Fumitremorgins and Relatives – from Tremorgenic Compounds to Valuable Anti-Cancer Drugs

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Abstract

Background: Fumitremorgins are mycotoxins but can also inhibit cancer cells and reverse their drug resistance.

Objective: The bioactivity of prenylated cyclo-Trp-Pro dipeptides and their derivatives concerning their application in anti-cancer therapies will be discussed.

Method: Reports on the discovery and assessment of this class of fungal compounds are compiled from literature using Google Scholar and PubMed. The bioactivities of the natural compounds are discussed with the aim of their improvement for cancer therapy.

Results: Although a number of compounds of this class has been found only a minority of them showed bioactivity in the applied bioassays. Fumitremorgins and related compounds are active against various cancer cells but they are also mycotoxins. Some of these natural compounds can arrest cancer cells in their cell cycle and some can block ABC-transporters and reverse resistance in chemotherapy. Structure activity relationships have been deduced leading to the prediction of highly active compounds. Several easily accessible derivatives of these natural products have been discovered being highly selective and non-toxic.

Conclusion: Sophisticated screening methods, high throughput screening, metabolic engineering, and synthetic biology are novel and promising technologies for the search for highly active drugs. Rapid gene sequencing in combination with engineered biosynthetic pathways should contribute substantially to novel pharmaceutics.

Keywords: diketopiperazines, cyclic dipeptides, fumitremorgin, cyclotryprostatin, norgeamide, anti-cancer, drug resistance, ABCG2 transporter

INTRODUCTION

Cyclic peptides usually have superior activities compared to their linear analogues because of their enhanced stability and conformational rigidity, factors improving their interaction with their cellular targets. Cyclic dipeptides, the smallest possible peptides, contain a 2,5-diketopiperazine formed by double condensation of two α-amino acids. Cyclic dipeptides are produced by many organisms for a multitude of functions [1, 2]. Often these diketopiperazine derivatives are further derivatized [3] and especially fungi have developed several pathways for prenylated and rearranged cyclic dipeptides resulting in a treasure trove of natural products [4]. The resulting compounds have many functions in the ecology of the producing organisms ranging from antibiotic [5], antiviral [6], antiprotozoal [7] activities to specific enzyme inhibitors [8]. The broad functionality of cyclic dipeptides led to the discovery of a large spectrum of potential medical applications and many secondary metabolites of this class have been demonstrated to have activities as antitumor [9], antihyperglycemic [10] or antiprion compounds [11] as well as inhibitors of opiod [12], GABAergic [13] or oxytocin receptors [14], only to name a few.

However, these compounds have been optimized for the special requirements of the producing organisms and not for applications in humans. This often results in severe side effects, a small therapeutic window or problems in the pharmacokinetics of the natural products. To take advantage of the often rather sophisticated mode of action of the natural product on the one hand and to optimize the compound for the special needs of drug delivery, to improve production of the compound and to suppress undesirable side effects on the other hand, chemical modifications are usually required. The concept of Diversity Oriented Synthesis (DOS) uses natural compounds as lead compounds already optimized over aeons by nature and generate substance libraries from these compounds [15]. Compounds from these libraries are then screened and further optimized according to the specific functions of the intended drugs.

This review deals with natural compounds based on prenylated cyclo-tryptophyl-proline I which are further rearranged, cyclised and oxidized leading to a multitude of products with complex stereochemistry
Fungi produce a large diversity of metabolites based on cyclo-tryptophyl-proline

The first compound of this class of fungal metabolites was isolated as a mycotoxin causing tremor in mammals [17]. The progressively deepening studies of fungal metabolites mainly from Penicillum and Aspergillus species revealed a complex diversity of secondary products based on cyclo-(Trp-Pro) 1. Starting from this cyclic dipeptide prenylation both at the benzene- and at the pyrrole-ring lead to several tryprostatins and brevianamides. Prenylation at C-2 adjacent to the nitrogen of the indol-ring can attach the prenyl-group in a regular way, producing tryprostatins or in a reversed order, giving various brevianamides. These irregularly prenylated brevianamides are then intermediates for an intra-molecular hetero-Diels-Alder reaction producing a large diversity of metabolites possessing the bicyclo[2,2,2]diazaoctane moiety. Determined by the relative position of the proton at the bridgehead of the bicyclic core in relation to the bridging secondary lactam most metabolites possess the syn-configuration. The rare anti-configuration is realized in the brevianamides, taichunamides and versicolamides. Different transition states during the hetero-Diels-Alder reaction result in syn- or anti-arrangements of the products; a process controlled by the involved [4+2] cycloadditionases [19] (Figure 1).

