 have different chemical structures and different mechanisms of action, with SQ109 targeting MmpL3 (an essential membrane transporter belonging to the resistance, nodulation and division [RND] family), whose main function (MmpL3) in MTB is to transport the trehalose monomycolate into the envelope thus

interfering with mycolic acid synthesis in the mycobacterial cell [44]. This membrane transporter (MmpL3) also assists with iron acquisition for mycobacteria survival, and together with Rv0203 plays an important role in mycobacterial heme uptake. Currently, MmpL3 is considered as one of the hottest targets in drug discovery against MTB, as several other compounds under preclinical investigation have also been reported to inhibit the transporter, such as the ureas in lead optimization stage (Fig. 3).

Other compounds have recently moved from phase I to phase II clinical trials. These include PNU-100480 (Sutezolid) (Fig. 3), a close analog of linezolid and AZD5847 (Fig. 3), a member of the oxazolidinone class.

Drug discovery against TB from natural products

The urgent need for the development of new drugs to help reduce the global burden of TB is well documented in the current biomedical literature [45–47]. Novel antimycobacterial scaffolds from natural products have recently been reviewed [51]. Uplekar et al. [48] points out that in order to attain the WHO's ambitious targets of 95% reduction in TB deaths and 90% reduction in TB incidence by 2035, the need for better and safer drug regimens to shorten treatment is key. Because natural products are a proven template for the development of new scaffolds of drugs, they have received considerable attention as potential anti-TB agents [46]. There are excellent reviews on antitubercular compounds derived from natural products [49–53]. In a recent review, Mdluli et al. [54] highlights some recent notable examples of natural product compounds that may prove to be useful leads for TB drug discovery. A number of medicinal plants with promising activity against TB have recently been reported [55]. Antimycobacterial bioactive chemical molecules have been found from many natural product skeletons, mainly from plant biodiversity, but also from other organisms, such as fungi and marine organisms. The plethora of structures reported to have anti-TB activity is summarized in a recent review focusing on naturally occurring compounds with reported growth inhibitory activity *in vitro* towards sensitive and resistant MTB [56]. Another noteworthy source of information is a recent comprehensive compilation of plant species for which promising anti-TB activity has been reported [57]. Considering that none of the several screened non-microbial natural products with activity against MTB has progressed towards the clinical trial stage in anti-TB drug development, it seems reasonable to evaluate the reasons for the failure. This could possibly be caused by: (i) low yields of purified compounds; (ii) structural complexity exhibited by natural products, such as the occurrence of multiple stereoisomers, e.g., triterpenes which contain ten or more chiral centers; (iii) most studies are purely academic and are not focused on drug development; (iv) low activity exhibited by the isolated compounds with MIC $\geqslant 1$ μg/ml; (v) the presence of pan inhibitors (non-specific compounds or pan-inhibitors); (vi) difficulties in isolating novel cidal compounds acting on new targets that can potentially reduce the duration of therapy; and (vii) difficulties in identification of anti-TB compounds with exceptional safety profiles without the

drug-drug-interaction problem presently confronting concurrent TB and HIV therapy. In addition, the current literature has no indication on the safety profile of isolated compounds as shown by the selectivity index (SI) (anti-TB activity vs. mammalian cytotoxic activity) [58], hence there is a need to evaluate the toxicological profile of purified and semi-purified natural products [59,60]. It will be a hard task to meet the aforementioned difficulties without increased funding for anti-TB drug discovery and construction of a more robust drug development pipeline through well-coordinated international efforts.

The classic pathway towards anti-TB drug discovery from natural products and indeed other infectious diseases must be able to overcome a number of challenges. The first is to reliably detect efficacious and safe hits and be able to identify already known compounds at the early stages of the drug discovery program. The second major challenge is the *de novo* structure elucidation of new molecular entities. The latter challenge has been revolutionized by current advances in spectroscopic techniques, specifically the high resolution neutron magnetic resonance (NMR) technologies. Many approaches have been developed to solve the major hurdle, but it still remains a major challenge in anti-TB drug discovery from natural products [58]. In order to impact the early phases of anti-TB drug discovery from natural products, innovative technologies need to be leveraged for rapid navigation of natural product hits through the detection, validation, isolation, hit-to-lead and lead optimization phases [46]. In the present review, the aforementioned bottlenecks are approached from a different perspective, so as to reflect on the truly interdisciplinary nature of the scientific challenges encountered at the initial phase of anti-TB drug discovery from natural products. Accordingly, the present review puts more emphasis on the recent advances in the field of mycobacteriology and natural product chemistry, specifically to provide an overview of the methods that are currently available, point out how both fields can impact the early phase of anti-TB drug discovery if seamlessly combined, obstacles faced even in an environment where mycobacteriologists and natural product chemists are working together and finally demonstrate some perspectives for drug discovery against TB from natural products.

The need for tuberculosis drug development

The needs, challenges and recent advances towards development of novel chemical molecules against TB have been reviewed recently [2]. Approximately 2 billion people of the world's population are latently infected with MTB and are at risk of reactivation to active disease [61]. Even though an inexpensive and effective quadruple drug therapy regimen comprising isoniazid, rifampicin, ethambutol and pyrazinamide was introduced 40 years ago, TB continues to spread in every corner of the globe [62]. TB remains a global emergency according to the seventeenth World Health Organization (WHO) report on the worldwide incidence of the disease [63]. Globally, there are approximately 8 million new cases and 2 million deaths yearly associated with TB; hence, the disease is responsible for more human mortality

than any other single microbial infection. A major break-through in TB therapy came after the introduction of strepto-mycin, followed by p-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampin (1963) over 40 years ago. The current treatment regimen has several drawbacks, including pro-longed treatment time to completely eradicate the bacteria (sterilization). This increases the opportunity for develop-ment of MTB-resistant strains documented in almost every country where the disease is prevalent. These obstacles, in addition to an increasing prevalence of MDR, XDR and cur-rently TDR strains, call for an urgent need to search for and develop novel agents against TB. Pulmonary TB remains a major health hazard in Asia, Africa and the Western Pacific region, despite its sharp decline in the Western world since the beginning of the 20th century [47]. A number of chal-lenges, including the lack of economic incentive due to the predominance of the disease in the developing world, have continued to face drug discovery towards TB. However, there has been a renewed interest by scientists, funding bodies and high-profile advocacy by the WHO's STOP TB department and other organizations towards discovery of new agents against TB, as well as the creation of a roadmap for their development [46]. These efforts have recently cul minated in the approval of two new drugs: delamanid (previously known as OPC67683) and bedaquiline (also known as TMC207 or R207910) for the treatment of MDR strains of MTB [45,64].

Bioassay-guided (bioactivity) fractionation

Recent reviews have described bioassay-guided fractionation in TB drug discovery programs [46,65]. Bioassay-directed frac-tionation is the state-of-the-art process that is currently being utilized to isolate and identify bioactive principles from natu-ral product crude extracts. This process consists of alternat-ing steps of evaluating the activity of natural products using bioassays and chemical fractionation; hence, multiple transi-tions of samples and mutual design of protocols at the mycobacteriology-natural product chemistry interface is required. The sensitivity of natural product fractionation pro-cedures has increased dramatically over the recent years due to enormous technological advancements in chromatograph and spectroscopy, opening new alleys not only for unstudied materials, but also for previously investigated genera, provid-ing access to unexpected chemical types and novel com-pounds [66]. Thus the development and application of new natural product chemistry methods is key in a bioassay-guided anti-TB drug discovery program. In order to provide valid guidance, the mycobacteriological assay is the second key point to be addressed and has to be chosen wisely with regard to the ultimate endpoint, i.e., the activity of the anti-TB agent against virulent MTB in vivo. Using this method, three potent antimycobacterial compounds have been isolated from Dracaena angustifolia [65].

High-throughput, inexpensive, time-saving assay using M. smegmatis

Mycobacterial strains can be broadly classified depending on their in vitro growth as follows: (i) fast-growing,

non-pathogenic strains; and (ii) slow-growing, pathogenic strains. Slow-growing pathogenic mycobacterium will be a difficult organism to screen a large number of candidates within a short period of time. Therefore, preference has been given to M. smegmatis mainly because of the following reasons: (i) it is non-pathogenic and can be handled easily; (ii) the growth rate of M. smegmatis is approximately eight times faster than MTB; (iii) M. smegmatis is widely used to understand the biology of MTB, such as in cell culture, gene expression and persistence in the face of nutrient starvation; and (iv) MTB has been found to display a drug susceptibility profile similar to MDR MTB [67]. Therefore, cell viability assay with M. smegmatis could serve as a 'surrogate' for MDR MTB. This bioassay usually serves to prioritize the candidates which can be tested further in more specific in vitro assays on pathogenic MTB, MDR and XDR strains. M. smegmatis has reportedly been used in primary screening for the selection of compounds with activity against MTB [68]. Recently, it has been reported that the susceptibility pattern of M. smegmatis to the two front-line essential drugs against TB – isoniazid and rifampicin – is identical to that of MDR clinical isolates of MTB [46]. The sensitivity of M. smegmatis based on screening should be extremely specific so that hits generated in this bioassay can be a potential target for both sensitive as well as MDR strains of MTB [68].

The target organism, M. tuberculosis

MTB, the actual etiologic agent for TB, is the ideal target organism in an anti-TB drug discovery effort. MTB $H_{37}Rv$ (ATCC 27294), a well-characterized virulent strain available from the American Type Culture Collection (ATCC, Rockville, MD), has a drug susceptibility profile which is quite similar to majority of those clinical MTB isolates which have not developed drug resistance as a result of prior treatment with one or more clinical TB drugs (susceptible clinical isolates). Testing of chemical molecules against drug-resistant and MDR strains of MTB (strains resulting from specific stepwise mutations to individual drugs) is not critical in primary screening, since these strains are not "superbugs", which are resistant to multiple anti-TB drugs by virtue of a single mechanism, such as the effusion pumps found in other bac-teria and, hence, would be expected to be susceptible to any novel compound, acting in a different site from that utilized by an existing anti-TB drug. Since MTB $H_{37}Rv$ is a virulent strain, it should only be handled in a biosafety level 3 labora-tory (BSL-3) that requires a pass-through autoclave, a negative air pressure relative to an anteroom and hallway, and a class 2 biosafety cabinet. Laboratory personnel working within the BSL-3 laboratories must be well-trained, must wear protective gear, and most importantly a respirator, which will minimize the risk of infection from aerosolized MTB. Most investigators in anti-TB drug discovery either collaborate with an institu-tion with a BSL-3 facility, or work with an avirulent surrogate organism, such as M. smegmatis (ATCC 19420), since few insti-tutions have a BSL-3 laboratory. The majority of natural pro-duct researchers have chosen to work with these rapidly growing, avirulent, saprophytic mycobacteria, erroneously

referred to as MTB 607 in several publications. However, *M. smegmatis* only possess a limited degree of similarity to MTB with regard to drug susceptibility. Alternatively, one can also use either MTB H$_{37}$Ra (ATCC 25177), or the commonly used vaccine strain, *Mycobacterium bovis* BCG (ATCC 35745), both of which are slow-growing and non-pathogenic and, most importantly, are more closely related to MTB H$_{37}$Rv than the rapidly growing mycobacteria with respect to drug susceptibility profile and genetic composition. To work with these strains, one only requires a class 2 biosafety cabinet and sound microbiological techniques [46].

