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FULL PAPER

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Target repurposing unravels avermectins and derivatives as novel antibiotics inhibiting energy-coupling factor transporters (ECFTs)

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Abstract

Energy-coupling factor transporters (ECFTs) are membrane-bound ATP-binding cassette (ABC) transporters in prokaryotes that are found in pathogens against which novel antibiotics are urgently needed. To date, just 54 inhibitors of three molecular-structural classes with mostly weak inhibitory activity are known. Target repurposing is a strategy that transfers knowledge gained from a well-studied protein family to under-studied targets of phylogenetic relation. Forty-eight human ABC transporters are known that may harbor structural motifs similar to ECFTs to which particularly multitarget compounds may bind. We assessed 31 multitarget compounds which together target the entire druggable human ABC transporter proteome against ECFTs, of which nine showed inhibitory activity (hit rate 29.0%) and four demonstrated moderate to strong inhibition of an ECFT (IC_{50} values between 4.28 and 50.2 µM) as well as antibacterial activity against ECFT-expressing *Streptococcus pneumoniae*. Here, ivermectin was the most potent candidate (MIC_{95}: 22.8 µM), and analysis of five

Abbreviations: ABA, abamectin; ABC, ATP-binding cassette; AMR, antimicrobial resistance; ATP, adenosine triphosphate; BEN, benzbromarone; D21, 9-deazaspurine 21[1]; DAS, dasatinib; DIP, dipyridamole; DMSO, dimethyl sulfoxide; DOR, doramectin; ECFTs, energy-coupling factor transporters; EMA, emamectin; EPR, eprinomectin; ERL, erlotinib; FolT, folic acid-(vitamin B_9)-specific S-component; FUR, furosemide; GEF, gefitinib; GLI, glibenclamide; IC_{50}, half-maximal inhibitory concentration; IMA, imatinib; IND, indomethacin; IVE, ivermectin; KO, Ko143; LAP, lapatinib; MIC_{50}, half-maximal growth inhibition concentration; MIC_{95}, minimum inhibition concentration; MK, MK-571; MON, montelukast; MOX, moxidectin; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; NEL, nelfinavir; NiaX, niacin-(vitamin B_3)-specific -S-component; NIL, nilotinib; P20, phenothiazine/purine 20[1]; PanT, pantothenic acid-(vitamin B_5)-specific S-component; PRA, pranlukast; PRO, probenecid; Q18, quinoline 18[2]; Q21, quinazoline 21[2]; Q22, quinoline 22[2]; Q26, quinoline 26[2]; RibU, riboflavin-(vitamin B_2)-specific S-component; RIT, ritonavir; SAQ, saquinavir; SAR, structure–activity relationships; SBP, sulphobromophthalein; SEM, standard error of the mean.; SUP, sulfinpyrazone; T14, thienopyrimidine 14[3]; VEP, verapamil; VIN, vinblastine.

Jörg Haupenthal and Muhammad Rafehi contributed equally to this study.

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ivermectin derivatives revealed moxidectin as one of the most potent ECFT-targeting antibacterial agents (IC_{50}: 2.23 µM; MIC_{95}: 2.91 µM). Distinct molecular-structural features of avermectins and derivatives as well as the differential biological response of the hit compounds in general provided first indications with respect to the structure–activity relationships and mode of action, respectively.

KEYWORDS
ABC transporter, avermectin, drug repurposing, ECF transporter, milbemycin

1 | INTRODUCTION

Resistance of virulent pathogens against available antibiotics, also known as "antimicrobial resistance" (AMR), is considered as one of the greatest socioeconomic threats to human mankind.[4] The phenomenon of AMR was already known and described for decades,[5–8] however, worldwide efforts for systematic analyses to understand the global scale of AMR as implemented in the "Global Antimicrobial Resistance and Use Surveillance System" (GLASS) started not before 2015.[4,9] A causal relationship between excessive antibiotic use and AMR could be drawn,[9] and systematic analyses revealed that the death of ~5 million people per year worldwide is associated with resistant pathogens of which >1 million could have survived, if proper antibacterial medication was available.[10] Worst-case estimates predict 10 million deaths per year in 2050, and a staggering 100 trillion USD in economic costs until then.[11] With the increase of antibiotics use and dosages, academic and industrial research need to catch up with AMR by identifying and validating novel potential bacterial drug targets as well as by developing novel antimicrobial agents,[5] for which the World Health Organization (WHO) has made a call in 2017.[12]