Fumitremorgins and related compounds got renewed awareness when in 1995 members of this compound class were shown to inhibit cell cycle progression of cancer cells at the G2/M phase. The mechanism of action is inhibition of the process of mitotubule assembly. Furthermore, in 1998 it was found that fumitremorgin C 32 was able to reverse multidrug resistance in breast cancer cells. Multidrug resistance is a growing problem both in treating microbes with antibiotics but also in chemotherapy of tumors. One mechanism of resistance is the upregulation of ATP-binding cassette (ABC) transporters, actively pumping the drug out of the cells [18]. In mammalian cells several ABC-transporters are known fulfilling various functions in healthy organisms, e.g. effluxing drugs at the blood–brain, blood-testis, and maternal-fetal barriers [19]. It is therefore important to block specifically ABC-transporters in cancer cells so that they become again sensitive to cytostatica. In breast cancer cells this specific ABC-transporter is the Breast Cancer Resistance Protein BCRP (ABCG2) which is specifically blocked by fumitremorgins [21]. Because of their ability to block selectively BCRP tryprostatins and fumitremorgin C became lead compounds for the development of improved and non-toxic BCRP-blockers.

The basis - prenylated cyclo-tryptophyl-prolines

A series of secondary metabolites from fungi, named brevianamides, range from brevianamide A 2 to brevianamide W 19 (Figure 2). Into the same group aspergilazaine A 20 can be placed. They are prenylated cyclo-tryptophyl-proline derivatives and brevianamide F is cyclo-(L-Trp-L-Pro) 1, the basis of all these metabolites. From the biosynthetic point of view brevianamides are of heterogeneous origin as brevianamides L-N do not even have the cyclo-(Trp-Pro) skeleton and, therefore, are not discussed here. The first brevianamides, brevianamide A 2, B 3 and E 11 from Penicillum brevicompactum, were reported by Birch and Wright [22] and later also found in several other Penicillum species [23,24]. Brevianamide C and D proofed to be artefacts of isolation. Brevianamide E 11 was one of the metabolites of P. brevicompactum [25] and deoxybrevianamide E 5 was characterized from Aspergillus usus [26]. However, not only fungi but also bacteria produce this type of secondary metabolites as brevianamide F 1 was isolated from a Streptomyces species [27,28]. Another marine-derived Streptomyces strain produced naseseazine A, and B but no biological activity could be found for any of them [29]. A. versicolor is a rich producer of brevianamides yielding brevianamide J 10 and K 12 (brevianamides L-N and O-P have diverging skeletons and are not discussed here) [30]. brevianamides Q 14 and R 15 [31] and brevianamides V 18 and W 19 [32] together with the rare 9ξ-O-2(2,3-dimethylbut-3-enyl)-derivative of brevianamide Q [33]. A marine isolate of A. taichungensis produced the dimeric diketopiperazine aspergilazaine A 20 [34] (Figure 2). From a strain of A. fumigatus tryprostatin A 2, tryprostatin B 3, closely related to brevianamides, and demethoxyfumitremorgin C 31 were isolated [35] as was 6-hydroxy-tryprostatin B 4 from another marine strain of the same species. From A. fumigatus, isolated from the North Sea, the highly active norgeamides A-D 21 - 24 were identified [36,37].

Most brevianamides did not have any biological activity in the various assays. Brevianamide S 13 was weakly active against Mycobacterium brevis BCG [38] and aspergilazaine A 20 showed weak activity against influenza virus H1N1. Only the prenylated cyclo-(L-Trp-L-Pro) derivatives tryprostatin A 2, 18-oxo-tryprostatin A 7, tryprostatin B 3, and N-prenyl-brevianamide F 6 were active against cancer cells lines. But tryprostatin A 2 does not only arrest the cell-cycle at the G2/M phase; it also disrupts the microtubule spindle leading to a specific inhibition at the M phase [39]. Even more interesting is its ability to block the ABC transporter BCRP, a major factor of drug resistance in breast cancer [40]. From the highly active norgeamides, norgeamide A 21 seems
to be the most active one against several carcinoma cell lines but not much has been published about these remarkable metabolites. Anti-cancer activity of the brevianamides seems to be focused on simple prenylated cyclo-(L-Trp-L-Pro) derivatives and coupled to other tightly defined parameters as mere hydroxylation of tryprostatin B 3 at C-6 abolishes bioactivity.