Challenges facing antimycobacterial drug discovery

The existence of MTB in different physiological states during infection, its pathogenesis and complex biology pose specific challenges for drug discovery against TB. The first major challenge is the perceived heterogeneity of the population of tubercle bacilli in the human host with respect to the metabolic state as reflected in the multiplication rate, which greatly impact on the choice of *in vitro* and *ex vivo* models used to screen new anti-TB compounds [69]. A particular outcome of the slow growth rate is ambiguity about the 'vulnerability' of the metabolic pathways, raising critical questions. For example: (i) which metabolic pathway is crucial for survival to a persisting organism? and (ii) during persistence, are metabolites required at much lower concentrations?

The second challenge is in identification of safe compounds for prolonged therapy. The most serious side effects of prolonged therapy are drug-drug interactions, since a compound-specific toxicity profile is usually addressed in the safety studies. This aspect can be studied very early in the drug discovery cascade, thus the focus of the unmet challenge shifts to identification of chemical entities with rapid kill kinetics, since this is fundamental for the quick reduction of the bacterial load and, eventually, sterilization. Hence, an anti-TB compound should be able to act on MTB in different metabolic states. It is worth noting that the success of target-based drug discovery is mainly dependent on the quality of the target and the level to which it has undergone validation [70]. Hence, from this perspective and while excluding the ribosome, the currently available repertoire of anti-TB drugs reveals only a small number of comprehensively validated targets, namely RNA polymerase, DNA gyrase, NADH-dependent enoyl-(acyl-carrier-protein) reductase, and ATP synthase [71,72]. Given the need to increase the chances of success at a time when attrition rates are quite high in the early phase of anti-TB drug discovery, lack of information on several validated targets poses severe limitations on the diversity of efforts, raising fundamental questions as to whether alternative 'lead generation' approaches are more suitable for anti-TB drug discovery. Fortunately, advances in genome sequencing technology can perhaps augment the ' whole-cell-screening' approaches since it can enable the identification of more targets [29,73]. It is worth noting that, whole-cell screening (phenotypic screening) clearly enables the early identification of compounds with killing ability (cidal activity) and their progression along the drug discovery

pathway. In addition, it also enables testing of cidality of compounds on different metabolic states of MTB and, thus, overcomes a key challenge early in the drug discovery path [74].

Combination of whole cell and target-based screening approaches

The inability to convert target inhibition into growth inhibition and eventually to bacterial cell death, which bedevils the target-driven approach, is circumvented by identifying compounds with potent anti-TB activity by whole-cell screening, which is clearly a feasible starting point. The whole-cell screening approach does not provide information in regard to mechanism of action and possible toxicity, which is especially relevant in anti-TB therapy because of the prolonged duration of treatment. This challenge can be partially mitigated by merging the whole-cell screening with the target-based approach by carrying out studies on mechanism of action and toxicity studies on the potent whole-cell active compound. This will facilitate identification of the pathway and/or target before extensive studies on medicinal chemistry are started. Even though further medicinal chemistry can, indeed, be driven by MIC-based structure activity relationship patterns, knowledge of the target and or mechanism of action would enable studies on possible mechanisms of toxicity [74]. Alternatively, another promising method is to use high-content screening systems [75], where a confocal microplate imaging reader is used to monitor inhibition of intracellular mycobacterial growth and possible cytotoxic effects, using infected macrophages simultaneously [74].

Anti-TB *in vitro* bioassays

Agar diffusion

The common disc or well-diffusion assays employed in many antimicrobial assays of natural products only indicate that there is growth inhibition at some unknown concentration along the concentration gradient, but are not quantitative when used to evaluate extracts or new compounds. The sizes of inhibition zones can only be interpreted as indicative of microbial susceptibility or resistance in a clinical setting with well-characterized antibiotics, since the size of the zone of inhibition depends upon both the rate of diffusion of the active agent and the rate of growth of the target organism. Diffusion assays with mycobacteria need to be avoided, since these organisms with a very lipid-rich, hydrophobic cell wall are often more susceptible to less-polar compounds [76]. Hence, non-polar compounds will diffuse more slowly than polar compounds of similar molecular weight in the aqueous agar medium resulting in relatively small inhibition zones, giving the erroneous impression of weak bioactivity. In addition, active low molecular weight, polar compounds may diffuse to equilibrium before colony growth of slow-growing mycobacteria is apparent, and if the concentration at equilibrium is below the MIC, then there will be no zone of inhibition; hence, the compound bioactivity will not be reflected [46].

Macro- and micro-agar dilution

Testing known concentrations of extracts, fractions or compounds in an agar medium allows for MIC value determination and the quantitation of bioactivity. The majority of mycobacterial strains, including MTB, will grow well on Middlebrook 7H11 agar supplemented with oleic acid, albumin, dextrose and catalase (OADC supplement, Difco), with the exception of a few fastidious species. Test samples can be added to the molten media (held at 50 °C) at 1% v/v final concentration and then either 100–200 μl medium to 96-well microplates, 1.5 ml to 24-well microplates, 4 ml to 6-well microplates or 20 ml added to standard 150 mm diameter Petri-dishes. Following the hardening of the medium, the inoculum can be spotted on the surface with a micro pipette. Suggested volumes of inoculum are: 1–5 μl for 96-well plates, 10 μl for 6- or 24-well plates and 100 μl (spread evenly) for standard Petri dishes. The plates are then incubated at 37 °C overnight, after which they can then be inverted for the remainder of the incubation period. The major disadvantage with such a bioassay is that it requires at least 18 days to visibly detect growth of the mycobacterial colonies [46].

Radiorespirometry

The inhibition or growth of MTB growth can be determined in a period of 1 week by evaluating the extent of oxidation of $[1–14^C]$ palmitic acid in a liquid mycobacterial Middlebrook 7H12 medium (BACTEC™ 460TB 12B) to $^{14}CO_2$, which is measured in the BACTEC 460 instrument [77]. Radiorespirometry was the method of choice in the developed world for the greater part of the 1980s and 1990s for clinical mycobacterial drug susceptibility testing since results were obtained more rapidly compared with conventional agar dilution methods. The relative activity of various samples can either be compared by testing at only one or two concentrations and determining the percentage inhibition of $^{14}CO_2$ production relative to drug-free controls [78], or multiple concentrations can be tested and an MIC calculated [79,80]. Readings can be taken at various time intervals, usually after every 24 h and, thus, this technique can provide a kinetic picture of mycobacterial growth or inhibition. The main drawback of this bioassay are the costs involved, including isotope disposal costs in some countries and the large volumes of medium required, which in turn requires a large amount of sample to be tested, usually in the range of 50–100 μl. More recent non-radiometric automated systems for clinical use utilize indicators of oxygen consumption [81], carbon dioxide production [82], or head space pressure [83] to determine mycobacterial growth or inhibition, but otherwise, they do have the same disadvantages as the BACTEC 460. These systems include the BACTEC TB-460 radiometric system (Becton Dickinson, Sparks, MD, USA) and, more recently, the mycobacterial growth indicator tube (MGIT) (Becton Dickinson) in both its automated and manual versions [84–87]. The radiorespirometric technique detects the metabolic activity of mycobacteria, as opposed to mycobacterial growth as colonies on a solid medium [46].

Micro-broth dilution

Evaluation of activity (susceptibility) of natural products in a 96-well microplate format offers the advantages of small sample requirements, low cost, and high-throughput, including the potential for automation. Mycobacteria are usually cultivated in Middlebrook 7H9 broth supplemented with 0.5% glycerol, 0.1% casitone, 0.05% Tween-80 and 10% OADC (oleic acid, albumin, dextrose and catalase), 7H9GC-Tween 80. The growth of many strains of mycobacteria can be quantitatively evaluated by turbidity in a liquid medium, but the tendency of mycobacteria to clump together makes this a difficult test. Crude extracts from natural products may in addition impart some turbidity to the culture medium, making interpretation of results difficult. The use of an oxidation–reduction indicator dye such as Alamar Blue (Trek Diagnostics, Westlake, Ohio) makes micro-broth dilution a more rapid and a sensitive bioassay. This method was first proposed by Yajko et al. [88] in a study that evaluated the activity of the first-line anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol and streptomycin) against clinical isolates of MTB. Alamar blue, a proprietary reagent, had been used previously to study both metabolism and viability in other microorganisms [89,90]. In addition, it has also been used to measure toxicity in both prokaryotic and eukaryotic cells [91]. The reagent (Alamar blue) is blue in color in the oxidized state, but it turns pink when reduced due to bacterial metabolism. The two colors (blue and pink) can easily be differentiated with the naked eye. The study of Yajko et al. [88] was important, since it showed for the first time that MICs of essential anti-TB drugs (isoniazid, rifampicin, streptomycin and ethambutol) could be determined following incubation of MTB isolates for only one to 2 weeks in the presence of the test drugs. This colorimetric method (Alamar blue) was also proposed by Collins and Franzblau [92] for use in a microplate format, microplate Alamar blue assay (MABA), for high-throughput screening of compounds against MTB and *Mycobacterium avium*, and by Shawar et al. [93], for rapid screening of natural products for activity against MTB. The results of the MABA can be read visually [94] and do not require any instruments. The reduced form of Alamar blue can also be quantitated colorimetrically by measuring absorbance at 570 nm (and subtracting absorbance at 600 nm; the peak for the oxidized form), or fluorometrically [77] by exciting at 530 nm and detecting emission at 590 nm; the latter mode has been shown to be more sensitive. For non-fluorometric readouts, micro-broth dilution tests can also be performed by using the non-proprietary resazurin [95,96] or tetrazolium dyes [97–100]. Hence, it is possible to conduct high-throughput, anti-TB assays in microplate format using a microplate spectrophotometer or microplate fluorometer, which are more quantitative bioassays capable of detecting partial inhibition, thus making it ideal for determination of the relative activity of fractions from natural product crude extracts using one or two concentrations.