Energy-coupling factor transporters (ECFTs) are a group of bacteria-specific transport proteins belonging to the superfamily of ATP-binding cassette (ABC) transporters.[13] At the expense of ATP, these heteromeric membrane proteins import structurally variable B-type vitamins (i.e., thiamine [vitamin B_1], riboflavin [vitamin B_2], niacin [viamin B_3], panthothenic acid [vitamin B_5], pyridoxin [vitamin B_6], biotin [vitamin B_7], folic acid [vitamin B_9], and cobalamin [vitamin B_{12}]; Supporting Information S2: Figure S1),[13,14] but also metal ions (i.e., cobalt and nickel)[13,14] into the cytosol, enabling both bacterial survival and growth. The broad substrate range of ECFTs is achieved by individual membrane-bound high-affinity substrate-binding proteins for each of the above-named intrinsic substrates ("S-components"—unique and distinctive features amongst ABC transporters in general) which are assembled together with two nucleotide-binding domains (NBDs) typical for ABC transporters in general and a membrane-spanning domain (MSD) typical for ECFTs ("T-component") in particular.[14–23] Since many pathogens do not express key proteins for the de novo synthesis of the above-mentioned essential vitamins, ECFTs have been proposed as novel and specific antimicrobial targets for advanced antimicrobial therapy,[14] and subsequent target validation studies provided first-in-field tool compounds.[20]

To date, 82 molecules are known to interact with ECFTs.[15–24] While 28 thiamine derivatives were identified as binders of the corresponding S-component only, the other 54 compounds demonstrated inhibition of ECFT-mediated transport,[15–20] of which six and three showed IC_{50} values lower than 10 and 5 µM, respectively.[17] The structural landscape of these 54 ECFT inhibitors is highly limited, with to date only three distinct structural classes. Figure 1 provides an overview of the currently explored structural landscape of ECFT inhibitors. The reason for the lack of small-molecule ligands of ECFTs may be on the one hand their relatively recent discovery as potential drug targets, but also target-specific peculiarities that hinder the successful targeted development of novel agents. The limitations in both the structural and bioactivity landscapes and the inability of medicinal chemistry efforts to generate highly potent and diverse tool as well as lead compounds despite the knowledge about ECFTs for recent years call for new approaches to discover novel scaffolds, bioactivity ranges, structure–activity relationships (SAR), and modes of modulation of these under-studied bacterial drug targets.

Target repurposing is a strategy to reuse knowledge of human drug targets and transfer this knowledge to nonhuman orthologs as a starting point for drug discovery purposes.[25–28] As ECFTs are ABC transporters, they contain structural features that are conserved within this superfamily of membrane proteins. These features may be the NBDs as the most conserved (and rather specific) parts of ABC transporters across species, but also the (more polyspecific) MSDs that represent the functional backbone of ABC transporters and were identified as the main small-molecule—target interaction sites in various structural studies.[29] Of particular interest are multitarget drugs that span their bioactivity across human ABC transporters, as they could address these conserved features and even under-studied targets, such as ECFTs.[30] Figure 2 provides a list of multitarget pan-ABC transporter modulators that we used within this study to further explore ECFTs.[1–3]

2 | RESULTS AND DISCUSSION

2.1 | Selection of pan-ABC transporter inhibitors

We searched several public databases (i.e., the National Center for Biotechnology Information [NCBI; https://www.ncbi.nlm.nih.gov]; PubChem [https://pubchem.ncbi.nlm.nih.gov], ChEMBL [https://www.