Formation of a central heterocyclic ring: cyclotryprostatins, fumitremorgins and verruculogenes

In 1972 verruculogen 38 was isolated from a strain of *P. verruculosum* derived from rotten peanuts which caused severe tremors when administered to mice [49]. The same mycotoxin and fumitremorgin B 40 were obtained from *A. caespitosus* [50] and *A. fumigatus* [51]; they proved to be genotoxic [49]. While searching for mycotoxins as cause for the outbreak of ryegrass staggars these two mycotoxins were also found in the culture of *P. piscarium* and other *Penicillium* species isolated from ryegrass [52]. From moulded maize silage *A. fumigatus* isolates produced fumitremorgin C 32 [66], verruculogen 38, epoxyfumitremorgin C 35 and TR-2 43 [47,53] while isolates of the same species from rice contained fumitremorgin A 36 and B 40 [45]. 12,13-Dihydroxy-fumitremorgin C 34 was first isolated from *A. fumigatus* [54] and later from a *Pseudoallescheria* sp. It has antibacterial activity [55] and is cytotoxic against HCT-116 tumour cells [56]. 13-oxoverruculogen 39 showed moderate activities against several cancer cells lines. Again from *A. fumigatus* cyclotryprostatins A 25, B 26, C 28 and D 29 were characterized and found to block the cell cycle at the G2/M phase [57]. In a marine-derived strain of the same species prenylcyclotryprostatin B 27, 20-hydroxycyclotryprostatin B = cyclotryprostatin E 30, 9-hydroxyfumitremorgin C 33, and 6-hydroxytryprostatin B 4 were discovered but they had no antiproliferative effects [58] (Figure 3).

Fumitremorgin C 32 gained much interest when it was found that it can reverse the resistance of cancer cells against cytotoxins. While fumitremorgin C alone was not toxic in concentrations below 80 μM to S1-M1 cells, in combination with mitoxantrone, 50% of the cells were killed at 0.35 μM. Fumitremorgins A 36 and B 40 were more toxic than fumitremorgin C killing 20% of the cells at 15 μM instead of 80 μM and were 20- and 14-times less active than fumitremorgin C in combination with mitoxantrone [59]. Only later it was discovered that fumitremorgins bind to ABCG2 and block the active export of the cytotoxins out of the cancer cells [60]. Like tryprostatin A 44 and B 48, fumitremorgin C 32 and demethoxyfumitremorgin C 31 arrest the mammalian cell cycle at the G2/M-phase [61].

Reviewing biological activities of this group of cyclo-(Trp-Pro) derivatives it is striking that the majority of cytotoxic products can be found in the cyclotryprostatin class. Furthermore, it also harbours one of the few inhibitors of BCRP known in this group of natural products. Although they are neurotoxins these compounds are not active against the human large cell lung carcinoma cell line NCI-H460 [62].

Rearrangement of tryprostatins leading to spiroyprostatins

Epoxidation of tryprostatin A 2 or B 3 in *A. fumigatus* leads to a rearrangement of substituents at the indolyl moiety resulting in the formation of spiroyprostatins. Spiroyprostatin A 44 and spiroyprostatin B 48 are the only spiroyprostatins showing considerable bioactivity and arresting the mammalian cell at the G2/M-phase [58,59]. A holothurian-derived *Aspergillus fumigatus* strain proved to be a rich source of cyclo-(L-Trp-L-Pro)-derivatives and coupled to other tightly defined parameters as mere hydroxylation of tryprostatin B 3 at C-6 abolishes bioactivity.

Carneamides, versicamides and austamides – tetrahydroazepine bearing natural compounds

Secondary metabolites of the carneamides and versicamides series are characterized by cyclo-(L-Trp-L-Pro) carrying two prenyl groups at the indolyl moiety with the one adjacent to the indolyl nitrogen attached in the reversed order. In most of these compounds the reversed prenyl moiety is cyclized yielding an additional tetrahydroazepine ring. Carneamides A-C 52 - 54 were obtained from *A. carneus* and did not have cytotoxic activities [66]. Dihydrocarneamide A 55 was isolated together with iso-notoamide B 84 from *Paecilomyces variotii* and both showed weak cytotoxic activities against the human large cell lung carcinoma cell line NCI-H460 [67]. From *A. versicolor* came versicamides A-H 56 - 63, but only versicamide H 63 exhibited moderate activities against HeLa and HCT-116 cancer cell lines [68]. Another *Aspergillus, A. ustus*, produced austamides 64 [69] which causes toxicosis in ducklings but has no cytotoxic activity. A marine *Penicillium* sp. gave deoxyisocarneamide 65, deoxydihydroisocarneamide 66 and 16β-hydroxy-17β-methoxy-deoxydihydroisocarneamide 67 but none of them possessed cytotoxic activities [70] (Figure 5). It seems that the formation of a 7-membered
ring instead of a 6-membered ring abates bioactivity. This is probably not only due to the different configuration caused by the tetrahydroazepine ring but also by the lack of the (2-methyl-1-propenyl)-side chain.