Nitrate reductase assay/Greiss method

Nitrate reductase assay (NRA), a new approach for the rapid colorimetric detection of drug resistance in TB or susceptibility of natural products against MTB is based on the capacity of MTB to reduce nitrate to nitrite. The NRA, also known as the Griess method, is an old method that has also been used to differentiate MTB from other species of mycobacterium [101]. This technique has been introduced for the rapid detection of drug resistance in TB [102], as well as in the evaluation of anti-TB activity of natural products. Following the incorporation of potassium nitrate in the culture medium, reduction of nitrate to nitrite can be detected using specific reagents, which produce a colored reaction. The sensitivity and specificity of NRA, compared with the BACTEC 460-TB system, were 100% and 100% for rifampicin, 97% and 96% for isoniazid, 95% and 83% for streptomycin, and 75% and 98% for ethambutol, respectively, when a panel of MTB strains with various resistance patterns was tested. The majority of results were available following incubation for 7 days, and NRA was able to identify most resistant and susceptible strains positively. Recently, two studies have described the use of the NRA directly with sputum samples. Musa et al. [103] evaluated the NRA for drug susceptibility testing (DST) of MTB directly on smear-positive sputum samples containing more than ten acid-fast bacilli (AFB) per microscopy field, while Solis et al. [104] compared the sensitivity and specificity of the direct NRA with the proportion method in Lowenstein Jensen (LJ) medium for determination of resistance to isoniazid (INH) and rifampicin (RIF) in clinical isolates of MTB. These two studies have shown the feasibility of implementing the NRA as a direct method for detecting drug-resistant clinical isolates of MTB in sputum samples [87]. Nitrate reductase assay (NRA) can also be used in susceptibility testing of MTB against natural products.

Flow cytometry

Flow cytometry was first used at the beginning of the 1980s to study the effects of antimicrobial agents in prokaryotes [105,106]. The number of scientific articles addressing the antimicrobial responses of bacteria (including mycobacteria), fungi, and parasites to antimicrobial agents considerably increased in the 1990s, due to interesting advances in the field of flow cytometry from microbiology laboratories [107]. Norden et al., has reported the use of both Fluorescein diacetate (FDA) (a non-fluorescent diacetyl fluorescein ester that becomes fluorescent upon hydrolysis by cytoplasmic esterases) staining and flow cytometry for susceptibility testing of MTB [108]. In addition, Pina-Vaz et al. stained MTB with SYTO 16 (a nucleic acid fluorescent stain that only penetrates into cells with severe lesions of the membrane) in the absence or presence of antimycobacterial drugs [109]. Flow cytometry is a promising technique which needs to be considered in the setting of a clinical mycobacterial laboratory, since it gives fast results, compared with the time needed to obtain susceptibility results of MTB using classical methodologies, which are currently too long.

The main disadvantage of this method is the high cost of equipment [105].

Reporter gene assays

Several species of firefly, beetle, crustacean, bacteria and the sea pansy have been used to clone genes which encode luciferase enzymes [110]. In addition, fluorescent proteins, such as the red fluorescent protein (RFP) and green fluorescent protein (GFP), have also been introduced into mycobacterial plasmids. These proteins permit rapid determination of bacterial viability by measuring the expression of an introduced fluorescent or luminescent protein [105]. These fluorescent proteins do not require an exogenous substrate, thus simplifying quantitation and enabling easy determination of growth and/or inhibition kinetics [111,112]. This method can be applied in a multi-well format with more convenient high throughput detection. Luciferase proteins from the firefly [113–115] and from *Vibrio harveyi* [116] utilize luciferin and n-decylaldehyde substrates, respectively, with n-decylaldehyde yielding a higher signal in mycobacteria. Luciferase enzymes are not ideal for kinetic measurements since they require the addition of a substrate, but they are potentially useful for susceptibility testing in MTB-infected macrophages [113] and in mice [116,117] since the luminescence measurements, performed in a luminometer, have a much higher signal-to-background ratio than is obtained from fluorescence assays. The major disadvantage of reporter gene assays is that their use for commercial applications is often limited by patent restrictions; hence, the number of mycobacteriology laboratories using this method for susceptibility testing of MTB against natural products is fairly small [105].

Dormant tubercle bacilli bioassay/low oxygen bioassay

The therapeutic challenges encountered in eradicating dormant tubercle bacilli in MTB infection is responsible for prolonged treatment of active disease [118], making TB control difficult [119]. Dormancy of tubercle bacilli has been identified as the principle cause for the majority of the problems associated with TB therapy [120]. Current anti-TB drugs cannot effectively kill the dormant forms of MTB [121], and the lack of a screening bioassay for chemical molecules with activity against dormant tubercle bacilli has been an obstacle towards the development of novel drugs against latent TB [121]. Currently, researchers are using non replicating mycobacteria [122] and hypoxic adapted (low oxygen adapted mycobacteria) mycobacteria that are subsequently exposed to test samples [123]. Under the test conditions, mycobacteria are in a state of dormancy. L.G. Wayne has devised Wayne's hypoxic model which is currently used for *in vitro* evaluation of new compounds; however, this method possesses a low throughput capability [121,124]. In addition, Cho et al. [125] has implemented a high-throughput, luminescence-based, low-oxygen-recovery assay for screening of compounds against non-replicating MTB using an MTB pFCAluxAB strain (this is the MTB H37Rv strain containing a plasmid with an acetamidase promoter driving a bacterial luciferase gene).

Recently, Khan and Sarkar [121], while using Wayne's hypoxic model and nitrate reductase activity in *M. bovis* BCG (Bacillus Calmette-Guérin) culture, have developed a dormant stage specific antitubercular screening protocol in microplate format [121].

High-performance liquid chromatography mycolic acid analysis

Identification of mycobacterial strains isolated from clinical specimens by mycolic acid analysis using HPLC and *p*-bromophenacyl bromide derivatizing reagent for UV detection is a well-established method [126,127]. The total area under the mycolic acid (TAMA) can be used as a good estimator of mycobacterial growth and also as a means of susceptibility testing of MTB, since a linear relationship between the TAMA chromatographic peaks of a culture of MTB and logCFU/mL has been found to exist [128,127]. Even though the reagents and supplies for HPLC are cheaper compared with those needed for the BACTEC radiometric method, the main drawback of this method is the initial cost of the equipment.

Numerical evaluation

It is important to design experimental evaluation protocols that are independent of the typical 2-fold dilution scheme that is commonly used in susceptibility testing of MTB for the purpose of establishing structure–activity relationships. A numerical evaluation scheme has been established that allows the determination of more precise MIC values and extrapolation of the precise endpoint (99% inhibition of mycobacterial metabolic activity in BACTEC and 90% growth inhibition in MABA) [129]. This scheme also allows for the quantitation of the bioassay-guided isolation protocol. The importance of quantification of bioactivity in search for novel anti-TB agents has also been emphasized by Eloff [130], pointing out that quantitation is a requirement for the detection of synergistic effects [46].

Macrophage bioassays

The intracellular activity of anti-mycobacterials has been evaluated using *in vitro* models of macrophage infection by *Mycobacterium* species. Macrophages can be sourced from humans, mice and rabbits. Different mycobacterial species (MTB H_{37}Ra, H_{37}Rv, Erdman, and clinical isolates, *M. bovis* BCG and *M. avium*) have been used, and hence a source of variability of results [131,105]. The monocytic cell line THP-1 (ATCC/TIB-202) obtained from American Type Culture Collection (Rockville, MD) can be used to examine the inhibitory activity of crude extracts, fractions and isolated compounds against intracellular bacilli. THP-1 cells (5×10^4 cells/ml) are treated with 100 nM of phorbol myristate acetate in a culture flask for 24 h to convert them into macrophages. These macrophages are incubated for 12 h with MTB H_{37}Ra at a multiplicity of infection of 1:100 for infection. Extracellular mycobacteria are removed by washing twice with phosphate-buffered saline and then adding fresh medium to adhered cells. Crude extracts, fractions and isolated compounds can then be added to these infected macrophages at different concentrations. 2-Nitroimidazole is used as the positive control. The effect of the test samples is monitored by determining the bacterial load within macrophages by lysing them with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$ and 10 mM KCl) and spreading the samples on Dubos agar plates at different time intervals to enumerate colonies after 21 days [121].

Anti-TB *ex vivo* bioassay

Patient Peripheral Blood Mononuclear Cell (PBMC) bioassay

Before novel formulations (hits) can proceed for proof of concept evaluation in animal models, efficacy against MTB can be tested further in an *ex vivo* model. In countries where TB is widely prevalent, Peripheral Blood Mononuclear Cell (PBMC) isolated from tubercular patients can be considered as a very good *ex vivo* model. The clinical efficacy of a compound will be better revealed following its evaluation on collections of a wide variety of patient samples in different stages of the infection with different strains of mycobacterium (MDR or XDR) in a diverse spectrum of disease situation. A series of immune pathological events happen following MTB infection as reported by several studies using animal models. For example, infected cells from active MTB patients have been shown to produce significant amounts of nitric oxide compared with non-infected cells [132–134]. In addition, IFN-gamma elevation is also observed in human PBMC infected with MTB. Animal models infected with MTB exhibit a gross down-regulation of gene expression associated with innate and adaptive immunity. In particular, a lower relative expression of key innate immunity related genes, including the Toll-like receptor genes (TLR genes 2 and 4), lack of differential expression of indicator adaptive immune gene transcripts (IFNG, IL2, IL4) and lower major histocompatibility complex class I (BOLA) and class II (BOLA-DRA) gene expression, has been shown to be consistent with innate immune gene expression in *M. bovis* (BTB)-infected animals [133]. This diversity in differential gene expression will affect the effect of drugs in PBMC isolated from patients in comparison with the non-tubercular counterparts. Hence, novel compounds tested on patient PBMC *ex vivo* bioassay before exploring the *in vivo* animal model will be more informative and cost-effective.

Anti-TB *in vivo* bioassay

Drug candidates (hits) for clinical evaluation must be active in an *in vivo* animal model of MTB infection at a dosage that can be well-tolerated in human subjects. Mice are usually infected via aerosol exposure to virulent strains of MTB, resulting in the deposition of low numbers of tubercle bacilli in the lungs [46]. Following multiplication of tubercle bacilli and host immune response, therapy is commenced either during the phase of rapid multiplication (up to 1 month) [135,136], or during the non-replicating/dormancy phase [119], which can last

for months. Recently, long-term models evaluating the sterilizing ability of novel compounds have been described [137]. *In vivo* models (non-human primates) that can be used to assess the activity of novel compounds in latent infection have recently been described [138].