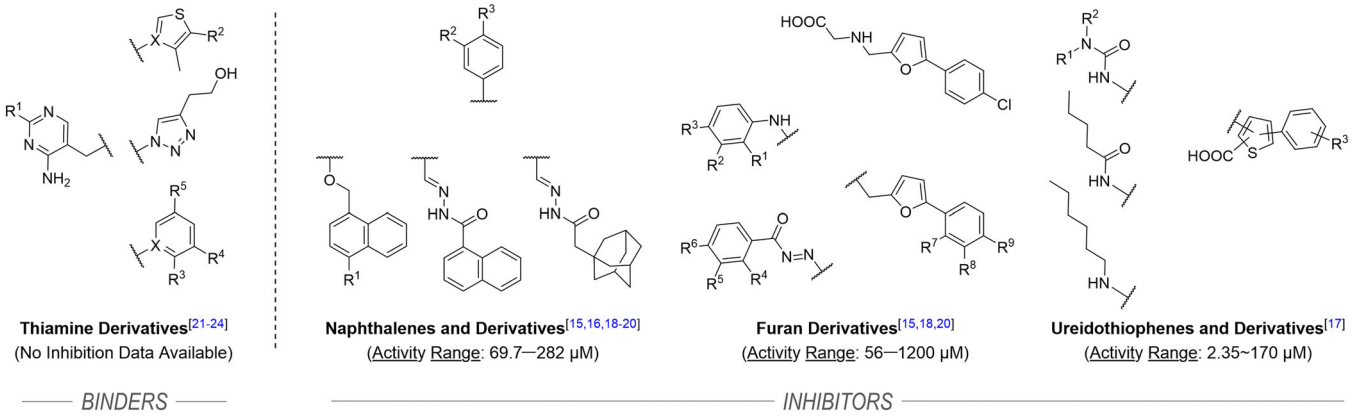

FIGURE 1 Molecular formulae of small-molecule binders and/or inhibitors of energy-coupling factor transporters (ECFTs): 28 thiamine derivatives (binders only, no inhibition data available)[21–24]; as well as six naphthalene,[15,16,18–20] six furan,[15,18,20] and 42 ureidothiophene derivatives (inhibitors).[17]