**Intramolecular hetero-Diels-Alder reactions lead to syn- and anti-bicyclo[2,2,2]diazaoctanes**

Notoamides are a series of secondary metabolites with a reversed prenyl group at the pyrol part. They have been named after Noto Peninsula, Japan, where the first notoamide producer has been collected. Notoamide A 80, B 81, C 73 and D 77 [90], notoamide E 69 [71], notoamide E2-E4 70 - 72 [2], notoamides F 85, G 86, H 82, I 87, J 76 and K 78 [72], notoamides L 89, M 74 and N 83 [73], and notamides O 90, P 79, Q 75 and R 88 [74] were all obtained from marine *Aspergillus* species. Notoamide S 68 [76, 77], notoamide T 91 [78], notoamide U 92 [79] and notoamide V 94 came from terrestrial *Aspergillus* species. These compounds vary from simple prenylated cyclo-(Trp-Pro) derivatives, e.g. notoamides C 73, E 69, J 76, S 68, over cyclized notoamides, e.g. notamides D 77, F 85, T 91, U 92, to rearranged compounds, e.g. notoamides A 80, B 81, L 89, O 90 (Figure 6). Of the tested notoamides only notoamides A 80, B 81, C 73, D 77 and I 87 showed cytotoxic activities against cancer cell lines.

Of interest are compounds harboring the bicyclo[2,2,2]diazaoctane unit because they are probably formed via an intramolecular hetero-Diels-Alder reaction. Stephacidins, sclerotiamide and notoamides possess a syn-configuration, while brevianamides and versicolamides possess the relative anti-configuration. The common syn-configuration and the rarer anti-configuration are determined by the relative position of the C–H proton at the bridgehead of the bicyclo[2,2,2]diazaoctane moiety in relation to the bridging secondary lactam. Stephacidin A 96 and B 98 have been isolated from *A. ochraceus*. They exhibit cytotoxicity against several human tumor cell lines and were most active against prostate testosterone-dependent LNCaP cells [80]. Interestingly, *A. ochraceus* and *A. protuberus* produced (+)-stephacidin A and (-)-notoamide B but *A. amoenaens* synthesized their enantiomers and both species produce (+)-versicolamide B 99, the 6-epimer of (-)-notoamide B [81], a unique trait in this group of compounds. From *A. ostianus* 21-hydroxy-stephacidin 97 was characterized but no bioactivity was reported [82]. The closely related aspergamides A 101 and B 102 [83, 84] can be regarded as intermediates in the formation of stephacidin A 96. Avrainvillamide 103 from *A. ochraceus* with syn-configuration at the bicyclo[2,2,2]diazaoctane core is an antibiotic against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Enterococcus faecalis*. It possesses cytotoxic activities against human colon HCT116, melanoma MALME-3 M, and *B. subtilis* and *T. atrovirens* breast cancer cells [85]. Syn-configuration was also determined for sclerotiamide 104 from *A. sclerotiorum* which causes signiﬁcant mortality in the corn earworm *Helicoverpa zea* [86, 87] (Figure 7).

Only relatively few of these bicyclo[2,2,2]diazaoctane derivatives possess the anti-configuration which is found in taichunamides, versicolamides, epi-stephacidin A, 6-epi-avrainvillamide but also in some notoamides. A series of these metabolites, named taichunamides A - E 105 – 109, F 93 and G 95 together with versicolamide B 99 and C 100, (-)-stephacidin A, (+)-6-epi-stephacidin A, 6-epi-avrainvillamide and notoamides U 92 and V 94 were isolated from the marine *A. taichungensis*. Despite the fascinating implications for the biosynthesis of the metabolites these compounds revealed little bioactivity. Only taichunamide F 93 and 6-epi-avrainvillamide inhibited at a concentration of 10 µM the chymotrypsin-like activity of the proteasome by 81 and 95%, respectively [88].

**Optimization of bioactive natural products**

The structures of these natural compounds have been optimized by the producing fungi for other reasons than for fighting cancer or blocking the ABCG2 transporter. This resulted in suboptimal function of the active metabolites in medical applications. To the main obstacles belong the limited production of the metabolites and the toxicity of several of them. One should remember here that fumitremorgins have been discovered for their ability to cause tremors in mammals and not for the destruction of cancer cells. As a consequence, several attempts have been made to produce less complex, cheaper, and less toxic compounds for clinical evaluations.