Toxicity evaluation

Toxicity is a leading cause of attrition of novel compounds at all stages of the drug development process [139,140]. *In vitro* toxicology studies are usually conducted before the first *in vivo* toxicity studies, usually to predict those compound-related toxicities that can limit the progression of a novel chemical molecule. Following the evaluation of novel compounds for activity against MTB, cytotoxicity to mammalian cell lines should be evaluated to determine if the compound is only toxic to mycobacterial cells (selective toxicity). There are several bioassays, including colorimetric methods based on the formation of formazan-like products [79,141], as well as the Alamar Blue dye and bioluminescent analysis of adenosine triphosphate (ATP), which appears to offer the answer to the demands of speed and simplicity, providing the required sensitivity to screen out for cytotoxicity [142]. Hence, the determination of general cytotoxicity is important in the course of drug discovery against MTB.

Selectivity and criteria for antimycobacterial activity

Studies on the selectivity index (SI) should be performed during the early phases of drug discovery against MTB. Based on the simultaneous determination of the general median cytotoxic concentration of a novel compound to a mammalian cell line (IC_{50}) and the lowest concentration inhibiting mycobacterial growth, the mycobacterial MIC selectivity index (SI) [135,143] can be determined as the ratio of both (i.e., IC_{50}/MIC), and taken into account throughout the bioactivity-guided fractionation of active principles in the drug discovery process. Relevant mycobacterial activity as defined by the Clinical and Laboratory Standards Institute (CLSI) relates to MIC values below 128 μg/ml (<128 μg/ml) for plant extracts, below 0.25% v/v for essential oils, and below 25 μM (<25 μM) for pure compounds [144]. A selectivity index of greater than 10 (>10) is considered to be of interest during the drug discovery process, especially to the pharmaceutical industry [145].

Natural products chemistry

Minor considerations

The majority of natural product collections usually start as crude extracts of fresh or dried material processed by different methods using various chemical solvents. Hydrophilic compounds are extracted using polar solvents, such as methanol, ethanol or ethyl-acetate, while lipophilic compounds are extracted using non-polar solvents, such as dichloromethane (DCM) or a mixture of DCM and methanol

(1:1). Crude natural product extracts are complex mixtures of perhaps hundreds of different compounds working together in synergy when the extract is administered as a whole. Discovery of natural product hits and their progression towards development includes extraction of the crude extract from the source, concentration, lyophilization (in cases where polar solvents have been used), fractionation and purification to yield a single bioactive compound. Chromatography is one of the most useful means for separation of complex compound mixtures, and also as a technique for both compound purification and identification. Chromatographic methods that are primarily used in isolation and identification of natural products include thin-layer chromatography (TLC), liquid column chromatography (LC), gas chromatography (GC), high-performance liquid chromatography (HPLC), fast protein liquid chromatography (FPLC), immobilized metal-ion affinity chromatography and antibody affinity chromatography [105].

Traditional bioassay-guided fractionation techniques may only be run for a few months in an intensive screening campaign, and the purification of active compounds may not be possible in that time frame; hence, they are generally regarded as being too slow to fit into the pace of high-throughput screening [146]. The chances for success in isolating a potent antimycobacterial compound from a semi-purified extract may or may not depend on the generated MIC values. The possibility exists that an extract with a relatively low MIC (high activity) may contain large amounts of only very few moderately active major compounds, while moderately active crude natural product extracts could contain minor constituents with high activity. An example for the former is the DCM extract of *Alpinia galanga* with an MIC of 1 μg/ml ($H_{37}Ra$, MABA), which contains 5% of acetoxychavicole acetate as the main active phytoconstituent with an MIC of 1 μg/ml. An example for the latter is the methanolic crude extract of *Ajuga remota* with an MIC of 100 μg/ml (98% inhibition in BACTEC/$H_{37}Rv$ at that concentration), which has been shown to contain 0.1% of ergosterol-5, 8-endoperoxide with an MIC of 1 μg/ml [147]. The combination of moderately active natural product crude extracts with synthetic analogs has been shown to bear great potential of increasing antimicrobial activity by two orders of magnitude, hence increasing the motivation for rigorous studies on extracts with moderate activity. In addition, any structural class of natural products that is consistently found to have activity against MTB shall be considered to be more attractive for further development as an anti-TB agent than a single compound with high potency, but no reported anti-TB activity of related natural analogs [46]. Hence, a rational drug discovery program against MTB should employ a bioassay-guided fractionation protocol that is capable of isolating minor constituents from a crude extract with interesting activity against the target organism, MTB. The major advantage of such a protocol is that isolation of large quantities of an entire series of structurally related anti-TB compounds is greatly enhanced, and hence basic structure–activity relationships can be established following isolation of primary hits using bioactivity-guided fractionation. In addition, such a protocol can help to prioritize classes of natural products for further evaluation towards lead compound identification for anti-TB activity. For example, carbazole alkaloids are a class of natural products

with moderate but consistent activity with a potential for development as lead compounds against MTB [148,149]. However, in the course of bioactivity-guided fractionation of *Micromelum hirsutum*, it is worth noting that the "best hit" approach focused further development on micromolide, the fatty acid lactone with an MIC of 1 μg/ml, versus its carbazole counterparts with lesser bioactivity (MICs of 16 to >128 μg/ml) [147].

Structure elucidation of natural products

Isolation, purification and structure elucidation of target compounds from complex crude extract mixtures are the major bottlenecks in natural products chemistry. Currently, the main spectroscopic tools for structure elucidation of natural products are nuclear magnetic resonance (NMR) and mass spectroscopic (MS) techniques, in addition to infrared (IR) and ultraviolet–visible spectrophotometric (UV–Vis) methods, which are also equally important [150]. Structure elucidation of natural products in small, sub-milligram quantities of material have currently been made possible by recent advances in NMR spectroscopy and MS techniques. The time line for dereplication, isolation and structure elucidation of natural individual compounds present in the crude extracts has been significantly reduced by the recent development in the hyphenated techniques, which combine separation technologies such as HPLC and solid-phase extraction (SPE) with NMR and MS techniques [151]. The sensitivity of modern hyphenated MS methods is in the nano or pictogram range; hence, it is well below the detection limit of a bioactive compound. Excellent reviews on these topics are available [46,152–154]. The major contributing factors in NMR are the superconducting magnet technology [155], micro- and cryoprobe technology [156–158], and the establishment of a myriad of multi-pulse experiments that cover all routine aspects of organic structure determination [159]. The need to analyze natural product quantities that are sufficient for the anti-TB bioassay (100 μg for a MABA anti-TB test) make sensitive ^{1}H NMR methods most useful for structural characterization of active compounds in a bioassay-guided drug discovery program. All proton-detecting experiments, such as 2D COSY, HSQC/HMBC and NOESY, are powerful tools in both the dereplication and structure elucidation of bioactive natural products besides the routine 1D proton NMR [160–162]. It is important to note that 1D NMR experiments that apply selective excitation pulses as part of classical COSY, TOCSY and NOESY sequences are particularly valuable sources of structural information [163–165]. For example, the sesquiterpene 2,10-bisaboladiene- 1,4-endoperoxide from *Rudbeckia laciniata*, an anti-TB natural product, was characterized using Gaussian-shaped pulses in a selective COSY experiment [46].

Dereplication and NMR fingerprinting of natural products

The driving force behind much phytochemical research is the discovery of new biologically active compounds with antimycobacterial activity. Bioassays, then, must be carried out in order to identify promising plant extracts, to guide the separation and isolation and finally to evaluate hit compounds. Dereplication (positive identification of known natural products) is not a trivial task [166–170] since comprehensive sets of standards are rarely available. Improved efficiency in dereplication of active principles is of dual importance. First, it lowers the overall efforts during bioassay-guided fractionation, since a relatively small number of (ubiquitous) constituents can blur the view of the natural products chemists for new compounds with desired activity. Secondly, it allows the concentration of resources on the elucidation of novel compounds. Dereplication, however, must be definitive, which in light of the structural complexity of natural products (e.g., multiple [stereo] isomers) places strong demands on the quality and comprehensiveness of the analytical data [46]. One simplified yet highly significant approach to this problem is to dereplicate compounds by ^{1}H NMR fingerprint analysis of their hyper complex proton signals [171–173]. This methodology makes use of the distinct fingerprint patterns of proton signals arising from the complex proton spin system contained in most natural products. An additional benefit of the aforementioned selective 1D NMR experiments is that they facilitate compound dereplication by providing coupling and/or shift-edited sub-spectra of the often crowded ^{1}H NMR spectra and are suitable to generate high-resolution data for ^{1}H NMR fingerprint analysis [46].

Countercurrent separation of natural products

Currently, the most common chromatographic methods applied in natural product separation include: adsorption chromatographic methods, such as TLC; LC; GC; HPLC; FPLC; immobilized metal-ion affinity chromatography; and antibody affinity chromatography [174,175]. In addition, partition chromatography is another separation technique that has so far been applied by rather few scientists [46]. Nevertheless, countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC), collectively known as countercurrent separation, are powerful tools in both the early and advanced stages of the fractionation process of crude natural product extracts [176]. There are recent reviews on this subject [46,177]. Modern CCC methods, such as high-speed countercurrent chromatography (HSCCC) and [fast] centrifugal partition chromatography ([F] CPC), reduce the difficulties involved in natural product drug discovery, i.e., expensive and time-consuming steps to isolate active constituents, and have the ability to attain high resolution [46]. Current instrumental developments have been summarized [178–180], and continuously updated information is available online [181]. The major advantages of countercurrent/partition chromatography evolve from the complete lack of a solid stationary phase, translating into the lack of any irreversible absorption [46], which is essential for a bioassay-directed search for anti-TB lead compounds. The chances of "losing" the anti-TB activity of a natural product during fractionation are eliminated [182], or at least reduced to the unavoidable possibility of degradation in solution at room temperature (c.f. with ubiquitous rotary evaporation at elevated temperatures), since CCC/CPC provides a means of loss-free fractionation, which in the course of bioassay-guided fractionation is particularly valuable. Dr. Yoichiro Ito (NHLBI/NIH), the inventor of modern CCC has recently created a timely and invaluable documentation of more than

30 years of experience in the field by formulating his 18 Golden Rules and Pitfalls in selecting optimum conditions for CCC [183]. Significant loss of activity between subsequent countercurrent/partition chromatographic steps can be confidently explained as several natural product constituents acting in synergy, which (synergy) has recently been identified as a major factor in explaining the overall antimicrobial activity of plant-derived agents [46]. For example, the significant antimicrobial effect of berberine alkaloids contained in the Berberis extracts has been associated with the flavonolignan 5V-methoxyhydnocarpin, which inhibits multidrug-resistance pumps [184]. While using the *Oplopanax horridus* (Devil's Club), it has been demonstrated that the polarity window can be chosen such that the activity could be enriched into a sub-fraction of less than 90% (w/w) [185]. This enabled the *in vivo* evaluation of a crude natural product with only one literature report on the anti-TB activity of hops constituents [186]. Recently, using CCC, the anti-TB polarity window of *O. horridus* has been shown to contain polyynes (polyacetylenes) by GC–MS analysis, in addition to other constituents. Moreover, it exhibited no cytotoxicity on Vero cells, which is not only a prerequisite for *in vivo* testing, but also came as a surprise since numerous literature reports have associated polyynes with cytotoxicity [46,187–194].