An attempt to produce cytotoxic compounds from spirotryprostatin derivatives failed because none of the spiro compounds containing the lactam 110 showed any activity. Only compounds with the lactam opened to the ethyl ester 111 had some activities against the human colon carcinoma MCF-7 cell line with an increase of activity towards methyl- and bromo-derivative [89]. Substitutions of the secondary amide of brevianamide F 1 led to compounds 112 and 113 with improved cytotoxicity against a number of cancer cell lines but with no ability to inhibit the cell cycle (Figure 8). Replacement of both the dimethyl vinyl group by 3,4,5-trimethoxyphenyl and proline by glycine in demethoxyfumitremorgin C 31 gave compound 114 with higher cytotoxicity (GI90, concentration required to inhibit the growth by 50%, 5.9 µM), the potential to inhibit the cell cycle and low toxicity (>400 mg kg⁻¹ in mice) [90]. Tryprostatin A and B having LL-configuration and are moderate inhibitors of tubulin polymerization. Their synthesized unnatural D,L-, D,D- and L,D-diastereomers lose this function completely. However, when tested in the NCI panel of 60 cancer cell lines tryprostatin A displayed inhibition of two cell lines with GI50 of 10 µM or less but D,L-tryprostatin A was in most cases more active. L,D-tryprostatin B was the most active isomer showing GI50 of 10 µM or less for 9 cell lines. All diastereomers were not general cytotoxins but possessed high selectivity across the individual cell lines [91]. The synthetic derivative 115 of
Overexpressing human ovarian xenograft T8 tumours. Administration of a single dose of 25 mg kg⁻¹ in the development of novel compounds. To this aim the pharmacokinetics, the structure activity relations between inhibitors and ABCG2. This should be used for a more rational approach to finding the pharmacophore of human ABCG2 inhibitors.

An exceptional characteristic of several fumitremorgins is their ability to block specifically the ABCG2 transporter [96]. In humans the ABC transporters can be divided into seven distinct subfamilies. They all act as exporters using energy from ATP hydrolysis and are involved in the export of metabolic products, vitamins, sterols but also in pumping xenobiotics and other toxins out of the cells. The human ABCG2 is organized in homo-oligomers and the major oligomeric unit of human ABCG2 in plasma membranes is a homo-dodecamer with three homo-tetramer units [97]. Human ABCG2 is normally expressed in several tissues, e.g. placenta, brain, prostate, intestine, testis, ovary, and liver [98]. ABCG2 was originally cloned from an Adriamycin-resistant breast cancer cell line and its over-expression has been connected with the resistance of cancer cells against cytostatics. Several clinical studies demonstrated the correlation between ABCG2 expression and prognosis but there were also reports where such a correlations could not be confirmed [99]. Despite this still somewhat unclear situation ABCG2 became a target for inhibitors resensitizing cancer cells for chemotherapy [100]. The selection of ABCG2 for inhibition was also fostered by the fact that in mice its knockout has no adverse effect suggesting that its physiological function is in hematopoietic stem cells to protect them from cytotoxic substrates [101].

Novel bridged brevianamide F 1 analogues have been synthesized in a search for novel ABCG2 inhibitors and evaluated for their bioactivities. While none of these products had antimicrobial activities and did not impair microtubule assembly, compounds 118 and 119 possessed moderate inhibition of ABCG2. From the comparison with inactive derivatives it was concluded that a bulky side chain was required for activity [102]. Desmethoxyfumitremorgin C analogues have been synthesized and tested for their ability to block ABCG2. The derivative 120 lacking only the side chain was moderately active but derivatives of this compound with primary α-amino acids, replacing proline were mostly inactive. Only compounds 121 - 124 showed weak activities when added in 1 μM concentration in combination with doxorubicin against resistant MES-SA/Dx5 cells [103]. The results of this study are somewhat difficult to judge because no positive control has been included for comparison. A similar study has been reported by van Loevezijn et al. but here the side chain of desmethoxyfumitremorgin C has also been varied. The resulting combinatorial library of 42 compounds has then been assessed for the potential to block ABCG2. The majority of compounds were more active than demethoxyfumitremorgin C and the most effective compounds were those with cyclohexyl, n-butyl or iso-butyl at C-3 and a linear lipophilic side chain at C-6. Because the tested compounds were all diastereomeric mixtures the most active compounds were separated into pure compounds yielding ko132 125CD and ko134 125CF as the most active drugs [104]. A derivative of ko134 with a methoxy function at the aromatic ring led to the even more active fumitremorgin C derivative ko143 126 [105]. Compounds ko132 and ko134 where 2-3 times more efficient than fumitremorgin C and reached activities similar to that of the broad inhibitor of ABC-transporters GF120918. The methoxy-derivative of ko134, ko143, was 4 times more efficient than ko134 with a therapeutic ration of about 1000 (activity versus toxicity). Ko143 has been tested in mice where it was nontoxic and inhibited intestinal ABCG2 when given orally [106] and it enhanced the efficacy of photodynamic therapy in colon cancer cells [107].