New perspectives

New and drug-resistant strains of MTB continue to emerge because of the remarkable genetic and adaptable plasticity of the microbiota. Natural products have been and will continue to be a rich source of novel drugs. New natural products chemistry tools and new mycobacteriological bioassays with relevance to MTB virulence are available and await eager employment by interdisciplinary research teams. The extended mycobacteriology toolbox allows for the detection of relevant biological activities at various levels corresponding to the disease, including mycobacterial growth (MABA), pathogen self-defense (LORA), host defense (macrophage), and *in vivo* (animal) regardless of the source of the natural product (plant, marine, animal or microorganisms). Researchers in all institutions will be able to contribute to the development of understanding and utilization of natural product resources, due to the continuous development of sensitive, rapid and inexpensive bioassays. Innovative procedures for preparative analysis can be tailored by taking advantage of new bioassays, new separation (CCC/CPC) and detection and identification (NMR) methods outlined in this review in order to improve access to and benefit from the favorable chemo diversity of nature. These methods will open new perspectives in the discovery of useful anti-TB agents when seamlessly combined. The increasing frequency of MDR-TB, XDR-TB and currently, totally TDR-TB, and limited therapeutic options emphasize the urgent need for novel drugs against TB.

Finally, it shall be noted that, from both the biological/mycobacteriological and the natural products chemistry perspective, the various aspects of the collaborative challenges faced during drug discovery against TB from natural products are applicable to other infectious diseases. It is believed that the interplay of co-developed innovative mycobacteriological and natural product chemistry methods on both ends will greatly impact the early phases of anti-TB drug discovery and increase the chances of success.

Conflict of interest

We have no conflict of interest to declare.

Acknowledgments

REFERENCES

[1] World Health Organization, WHO Global Tuberculosis Report 2014, <http://www.who.int/ tdr/news/2014/global-TB-report/ en/>, 2014.

[2] C. Lienhardt et al, New drugs for the treatment of tuberculosis: needs, challenges, promise and prospects for the future, J. Infect. Dis. 205 (2012) S241–S249.

[3] G. Riccardi, M.R. Pasca, Trends in discovery of new drugs for tuberculosis therapy, J. Antibiot. 67 (2014) 655–659.

[4] K.D. Green, S. Garneau-Tsodikova, Resistance in tuberculosis: what do we know and where can we go?, Front Microbiol. 4 (2013) 208.

[5] D. Blanco, E. Perez-Herran, M. Cacho, L. Ballell, J. Castro, R. González del Río, et al, *Mycobacterium tuberculosis* gyrase inhibitors (MGI) as a new class of antitubercular drugs, J. Antimicrob. Agents Chemother. (2015), http://dx.doi.org/ 10.1128/AAC.03913-14.

[6] G. Sotgiu, A. Spanevello, G.B. Migliori, In vitro susceptibility testing and totally drug-resistant tuberculosis, Eur. Respir. J. 42 (2013) 292.

[7] Z.F. Udwadia, R.A. Amale, K.K. Ajbani, C. Rodrigues, Totally drug-resistant tuberculosis in India, Clin. Infect. Dis. 54 (2012) 579–581.

[8] A.A. Velayati et al, Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran, Chest 136 (2009) 420–425.

[9] G.B. Migliori et al, Totally drug-resistant and extremely drug-resistant tuberculosis: the same disease?, Clin Infect. Dis. 54 (2012) 1379–1380.

[10] G. Riccardi, M.R. Pasca, S. Buroni, *Mycobacterium tuberculosis*: drug resistance and future perspectives, Future Microbiol. 4 (2009) 597–614.

[11] C. Vilchèze, W.R. Jacobs Jr., The mechanism of isoniazid killing: clarity through the scope of genetics, Annu. Rev. Microbiol. 61 (2007) 35–50.

[12] L.Q. Wang, C.N. Falany, M.O. James, Triclosan as a substrate and inhibitor of 30-phosphoadenosine 50-phosphosulfate-sulfotransferase and UDP-glucuronosyl transferase in human liver fractions, Drug Metab. Dispos. 32 (2004) 1162–1169.

[13] J.S. Freundlich et al, Triclosan derivatives: towards potent inhibitors of drug-sensitive and drug-resistant *Mycobacterium tuberculosis*, Chem. Med. Chem. 4 (2009) 241–248.

[14] T. Matviiuk et al, Synthesis of 3-heteryl substituted pyrrolidine-2,5-diones via catalytic Michael reaction and evaluation of their inhibitory activity against InhA and *Mycobacterium tuberculosis*, Eur. J. Med. Chem. 71 (2014) 46–52.

[15] R.C. Hartkoorn et al, Towards a new tuberculosis drug: pyridomycin – nature's isoniazid, EMBO Mol. Med. 4 (2012) 1032–1042.

[16] A. Scorpio et al, Characterization of pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 41 (1997) 540–543.

[17] W. Shi et al, Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*, Science 333 (2011) 1630–1632.

[18] M. Saguy et al, Ribosomal protein S1 influences trans-translation *in vitro* and *in vivo*, Nucleic Acids Res. 35 (2007) 2368–2376.

[19] Y. Zhang et al, Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid, J. Antimicrob. Chemother. 52 (2003) 790–795.

[20] W. McDermott, R. Tompsett, Activation of pyrazinamide and nicotinamide in acidic environments *in vitro*, Am. Rev. Tuberc. 70 (1954) 748–754.

[21] R.M. McCune, F.M. Feldmann, H.P. Lambert, W. McDermott, Microbial persistence. I. The capacity of tubercle bacilli to survive sterilization in mouse tissues, J. Exp. Med. 123 (1966) 445–468.

[22] B.A. Wolucka, Biosynthesis of D-arabinose in mycobacteria – a novel bacterial pathway with implications for antimycobacterial therapy, FEBS J. 275 (2008) 2691–2711.

[23] A. Telenti et al, The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol, Nat. Med. 3 (1997) 567–570.

[24] A. Telenti et al, Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*, Lancet 341 (1993) 647–650.

[25] P. Sensi, History of the development of rifampin, Rev. Infect. Dis. 5 (1983) S402–S406.

[26] J. Mukhopadhyay et al, The RNA polymerase "switch region" is a target for inhibitors, Cell 135 (2008) 295–307.

[27] P. Sensi, Approaches to the development of new antituberculosis drugs, Rev. Infect. Dis. 11 (1989) S467–S470.

[28] E.J. North, M. Jackson, R.E. Lee, New approaches to target the mycolic acid biosynthesis pathway for the development of tuberculosis therapeutics, Curr. Pharm. Des. 20 (2014) 4357–4378.

[29] K. Andries et al, A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*, Science 307 (2005) 223–227.

[30] B. Chan, T.M. Khadem, J. Brown, A review of tuberculosis: focus on bedaquiline, Am. J. Health Syst. Pharm. 70 (2013) 1984–1994.

[31] A.C. Haagsma et al, Selectivity of TMC207 towards mycobacterial ATP synthase compared with that towards the eukaryotic homologue, Antimicrob. Agents Chemother. 53 (2009) 1290–1292.

[32] E.B. Chahine, L.R. Karaoui, H. Mansour, Bedaquiline: a novel diarylquinoline for multidrug-resistant tuberculosis, Ann. Pharmacother. 48 (2014) 107–115.

[33] R. Singh et al, PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release, Science 322 (2008) 1392–1395.

[34] U. Manjunatha, H.I. Boshoff, C.E. Barry, The mechanism of action of PA-824: novel insights from transcriptional profiling, Commun. Integr. Biol. 2 (2009) 215–218.

[35] A.H. Diacon et al, Early bactericidal activity and pharmacokinetics of PA-824 in smear-positive tuberculosis patients, Antimicrob. Agents Chemother. 54 (2010) 3402–3407.

[36] M.T. Gler et al, Delamanid for multidrug-resistant pulmonary tuberculosis, N. Engl. J. Med. 366 (2012) 2151–2160.

[37] Q. Zhang, Y. Liu, S. Tang, W. Sha, H. Xiao, Clinical benefit of delamanid (OPC-67683) in the treatment of multidrug-resistant tuberculosis patients in China, Cell Biochem. Biophys. 67 (2013) 957–963.

[38] S.S. Munsiff, C. Kambili, S.D. Ahuja, Rifapentine for the treatment of pulmonary tuberculosis, Clin. Infect. Dis. 43 (2006) 1468–1475.

[39] J.G. Chan, X. Bai, D. Traini, An update on the use of rifapentine for tuberculosis therapy, Expert Opin. Drug Deliv. 11 (2014) 421–431.

[40] T.R. Sterling, M.E. Villarino, A.S. Borisov, N. Shang, F. Gordin, E. Bliven-Sizemoreet, et al, Three months of rifapentine and isoniazid for latent tuberculosis infection, N. Engl. J. Med. 365 (2011) 2155–66.

[41] K.E. Dooley et al, Safety and pharmacokinetics of escalating daily doses of the antituberculosis drug rifapentine in healthy volunteers, Clin. Pharmacol. Ther. 91 (2012) 881–888.

[42] K. Tahlan et al, SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 56 (2012) 1797–1809.

[43] K.A. Sacksteder, M. Protopopova, C.E. Barry, K. Andriesm, C.A. Nacy, Discovery and development of SQ109: a new antitubercular drug with a novel mechanism of action, Future Microbiol. 7 (2012) 823–837.

[44] C.P. Owens et al, The *Mycobacterium tuberculosis* secreted protein Rv0203 transfers heme to membrane proteins MmpL3 and MmpL11, J. Biol. Chem. 288 (2013) 21714–21728.

[45] A. Zumla, P. Nahid, S.T. Cole, Advances in the development of new tuberculosis drugs and treatment regimens, Nat. Rev. 12 (2015) 388–404.

[46] G.F. Pauli, R.J. Case, T. Inui, Y. Wang, S. Cho, N.H. Fischer, et al, New perspectives on natural products in TB drug research, Life Sci. 78 (2005) 485–494.