Although with ko143 a very active and highly specific compounds has been discovered it has the disadvantage of an ester moiety which is prone for hydrolysis leading to inactivation. For its demethoxy-derivative ko134 instability in rat plasma has been reported [108]. To overcome this disadvantage several demethoxyfumitremorgin C derivatives with saturated side chain and opened proline ring have been synthesized and tested for their ability to inhibit the ABC-transporters ABCG2, ABCB1 and ABCC1. It could be demonstrated that the diketopiperazine ring is essential for activity and that there were rather rigid requirements for the stereochemistry as only (3S,6S,12aS)-compounds showed any inhibition of ABCG2 while their (3S,6R,12aS)-isomers were completely inactive. However, this was only true in the case of ABCG2 while the transporters ABCB1 and ABCC1 did not have such diastereomeric requirements. Derivatives 127 and 128 possessed excellent activity towards ABCG2 inhibition but the selectivity was worse than that of ko134; derivatives 129, 130 and 131 had good selectivity but were slightly less active. Compound 133 had the highest selectivity for ABCG2 and was here 19- resp. 62-fold more active than against ABCC1 or ABCB1. At 1 μM compound 133 reversed ABCG2-mediated resistance in P388/BCRP cells and had low cytotoxicity reaching the same potential as ko134 125CF [109]. Such a tight control of inhibition of ABCG2 by the chirality of active drugs is surprising considering the reported broad substrate specificities of ABC transporters [107] (Figure 8).

Finding the pharmacophore of human ABCG2 inhibitors

All these studies obtained from natural products and their derivatives brought invaluable information on the structure activity relations between inhibitors and ABCG2. This should be used for a more rational approach in the development of novel compounds. To this aim the pharmacokinetics and tissue distribution of fumitremorgin C 32 was assessed after intravenous application to female SCID mice bearing the ABCG2-overexpressing human ovarian xenograft T8 tumours. Administration of a single dose of 25 mg kg⁻¹ had no toxic
side effects. Fumitremorgin C was widely distributed in all tissues with highest concentrations in lung, followed by liver and kidney; its main elimination was by hepatic metabolism. It had no effect on the expression of ABCG2 in T8 tumours as determined by RT-PCR [108].

To assess the structural space of ABCG2 inhibitors 123 compounds were tested for their effects on mitoxantrone efflux in Saos-2 cells transfected with human wild-type ABCG2. For compounds known to inhibit other ABC transporters a three times higher frequency for ABCG2 inhibitors was observed. When a quantitative structure-activity relationship (QSAR) model with 152 descriptors of molecular structure was tested it turned out that only two parameters, logD_{pH} and molecular polarizability, were sufficient to predict 83% of ABCG2 inhibitors correctly. Both descriptors are highly correlated to the passive membrane permeability. Lipophilicity is an important parameter for membrane partitioning and an attempt to study a lipophilicity-independent subset of the compounds revealed, in addition to lipophilicity, descriptors related to π-electron energies and the abundance of nitrogen atoms as being important for the discrimination of inhibitors from non-inhibitors. This indicates that hydrogen bonds and interactions involving π-electron systems are involved in inhibitor binding to ABCG2 [109]. In a study it has been demonstrated how important it is to assess compounds under standardized conditions for the generation of data further used in QSAR models. Here, an attempt has been made to combine different SAR data into one model but the study came to the conclusion that this was not possible due to the heterogeneity of conditions employed [110].

Beside these attempts to establish QSAR models alternatives have been presented for the identification of novel ABCG2 inhibitors. Zhang et al. exploited the fact that luciferin is also a substrate for ABCG2. They engineered HEK293 cells to express ABCG2 and luciferase which uses luciferin causing bioluminescence. Such cells can be applied in a high-throughput screen for ABCG2 inhibitors. The readout is an increased bioluminescence caused by the blockage of luciferin export by the ABCG2 inhibitor. Using this system the authors screened a drug library that includes drugs approved by the Food and Drug Administration (FDA) and drug candidates from phase II clinical trials. By finding, e.g. that silver nitrate is also an ABCG2 inhibitor, the authors also demonstrated nicely in their study that such an approach is a valuable screening method which, however, needs further verification by established in vitro methods and additional assessment of both the toxicity of the compounds and their specificity [111].

An alternative to the screening of substance libraries is the combination of enzymes from different microorganisms and different pathways with the aim of generating novel compounds [112]. Entering the area of synthetic biology Wunsch et al. coexpressed the non-ribosomal peptide synthetase ftmPS from Neosartorya fischeri with three cyclic dipeptide reverse prenyltransferase from different biosynthetic gene clusters in Aspergillus nidulans. As expected expression of ftmPS yielded brevianamide F1, coexpression of ftmPS with cdpC2PT for the reverse C2-prenyltransferase from N. fischeri led to the corresponding prenylated compounds deoxybrevianamide E5. When ftmPS was coexpressed with cdpNPT, the reverse C3-prenyltransferase from A. fumigatus, reverse prenylation took place at C-3 with a concurrent ring closure between C-2 and the diketopiperazine. Interestingly, coexpression of ftmPS with cdpC3PT for the reverse C3-prenyltransferase from N. fischeri yielded the regularly prenylated N1-derivative together with the C2 and C3-prenylation products [113]. This approach has been developed further using different tryptophan derivatives and various prenyltransferases from fungi [114]. Such a combination of genes has its limitation in the compatibility of new pathways and the toxicity of novel compounds to the producing cells. This limitation can be overcome by in vitro systems using enzymes spotted on microarrays. The miniaturization of such devices allows the application of several substrates in combination with several enzymes. The resulting considerable large substance libraries can then be screened for any given bioactivity which has then to be confirmed by synthesis on a larger scale followed by isolation and characterization of the active product [115, 116].