[47] P.P. Mitra, Drug discovery in tuberculosis: a molecular approach, Indian J. Tuberc. 59 (2012) 194–206.

[48] M. Uplekar, D. Weil, K. Lonnroth, E. Jaramillo, C. Lienhardt, H. Monica Dias, et al, WHO's new end TB strategy, Lancet (2015), http://dx.doi.org/10.1016/S0140-6736(15)60570-0.

[49] I.M.R. Uchida, Y. Takakusagi, et al, Lariatins, novel anti-mycobacterial peptides with a lasso structure, produced by Rhodococcus jostii K01–B0171, J. Antibiot. (Tokyo) 60 (2007) 357–363.

[50] C.E. Salomon, L.E. Schmidt, Natural products as leads for tuberculosis drug development, Curr. Top. Med. Chem. 12 (2012) 735–765.

[51] J.D. Guzman, A. Gupta, F. Bucar, et al, Antimycobacterials from natural sources: ancient times, antibiotic era and novel scaffolds, Front. Biosci. (Landmark Ed.) 17 (2012) 1861–1881.

[52] H.A. Kirst, Developing new antibacterials through natural product research, Expert Opin. Drug Discov. 8 (2013) 479–493.

[53] S.B. Singh, K. Young, L. Miesel, Screening strategies for discovery of antibacterial natural products, Expert Rev. Anti Infect. Ther. 9 (2011) 589–613.

[54] K. Mdluli, T. Kaneko, A. Upton, Tuberculosis drug discovery and emerging targets, Ann. NY. Acad. Sci. 1323 (2014) 56–75.

[55] J.M. Nguta, R. Appiah-Opong, A.K. Nyarko, D. Yeboah-Manu, G.A. Addo, Medicinal plants used to treat TB in Ghana, Int. J. Mycobacteriol. 4 (2) (2015) 116–123.

[56] A. García, V. Bocanegra-García, J.P. Palma-Nicolás, G. Rivera, Recent advances in antitubercular natural products, Eur. J. Med. Chem. 49 (2012) 1–23.

[57] D.J. Newman, G.M. Cragg, K.M. Snader, The influence of natural products upon drug discovery, Nat. Prod. Rep. 17 (2000) 215–234.

[58] O. Potterat, M. Hamburger, Natural products in drug discovery – concepts and approaches for tracking bioactivity, Curr. Org. Chem. 10 (2006) 899–920.

[59] J.M. Nguta, J.M. Mbaria, D.W. Gakuya, P.K. Gathumbi, S.G. Kiama, Antimalarial herbal remedies of Msambweni, Kenya, J. Ethnopharmacol. 128 (2010) 424–432.

[60] J. Nguta, J.M. Mbaria, Brine shrimp toxicity and antimalarial activity of some plants traditionally used in treatment of malaria in Msambweni district of Kenya, J. Ethnopharmacol. 148 (2013) 988–992.

[61] R. Diel, R. Loddenkemper, J. Zellweger, G. Sotgiu, L. D'Ambrosio, R. Centis, et al, Old ideas to innovate TB control: preventive treatment to achieve elimination, Eur. Respir. 42 (2013) 785–801.

[62] M. Raviglione, B. Marais, K. Floyd, K. Lönnroth, H. Getahun, G.B. Migliori, et al, Scaling up interventions to achieve global tuberculosis control: progress and new developments, Lancet 379 (2012) 1902–1913.

[63] World Health Organization, WHO Global Tuberculosis Report 2012, <http://www.who.int/ tdr/news/2012/global-TB-report/en/>, 2012.

[64] J. Cohen, Infectious disease. Approval of novel TB drug celebrated-with restraint, Science 339 (2013) 130.

[65] N.N. Ibekwe, S.J. Ameh, Plant natural products research in tuberculosis drug discovery and development: a situation report with focus on Nigerian biodiversity, Afr. J. Biotechnol. 13 (23) (2014) 2307–2320.

[66] J.D. Phillipson, A matter of some sensitivity, Phytochemistry 38 (1995) 1319–1343.

[67] V. Chaturvedi, N. Dwivedi, R.P. Tripathi, S.J. Sinha, Evaluation of Mycobacterium smegmatis as a possible surrogate screen for selecting molecules active against multi-drug resistant Mycobacterium tuberculosis, Gen. Appl. Microbiol. 52 (6) (2007) 333–337.

[68] R. Wang, E.M. Marcotte, The proteomic response of Mycobacterium smegmatis to anti-tuberculosis drugs suggests targeted pathways, J. Proteome Res. 78 (2008) 855–865.

[69] D.B. Young et al, Confronting the scientific obstacles to global control of tuberculosis, J. Clin. Invest. 118 (2008) 1255–1265.

[70] T.S. Balganesh, B.J. Furr, Molecular approaches to target discovery – evaluating targets for anti-tuberculosis drug discovery programmes, Infect. Disord. Drug Targets 7 (2007) 120–126.

[71] R. Shi et al, Overview of anti-tuberculosis (TB) drugs and their resistance mechanisms, Mini Rev. Med. Chem. 7 (2007) 1177–1185.

[72] S.T. Cole, P.M. Alzari, Towards new tuberculosis drugs, Biochem. Soc. Trans. 35 (2007) 1321–1324.

[73] U.H. Manjunatha et al, Identification of a nitroimidazo-oxazine specific protein involved in PA-824 resistance in Mycobacterium tuberculosis, Proc. Natl. Acad. Sci. USA 103 (2006) 431–436.

[74] T.S. Balganesh, P.M. Alzari, S.T. Cole, Rising standards for tuberculosis drug development, Trends Pharmacol. Sci. 29 (2008) 576–581.

[75] V.C. Abraham et al, High content screening applied to large scale cell biology, Trends Biotechnol. 22 (2004) 15–22.

[76] N.D. Connell, H. Nikaido, in: B.R. Bloom (Ed.), Tuberculosis: Pathogenesis, Protection and Control, ASM Press, Washington, D.C., 1994, pp. 333–352.

[77] L. Collins, S. Franzblau, Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium, J. Antimicrob. Agents Chemother. 41 (1997) 1004–1009.

[78] C.L. Cantrell, N.H. Fischer, L. Urbatsch, M. McGuire, S.G. Franzblau, Antimycobacterial crude plant extracts from South, Central, and North America, Phytomedicine 5 (1998) 139–147.

[79] C.L. Cantrell, T. Lu, F.R. Fronczek, N.H. Fischer, L.B. Adams, S.G. Franzblau, Antimycobacterial cycloartanes from Borrichia frutescens, J. Nat. Prod. 59 (1996) 1131–1136.

[80] M.S. Rajab, C.L. Cantrell, S.G. Franzblau, N.H. Fischer, Antimycobacterial activity of (E)-phytol and derivatives: a preliminary structure activity study, Planta Med. 64 (1998) 2–4.

[81] C.A. Sanders, R.R. Nieda, E.P. Desmond, Validation of the use of Middlebrook 7H10 agar, BACTEC MGIT 960, and BACTEC 460 12B media for testing the susceptibility of Mycobacterium tuberculosis to levofloxacin, J. Clin. Microbiol. 42 (2004) 5225–5228.

[82] M.S. Diaz-Infantes, M.J. Ruiz-Serrano, L. Martinez-Sanchez, A. Ortega, E. Bouza, Evaluation of the MB/BacT mycobacterium detection system for susceptibility testing of Mycobacterium tuberculosis, J. Clin. Microbiol. 38 (2000) 1988–1999.

[83] P. Ruiz, F.J. Zerolo, M.J. Casal, Comparison of susceptibility testing of Mycobacterium tuberculosis using the ESP culture system II with that using the BACTEC method, J. Clin. Microbiol. 38 (2000) 4663–4664.

[84] G.D. Roberts, N.L. Goodman, L. Heifets, Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of Mycobacterium tuberculosis from acid-fast smear-positive specimens, J. Clin. Microbiol. 18 (1983) 689–696.

[85] J.C. Palomino, H. Traore, K. Fissette, F. Portaels, Evaluation of mycobacteria growth indicator tube (MGIT) for drug susceptibility testing of Mycobacterium tuberculosis, Int. J. Tuberc. Lung Dis. 3 (1999) 344–348.

[86] I.S. Johansen, V.O. Thomsen, M. Marjamaki, A. Sosnovskaja, B. Lundgren, Rapid, automated, non-radiometric susceptibility testing of Mycobacterium tuberculosis complex to four first-line anti-tuberculous drugs used in standard short-course chemotherapy, Diagn. Microbiol. Infect. Dis. 50 (2004) 103–107.

[87] J.C. Palomino, A. Martin, F. Portaels, Rapid drug resistance detection in Mycobacterium tuberculosis: a review of colourimetric methods, Clin. Microbiol. Infect. 13 (2007) 754–762.

[88] D.M. Yajko, J.J. Madej, M.V. Lancaster, Colorimetric method for determining MICs of antimicrobial agents for Mycobacterium tuberculosis, J. Clin. Microbiol. 33 (1995) 2324–2327.

[89] S.M. Novak, J. Hindler, D.A. Bruckner, Reliability of two novel methods, alamar and Etest, for detection of methicillin-resistant Staphylococcus aureus, J. Clin. Microbiol. 31 (1993) 3056–3057.

[90] M.A. Pfaller, A.L. Barry, Evaluation of a novel colorimetric broth microdilution method for antifungal susceptibility testing of yeast isolates, J. Clin. Microbiol. 32 (1994) 1992–1996.

[91] S.A. Ahmed, R.M. Gogal Jr., J.E. Walsh, A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to (3H) thymidine incorporation assay, J. Immunol. Methods 170 (1994) 211–224.

[92] L. Collins, S.G. Franzblau, Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of

compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*, Antimicrob. Agents Chemother. 41 (1997) 1004–1009.

[93] R.M. Shawar, D.J. Humble, J.M.V. Dalfsen, Rapid screening of natural products for antimycobacterial activity by using luciferase-expressing strains of *Mycobacterium bovis* BCG and *Mycobacterium intracellulare*, Antimicrob. Agents Chemother. 41 (1997) 570–574.

[94] S.G. Franzblau, R.S. Witzig, J.C. McLaughlin, P. Torres, G. Madico, A. Hernandez, et al, Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate alamar blue assay, J. Clin. Microbiol. 36 (1998) 362–366.

[95] A. Martin, M. Camacho, F. Portaels, J.C. Palomino, Resazurin microtiter assay plate testing of *Mycobacterium tuberculosis* susceptibilities to second-line drugs: rapid, simple, and inexpensive method, Antimicrob. Agents Chemother. 47 (2003) 3616–3619.

[96] J.C. Palomino, A. Martin, M. Camacho, H. Guerra, J. Swings, F. Portaels, Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 46 (2002) 2720–2722.