Although these techniques are excellent tools for generating, screening and assessing novel natural products and their derivative they bear a number of pitfalls. For example, a 50-fold higher activity was observed for chemically synthesized tryprostatin B3 compared to the biosynthesized one. A search for an explanation for this discrepancy revealed that tryprostatin in the solvent dimethyl sulfoxide is slowly oxidized to a mixture of products when exposed to air. Obviously, the increased cytotoxicity was caused not by tryprostatin B but by its unknown byproducts [117]. Furthermore, all this techniques are using in vitro assays giving results which have to be validated in animal models. But even before going into in vivo experiments one has always to be aware that these are models which are not identical to humans. This is also the case for Berp1/Abcg2 of the mouse which differs slightly from it human orthologue BCRP/ABCG2. Comparing two different cell lines from mice and from humans it could be shown that fumitremorgin C32 more potent inhibited the human transporter, resulting in a significantly lower IC_{50} value compared to the murine analogue. Cytotoxicity of fumitremorgin C in human cells was also found to be much lower than in mouse cells, indicating an overall higher sensitivity of human ABCG2 compared to murine Abcg2 [118].

CONCLUSION
When fumitremorgin C 32 was found it was first seen only as a toxin and later as anti-cancer agent. Further studies revealed it to be a member of a still growing class of cyclo-tryptophyl-proline derivatives mainly produced by fungi of the genera Aspergillus, Penicillium, and Malbranchea. For most of these natural products no bioactivity could be found but it is highly unlikely that organisms use resources and energy for the production of useless compounds. Therefore, it can be assumed that all of these secondary metabolites are bioactive but we have not identified yet their targets and ecological functions. The most interesting known bioactivities in this class of compounds are directed against cancer cells and cytotoxicity against cancer cell lines, e.g. by norgaemin A 21 and stephacidin A 96, arrest of the cell cycle, e.g. by tryprostatin A 2 and cyclotryprostatin A 25, and inhibition of the ABCG2 transporter, also known as Breast Cancer Resistance Protein (BCRP), e.g. by fumitremorgin C 32 and tryprostatin A 2, have been reported. The challenge was to turn mycotoxins into valuable anticancer drugs. This is a very complex process requiring among other things novel derivatives and their characterizations, improved detailed knowledge of the target, the establishment of structure-activity relations and the assessment of the pharmacokinetics of the most active compounds in animal models. The results are derivatives of fumitremorgin C, like ko143 126, which are easier and cheaper to produce, lack the neutrotoxicity of fumitremorgin C, are about 10 times more active and have a much larger therapeutic ratio due to their low toxicity. Still, this is only the beginning and more detailed animal studies have to demonstrate the value of these compounds in the treatment of tumours.

The synthetic derivative of fumitremorgin C, ko143 126, is not the only ABCG2 inhibitor identified so far but, although a number of inhibitors of ABC transporters are known, only few of them are specific for ABCG2 [130]. One of them is compound 134 with a quinazoline core possess sensitivity and selectivity similar to ko143 towards ABCG2 [132]. The structurally unrelated benzothiazole-triazine derivative PZ-39 135 has also been found to be a non-toxic and specific ABCG2 inhibitor which also accelerates ABCG2 degradation [121] as has been shown for novobiocin [122] or the flavon 6-prenylchrysin [123]. Only recently the chromosome derivative 136 has been reported as specific ABCG2 inhibitor with low toxicity [124] (Figure 9). A number of ABCG2 inhibitors have been described in patents but the available pharmacological data are too preliminary to predict the outcome of in vivo studies [122].

When treating tumours one has to keep in mind that cancer cells have high mutation rates. One consequence are mutations also in ABC-transporters. From ABCG2 often mutations have been found at position 482 where arginine has been exchanged with glycine or threonine (R482G and R482T), affecting the substrate specificity of the protein. This leads to resistance to a variety of chemotherapeutic agents. The mutations not only cause changes in the specificity towards the cytotoxins but also towards inhibitors of ABCG2. Interestingly, the activity of fumitremorgin C does not change between the wild type and the mutant protein [128].