[97] G. Abate, R.N. Mshana, H. Miorner, Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*, Int. J. Tuberc. Lung Dis. 2 (1998) 1011–1016.

[98] L. Caviedes, J. Delgado, R.H. Gilman, Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*, J. Clin. Microbiol. 40 (2002) 1873–1874.

[99] S. Foongladda, D. Roengsanthia, W. Arjrattanakool, C. Chuchottaworn, A. Chaiprasert, S.G. Franzblau, Rapid and simple MTT method for rifampicin and isoniazid susceptibility testing of *Mycobacterium tuberculosis*, Int. J. Tuberc. Lung Dis. 6 (2002) 1118–1122.

[100] R.N. Mshana, G. Tadesse, G. Abate, H. Miorner, Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant *Mycobacterium tuberculosis*, J. Clin. Microbiol. 36 (1998) 1214–1219.

[101] T.K. Kent, G.P. Kubica, Public health mycobacteriology: a guide for level III laboratories, Centers for Disease Control, Atlanta, 1985.

[102] K.A. Angeby, S.E. Hoffner, Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay, J. Clin. Microbiol. 40 (2002) 553–555.

[103] H.R. Musa, M. Ambroggi, A. Souto, K.A. Angeby, Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy positive sputum samples, J. Clin. Microbiol. 43 (2005) 3159–3161.

[104] L.A. Solis, S.S. Shin, L.L. Han, F. Llanos, M. Stowell, A. Sloutsky, Validation of a rapid method for detection of *M. tuberculosis* resistance to isoniazid and rifampin in Lima, Peru, Int. J. Tuberc. Lung Dis. 9 (2005) 760–764.

[105] J.G. Bueno, V.V. Sánchez Kouznetsov, Antimycobacterial susceptibility testing methods for natural products research, Braz. J. Microbiol. 41 (2010) 270–277.

[106] H.B. Steen, E. Boye, K. Skarstad, B. Bloom, T. Godal, S. Mustafa, Applications of flow cytometry on bacteria: cell cycle kinetics, drug effects, and quantitation of antibody binding, Cytometry 2 (4) (1982) 249–257.

[107] A. Alvarez-Barrientos, J. Arroyo, R. Canton, C. Nombela, M. Sanchez-Perez, Applications of flow cytometry to clinical microbiology, Clin. Microbiol. Rev. 13 (2) (2000) 167–195.

[108] M.A. Norden, T.A. Kurzynski, S.E. Bownds, S.M. Callister, R.F. Schell, Rapid susceptibility testing of M. tuberculosis ($H_{37}Ra$) by flow cytometry, J. Clin. Microbiol. 33 (5) (1995) 1231–1237.

[109] C. Pina-Vaz, S. Costa-de-Oliveira, A.G. Rodrigues, Safe susceptibility testing of M. tuberculosis by flow cytometry with the fluorescent nucleic acid stain SYTO 16, J. Med. Microbiol. 54 (Pt. 1) (2005) 77–81.

[110] D.C. New, D.M. Miller-Martini, Y.H. Wong, Reporter gene assays and their applications to bioassays of natural products, Phytother. Res. 17 (5) (2003) 439–448.

[111] C. Changsen, S.G. Franzblau, P. Palittapongarnpim, Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter, Antimicrob. Agents Chemother. 47 (2003) 3682–3687.

[112] L.A. Collins, M.N. Torrero, S.G. Franzblau, Green fluorescent protein reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 42 (1998) 344–347.

[113] T.M. Arain, A.E. Resconi, M.J. Hickey, C.K. Stover, Bioluminescence screening *in vitro* (Bio-Siv) assays for high-volume antimycobacterial drug discovery, Antimicrob. Agents Chemother. 40 (1996) 1536–1541.

[114] R.C. Cooksey, J.T. Crawford, W.R.J. Jacobs, T.M. Shinnick, A rapid method for screening antimicrobial agents for activities against a strain of *Mycobacterium tuberculosis* expressing firefly luciferase, Antimicrob. Agents Chemother. 37 (1993) 1348–1352.

[115] R.M. Shawar, D.J. Humble, J.M.V. Dalfsen, C.K. Stover, M.J. Hickey, S. Steele, et al, Rapid screening of natural products for antimycobacterial activity by using luciferase-expressing strains of *Mycobacterium bovis BCG* and *Mycobacterium intracellulare*, Antimicrob. Agents Chemother. 41 (1997) 570–574.

[116] V.A. Snewin, M.P. Gares, P.O. Gaora, Z. Hasan, I.N. Brown, D.B. Young, Assessment of immunity to mycobacterial infection with luciferase reporter constructs, Infect. Immun. 67 (1999) 4586–4593.

[117] C.K. Stover, P. Warrener, D.R.V. Devanter, D.R. Sherman, T.M. Arain, M.H. Langhorne, et al, A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis, Nature 405 (2000) 962–966.

[118] H.I. Boshoff, C.E. Barry, Tuberculosis metabolism and respiration in the absence of growth, Nat. Rev. Microbiol. 3 (2005) 70–80.

[119] T. Dick, Dormant tubercle bacilli: the key to more effective TB chemotherapy?, J Antimicrob. Chemother. 47 (1) (2001) 117–118.

[120] L.G. Wayne, C.D. Sohaskey, Nonreplicating persistence of M. tuberculosis, Annu. Rev. Microbiol. 55 (2001) 139–163.

[121] A. Khan, D. Sakhar, A simple whole cell based high throughput screening protocol using M. bovis BCG for inhibitors against dormant and active tubercle bacilli, J. Microbiol. Methods 73 (1) (2008) 62–68.

[122] Z. Sun, Y. Zhang, Antituberculosis activity of certain antifungal and antihelmintic drugs, Tuberc. Lung Dis. 79 (1999) 319–320.

[123] Y. Li, S.G. Franzblau, Microplate assay for testing bactericidal activity of compounds against non-growing Mycobacterium tuberculosis, in: Inter-Sci. Conf. Antimicrob. Agents. Chemother. 39th, Abstract, vol. 863, 1999, p. 814.

[124] L.G. Wayne, L.G. Hayes, An *in vitro* model for sequential study of shiftdown of M. tuberculosis through two stages of nonreplicating persistence, Infect. Immun. 64 (6) (1996) 2062–2069.

[125] S.H. Cho, S. Warit, B. Wan, C.H. Hwang, G.F. Pauli, S.G. Franzblau, Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating M.

tuberculosis, Antimicrob. Agents Chemother. 51 (2007) 1380–1385.

[126] S.L. Baldwin, C. D'Souza, W.R.R. Butler, L.S. Guthertz, Mycolic acid analysis by high performance liquid chromatography for identification of *Mycobacterium* species, Clin. Microbiol. Rev. 14 (4) (2001) 704–726.

[127] J.M. Viader-Salvado, E. Garza-Gonzalez, R. Valdez-Leal, M.A. Del-Bosque-Moncayo, R. Tijerina-Menchaca, M. Guerrero-Olazaran, Mycolic acid index susceptibility method for *M. tuberculosis*, J. Clin. Microbiol. 39 (7) (2001) 2642–2645.

[128] E. Garza-Gonzalez, M. Guerrero-Olazaran, R. Tijerina-Menchaca, J.M. Viader-Salvado, Determination of drug susceptibility of *M. tuberculosis* through mycolic acid analysis, J. Clin. Microbiol. 35 (5) (1997) 1287–1289.

[129] G.F. Pauli, N.H. Fischer, A.N. Biswas, S.G. Franzblau, A refined anti-TB bioassay for natural products, 39th Annual Meeting of the American Society for Pharmacognosy O: 24, American Society for Pharmacognosy, Orlando, Florida, 1998.

[130] J.N. Eloff, Quantification of the bioactivity of plant extracts during screening and bioassay guided fractionation, Phytomedicine 11 (2004) 370–371.

[131] P. Lukey, E. Hooker, Macrophage virulence assays, in: N. Stoker, T. Parish (Eds.), *M. tuberculosis* Protocols, Humana Press, New Jersey, USA, 2001, pp. 271–280.

[132] R. Singh, U. Manjunatha, H.I. Boshoff, Y.H. Ha, P. Niyomrattanakit, R. Ledwidge, et al, PA-824 kills non-replicating *Mycobacterium tuberculosis* by intracellular NO release, Science 28 (2008) 337–338.

[133] S. Sharma, M. Sharma, M. Bose, *Mycobacterium tuberculosis* infection of human monocyte-derived macrophages leads to apoptosis of T cells, Immunol. Cell Biol. 87 (2009) 226–234.

[134] M.I. Voskuil, D. Schnappinger, K.C. Visconti, M.I. Harrell, G.M. Dolganov, D.R. Sherman, et al, Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program, J. Exp. Med. 198 (5) (2003) 705–713.

[135] K. Falzari, Z. Zhu, D. Pan, H. Liu, P. Hongmanee, S.G. Franzblau, *In vitro* and *in vivo* activities of macrolide derivatives against *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 49 (2005) 1447–1454.

[136] B.P. Kelly, S.K. Furney, M.T. Jessen, I.M. Orme, Low-dose aerosol infection model for testing drugs for efficacy against *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 40 (1996) 2809–2812.

[137] E.L. Nuermberger, T. Yoshimatsu, S. Tyagi, R.J. O'Brien, A.N. Vernon, R.E. Chaisson, et al, Moxifloxacin-containing regimen greatly reduces time to culture conversion in murine tuberculosis, Am. J. Respir. Care Med. 169 (3) (2004) 421–426.

[138] P.L. Lin, J.A.L. Flynn, Understanding latent tuberculosis: a moving target, J. Immunol. 185 (2010) 15–22.

[139] F.A. Barile, Principles of Toxicology Testing, CRC Press, Boca Raton, USA, 2008.

[140] J.A. Kramer, J.E. Sagartz, D.L. Morris, The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates, Nat. Rev. Drug Discov. 6 (8) (2007) 636–649.

[141] B. Arechabala, C. Coiffard, P. Rivalland, L.J. Coiffard, Y. de Roeck-Holtzhauer, Comparison of cytotoxicity of various surfactants tested on normal human fibroblast cultures using the neutral red test, MTT assay and LDH release, J. Appl. Toxicol. 19 (1999) 163–165.

[142] K. Slater, Cytotoxicity tests for high-throughput drug discovery, Curr. Opin. Biotechnol. 12 (2001) 70–74.

[143] I. Orme, Search for new drugs for treatment of tuberculosis, Antimicrob. Agents Chemother. 45 (2001) 1943–1946.