OUTLOOK

Considerable progress has been made in the design of potential drugs inhibiting specifically ABCG2 transporter, one of the main mechanisms for tumour resistance. With the discovery of ko143 126 and related compounds it could be shown that a mycotoxin causing tremors in mammals can be improved to a non-toxic and specific inhibitor. The studies leading to these compounds, however, also demonstrated that there still exists a large reservoir of related and probably even more active compounds which remains to be characterized [127]. Combining natural compounds with combinatorial chemistry will be a powerful tool for their discovery. Here, not only novel chemical syntheses but also combinations of biochemistry and classical organic chemistry can lead to major novel findings [128]. X-ray studies revealed conformational changes of ABCG2 when binding to the antineoplastic agent mitoxantrone [129] but only very recently the first three-dimensional structure of human ABCG2 became available [130]. This structure and its refinement will hopefully lead to a deeper understanding of the interaction of inhibitors with ABCG2 and the effect of mutations on the substrate specificity of the protein. This in turn will then allow considerably improved SAR and QSAR models for ABCG2 inhibitors.

Finding ABCG2 inhibitors is only the first step on a long way to clinical applications. Before any clinical trials ADMET (absorption, distribution, metabolism, and excretion - toxicity in pharmacokinetics) of the potential drug is needed. The rapidly progressing chip technology led to the realization of a miniaturized 3D cell-culture array for high-throughput toxicity screening of drug candidates. Here, human cells are encapsulated in alginate gels positioned on a functionalized glass slide for screening against various compounds in combination with another array carrying a mixture of three P450-oxidases designed to emulate the human liver. In this combination both the activity of the compounds and the toxicity of their metabolites can be determined [131]. As already stated above in the section on SAR, standardized test protocols are needed to allow the comparison of the individual bioactive compounds.

The preclinical characterization also comprises in vivo studies in several animal models. Only very few ABCG2 inhibitors have yet been tested in animal models but the few results reported seem to support the notion that blocking the efflux pumps in cancer cells improves the effect of cytostatica in tumours [132]. This contrasts with the relatively large number of failed clinical trials for inhibitors of other ABC transporters. More research
into the problem of the specific functions of ABC transporters in the mammalian organism is needed to answer
the question how specific an inhibitor of an ABC transporter has to be for application in cancer treatments.

But not only the characterization and testing of novel ABCG2 inhibitors has to be done, for their application
the classification of tumours in patients has also to be improved further. As cancer is a highly complex disease
personalized medicine involving the still rapidly evolving sequencing technologies of nucleic acids and other
analytic approaches will lead to a finer grouping of the individual patients. This in turn will hopefully reduce the
number of failures in clinical studies \cite{133} and finally lead to the application of ABCG2 inhibitors in cancer
therapy.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.
Figure 1: Biosynthetic pathways leading from brevianamide F 1 to various cyclic and rearranged metabolites. Intramolecular hetero-Diels-Alder reactions lead from desoxybrevianamide E 5 or its postulated rearrangement product to stephacidin A or brevianamide. The selectivity of the catalyzing [4+2]-cycloadditionase decides whether an exo- or endo-transition state is involved leading either to stephacidin 96 or (-)-6-epi-stephacidin or to brevianamide A 8 or B 9, respectively.
Figure 2: The large but heterogeneous group of brevianamides and the related norgeamides and aspergilazine A.
Figure 3: Cyclotryprostatines and fumitremorgines have an additional central heterocycle.
Figure 4: Epoxidation followed by rearrangements leads to the small group of spirotroprostatins.
Figure 5: Cyclization of the reverse hemiterpene moiety leads to the class of versicamides with an additional tetrahydroazepine ring.
Figure 6: The large but structurally heterogeneous group of notoamides. The individual notoamides have been structurally grouped here which differs to the naming of notoamides resulting historically from the sequence of their discovery.
Figure 7: A rare intramolecular hetero-Diels-Alder reaction is the key step to taichunamides, versicolamides, stephacidins and related metabolites.
Figure 8: Highly active and specific cytotoxic compound and ABCG2-inhibitors derived from fumitremorgins and related fungal metabolites.
Figure 9: Examples of some other specific ABCG2 inhibitors not derived from fumitremorgins.

REFERENCES
Aspergillus fumigatus stem of carbon formation reveal biosynthetic pathway crosstalk.


The structures of the norgaamides were published via the Internet detailing the research performed by the Hans-Knöll Institute. www.hki-jena.de.


Transport and resistance in cancer chemotherapy. [Peng, H.; Dong, Z.; Qi, J.; Yang, Y.; Liu, Y.; Li, Z.; Xu, J.; Zhang, J.]

A novel two mode-acting inhibitor of ABCG2-mediated multidrug resistance in cells transfected with the breast cancer resistance protein. [Cancer Res., 2000, 60, 47-50.]


Wauters, I.; Goossens, H.; Delbeke, E.; Maylaert, K.; Roman, B.I.; Van Hecke, K.; Van Speybroeck, V.; Stevens, C.V. Beyond the diketopiperazin family with alternatively bridged brevianamide F analogues. [J. Org. Chem., 2015, 80, 8046-8054.]