[144] P. Cos, A.J. Vlietinck, D.V. Berghe, L. Maes, Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of concept', J. Ethnopharmacol. 106 (3) (2006) 290–302.

[145] S. Gibbons, Phytochemicals for bacterial resistance: strengths, weaknesses and opportunities, Planta Med. 74 (6) (2008) 594–602.

[146] A.L. Harvey, Natural products as a screening resource, Curr. Opin. Chem. Biol. 11 (5) (2007) 480–484.

[147] C.L. Cantrell, M.S. Rajab, S.G. Franzblau, F.R. Fronczek, N.H. Fischer, Antimycobacterial ergosterol-5,8-endoperoxide from *Ajuga remota*, Planta Med. 65 (1999) 732–734.

[148] C. Ma, R. Case, Y. Wang, H. Zhang, G. Tan, N. Van Hung, et al, Antituberculosis compounds from *Micromelum hirsutum*, Planta Med. 71 (2005) 261–267.

[149] A. Sunthitikawinsakul, N. Kongkathip, B. Kongkathip, S. Phonnakhu, J.W. Daly, T.F. Spande, et al, Coumarins and carbazoles from *Clausena excavata* exhibited antimycobacterial activities, Planta Med. 69 (2003) 155–157.

[150] L. Cseke, W. Setzer, B. Vogler, A. Kirakosyan, B. Kaufman, Traditional, analytical, and preparative separations of natural products, in: L. Cseke, A. Kirakosyan, B. Kaufman, S. Warber, J. Duke, H. Brielmann (Eds.), Natural Products From Plants, CRC Press, Boca Raton, USA, 2006, pp. 264–317.

[151] K.S. Lam, New aspects of natural products in drug discovery, Trends Microbiol. 15 (6) (2007) 279–289.

[152] J.L. Wolfender, K. Ndjoko, K. Hostettmann, LC/NMR in natural products chemistry, Curr. Org. Chem. 2 (1998) 575–596.

[153] J.L. Wolfender, K. Ndjoko, K. Hostettmann, The potential of LC-NMR in phytochemical analysis, Phytochem. Anal. 12 (2001) 2–22.

[154] J.L. Wolfender, K. Ndjoko, K. Hostettmann, Liquid chromatography with ultraviolet absorbance–mass spectrometric detection and with nuclear magnetic resonance spectrometry: a powerful combination for the on-line structural investigation of plant metabolites, J. Chromatogr. A 1000 (2003) 437–455.

[155] J. Haase, D. Eckert, H. Siegel, H. Eschrig, K.H. Mueller, F. Steglich, Nuclear magnetic resonance in pulsed high-field magnets, Concepts Magn. Reson. B 19B (2003) 9–13.

[156] R.C. Crouch, W. Llanos, K.G. Mehr, C.E. Hadden, D.J. Russell, G.E. Martin, Applications of cryogenic NMR probe technology to long range 1H–15N 2D NMR studies at natural abundance, Magn. Reson. Chem. 39 (2001) 555–558.

[157] G.E. Martin, C.E. Hadden, D.J. Russell, B.D. Kaluzny, J.E. Guido, W.K. Duholke, et al, Identification of degradants of a complex alkaloid using NMR cryoprobe technology and ACD/structure elucidator, J. Heterocycl. Chem. 39 (2002) 1241–1250.

[158] D.L. Olson, M.E. Lacey, J.V. Sweedler, High-resolution microcoil NMR for analysis of mass-limited, nanoliter samples, Anal. Chem. 70 (1998) 645–650.

[159] S. Braun, H.O. Kalinowski, S. Berger, 150 and More Basic NMR Experiments, Wiley-VCH, Weinheim, 1998. 596.

[160] P. Crews, J. Rodríguez, M. Jaspars, Organic Structure Analysis, Oxford University Press, New York, 1998 (p. xxiv 552 pp.).

[161] M. Jaspars, Computer assisted structure elucidation of natural products using two dimensional NMR spectroscopy, Nat. Prod. Rep. 16 (1999) 241–248.

[162] W.F. Reynolds, R.G. Enriquez, Choosing the best pulse sequences, acquisition parameters, post-acquisition processing strategies, and probes for natural product structure elucidation by NMR spectroscopy, J. Nat. Prod. 65 (2002) 221–244.

[163] A. Bax, K.A. Farley, G.S. Walker, Increased HMBC sensitivity for correlating poorly resolved proton multiplets to carbon-13 using selective or semi-selective pulses, J. Magn. Reson. 119 (1996) 134–138.

[164] G.F. Pauli, R. Frohlich, Chiral key positions in Uzara steroids, Phytochem. Anal. 11 (2000) 79–89.

[165] D. Uhrin, A. Mele, R. Dwek, Selective inverse correlation by using chemical shift selective filters and selective pulses, J. Magn. Reson. Ser. A 101 (1993) 98–102.

[166] J. Bradshaw, D. Butina, A.J. Dunn, R.H. Green, M. Hajek, M.M. Jones, et al, A rapid and facile method for the dereplication of purified natural products, J. Nat. Prod. 64 (2001) 1541–1544.

[167] G.A. Cordell, Y.G. Shin, Finding the needle in the haystack. The dereplication of natural product extracts, Pure Appl. Chem. 71 (1999) 1089–1094.

[168] D.G. Corley, R.C. Durley, Strategies for a database dereplication of natural products, J. Nat. Prod. 57 (1994) 1484–1490.

[169] D. Kinghorn, N. Farnsworth, D. Soejarto, G. Cordell, S. Swanson, J. Pezzuto, et al, Novel strategies for the discovery of plant-derived anticancer agents, Pharm. Biol. (Lisse, Netherlands) 41 (2003) 53–67.

[170] C. Steinbeck, Recent developments in automated structure elucidation of natural products, Nat. Prod. Rep. 21 (2004) 512–518.

[171] L.R. Chadwick, G.F. Pauli, H.H.S. Fong, E. Chou, J. Burdette, C. Overk, et al, 1H NMR Spectra of Simple Molecules-Hyper-Complex Signals in Antioxidant and Estrogenic Phenolics from Humulus lupulus L, American Society of Pharmacognosy, Phoenix, AZ, 2004 (ASP 2004 O:14).

[172] B. Jaki, L. Chadwick, D. Seigler, G.F. Pauli, J Code Cracking in the Fingerprint Dereplication of Small Molecules, SMASH Organizational Committee, Small Molecules Are Still Hot, Verona Italy, 2003 (SMASH 2003 P: 12).

[173] B. Jaki, S.G. Franzblau, G.F. Pauli, An NMR method towards routine chiral determination of natural products, Phytochem. Anal. 15 (2004) 213–219.

[174] J.B. Harborne, Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, Chapman and Hall, London, New York, 1998 (p. xiii 302).

[175] K. Hostettmann, M. Hostettmann, A. Marston, Preparative Chromatography Techniques: Applications in Natural Product Isolation, Springer-Verlag, Berlin, New York, 1998 (p. x 244).

[176] K. Hostettmann, A. Marston, Countercurrent chromatography in the preparative separation of plant-derived natural products, J. Liquid Chromatogr. Relat. Technol. 24 (2001) 1711–1721.

[177] G.F. Pauli, S.M. Pro, J.B. Friesen, Countercurrent separation of natural products, J. Nat. Prod. 71 (8) (2008) 1489–1508.

[178] A. Berthod, Countercurrent Chromatography: The Support-Free Liquid Phase, Elsevier, Amsterdam, Boston, 2002 (p. xxiv 397 pp.).

[179] A.P. Foucault, Centrifugal Partition Chromatography, Dekker, New York, 1995 (p. x 415).

[180] L. Marchal, J. Legrand, A. Foucault, Centrifugal partition chromatography: a survey of its history, and our recent advances in the field, Chem. Rec. 3 (2003) 133–143.

[181] Countercurrent Chromatography Portal, <http://www.counter-current.org>.

[182] K.A. Alvi, Screening natural products: bioassay-directed isolation of active components by dual-mode CCC, J. Liquid Chromatogr. Relat. Technol. 24 (2001) 1765–1773.

[183] Y. Ito, Golden rules and pitfalls in selecting optimum conditions for high-speed countercurrent chromatography, J. Chromatogr. A 1065 (2005) 145–168.

[184] F.R. Stermitz, P. Lorenz, J.N. Tawara, L.A. Zenewicz, K. Lewis, Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5 V-methoxyhydnocarpin, a multidrug pump inhibitor, Proc. Natl. Acad. Sci. USA 97 (2000) 1433–1437.

[185] T. Inui, R. Case, E. Chou, D. Soejarto, H. Fong, S. Franzblau, et al, CCC in the phytochemical analysis of anti-TB ethnobotanicals, J. Liquid Chromatogr. Relat. Technol. 28 (2005) 2017–2028.

[186] Y.C. Chin, H.H. Anderson, G. Alderton, J. Lewis, Antituberculous activity and toxicity of lupulon for the mouse, Proc. Soc. Exp. Biol. Med. 70 (1949) 158–162.

[187] B.Z. Ahn, S.I. Kim, Y.H. Lee, Acetylpanaxydol and panaxydol chlorohydrin, two new polyynes from Korean ginseng with cytotoxic activity against L1210 cells, Arch. Pharm. 322 (1989) 223–226.

[188] J.B. Hudson, E.A. Graham, R. Rossi, A. Carpita, D. Neri, G.H.N. Towers, Biological activities of terthiophenes and polyynes from the Asteraceae, Planta Med. 59 (1993) 447–450.

[189] A. Ito, B. Cui, D. Chavez, H.B. Chai, Y.G. Shin, K. Kawanishi, et al, Cytotoxic polyacetylenes from the twigs of Ochanostachys amentacea, J. Nat. Prod. 64 (2001) 246–248.

[190] S.I. Kim, K.S. Kang, H. Kim, B.Z. Ahn, Panaxyne, a new cytotoxic polyyne from Panax ginseng root against L1210 cell, Saengyak. Hakhoechi. 20 (1989) 71–75.

[191] S.I. Kim, K.S. Kang, Y.H. Lee, Panaxyne epoxide, a new cytotoxic polyyne from Panax ginseng root against L1210 cells, Arch. Pharmacal Res. 12 (1989) 48–51.

[192] N.B. Perry, E.M. Span, C. Zidorn, Aciphyllal-a C34-polyacetylene from Aciphylla scott-thomsonii (Apiaceae), Tetrahedron Lett. 42 (2001) 4325–4328.

[193] R. Mahajan, Bedaquiline: first FDA-approved tuberculosis drug in 40 years, Int. J. Appl. Basic Med. Res. 3 (2013) 1–2.

[194] I.M. Orme, Vaccine development for tuberculosis: current progress, Drugs 73 (2013) 1015–1024.