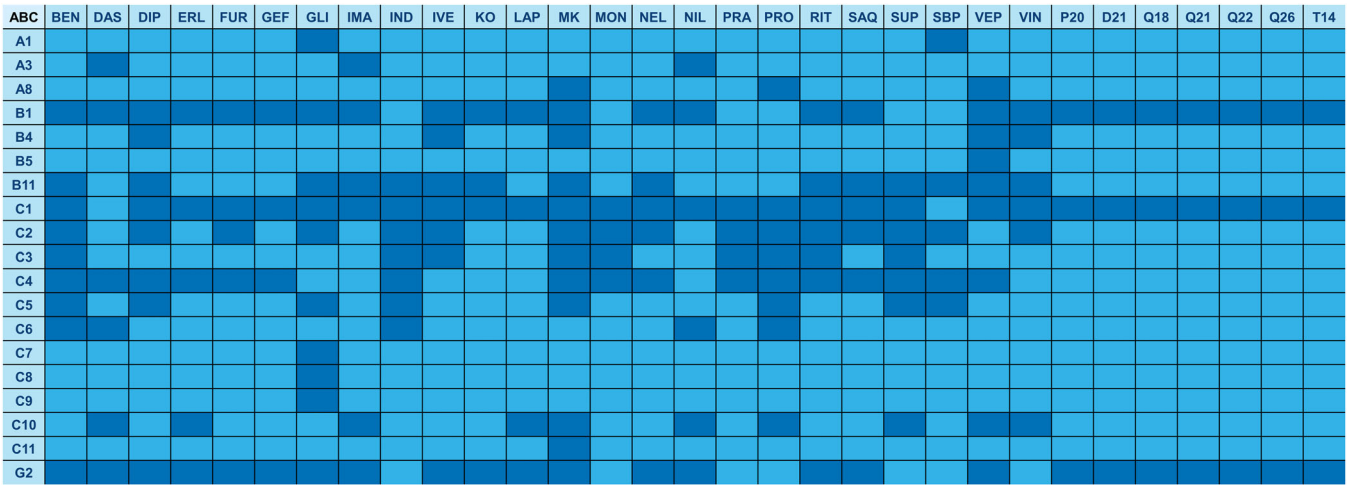

FIGURE 2 Selected 31 multitarget modulators of human ATP-binding cassette (ABC) transporters as reported in the literature[1–3]; light blue: not targeting respective ABC transporter and/or no data available; dark blue: targeting respective ABC transporter. Given are abbreviations as used within this report and the target names of ABC transporters belonging to the currently druggable ABC transporter proteome. Supporting Information S1: Table S1 provides the entire list of 280 identified pan-ABC transporter modulators, and Supporting Information S2: Figure S2 visualizes the molecular structures of all compounds assessed within this study including their used abbreviation.

ebi.ac.uk/chembl], UniProt [https://www.uniprot.org], DrugBank [https://go.drugbank.com], and IUPHAR/BPS Guide to PHARMACOLOGY [https://www.guidetopharmacology.org]) as well as associated literature and datasets on ABC transporter modulators[31–37] for compounds that targeted multiple human ABC transporters. We found 280 modulators (i.e., inhibitors, activators, substrates, *etc*; summarized in Supporting Information S1: Table S1) which targeted at least three ABC transporters and/or ABC transporter-expressing cells. We selected 31 candidates (Figure 2) for testing against ECFTs based on their polypharmacological profiles (for commercially available and commonly known drugs and drug-like compounds: ≥4 ABC transporter targets; for newly developed "focused pan-ABC transporter inhibitors"[38] with limited assessment: ≥3 ABC transporter targets), pharmacological diversity (all compounds together must target the entire ABC transporter proteome), structural diversity (particularly to identify novel scaffolds not presented in Figure 1 and Supporting Information S2: Figure S1), literary diversity (taking both approved drugs known for long time for their polypharmacology as well as recently developed "focused pan-ABC transporter inhibitors"[38] into account),[1–3] as well as commercial availability and affordability.

2.2 | Analysis of inhibitory activity against an ECFT

To evaluate the inhibitory activity of the compounds (at 10 and 50 μM) against ECFTs, we subjected them to our recently established whole-cell-based bacterial uptake assay using *Lactobacillus casei* (*L. casei*) as a model. In *L. casei*, the ECF transporter along with the folic acid-(vitamin B_9)-specific S-component (FolT) are constitutively expressed and therefore do not require an artificial, for example, plasmid-based, expression system.[18] As can be seen from the initial

screening results in Figure 3a, nine out of 31 compounds could be identified as hit molecules (≥20% inhibition at 10 and/or 50 µM). This equals a hit rate of 29.0% which is exceptional for four reasons: first, despite the use of 31 compounds with a common pharmacological basis (multitarget human ABC transporter modulation), the translation of their pharmacology into a target landscape of specific transporters (i.e., ECFTs), particularly of a different species, is not obvious. Specifically, the structural distinction of ECFTs to other ABC transporters lowers the likelihood of hits; second, the molecular-structural diversity of the chosen compounds may have increased the general chance of hit compounds but actually decreased the chance of a high hit rate. In this sense, a much lower hit rate could be expected; third, from a general point of view, serendipitous screenings for specific targets usually result in much lower hit rates; and fourth, medicinal chemistry efforts have not been able to provide a large number of ECFT inhibitors despite the knowledge about ECFTs as potential pharmacological drug targets. Thus, target repurposing significantly increased the landscape of ECFT inhibitors.

Four hits (benzbromarone [BEN], ivermectin [IVE], MK-571 [MK], and pranlukast [PRA]) showed at least 50% inhibition at 50 µM, most of them with significant inhibition already at 10 µM, suggesting half-maximal inhibitory concentration (IC_{50}) values equal or below 50 µM. This finding is also surprising considering that just 32 of the 54 known inhibitors of ECFTs exert their bioactivity below or equal 50 µM, and all of them belong to the structural class of ureidothiophenes.[17] Focusing on structural diversity, the most striking finding is that all nine hit molecules beard in total 11 different heteroaromatic scaffolds that were not found in ECFT inhibitors yet (i.e., benzofuran, β-carboline,

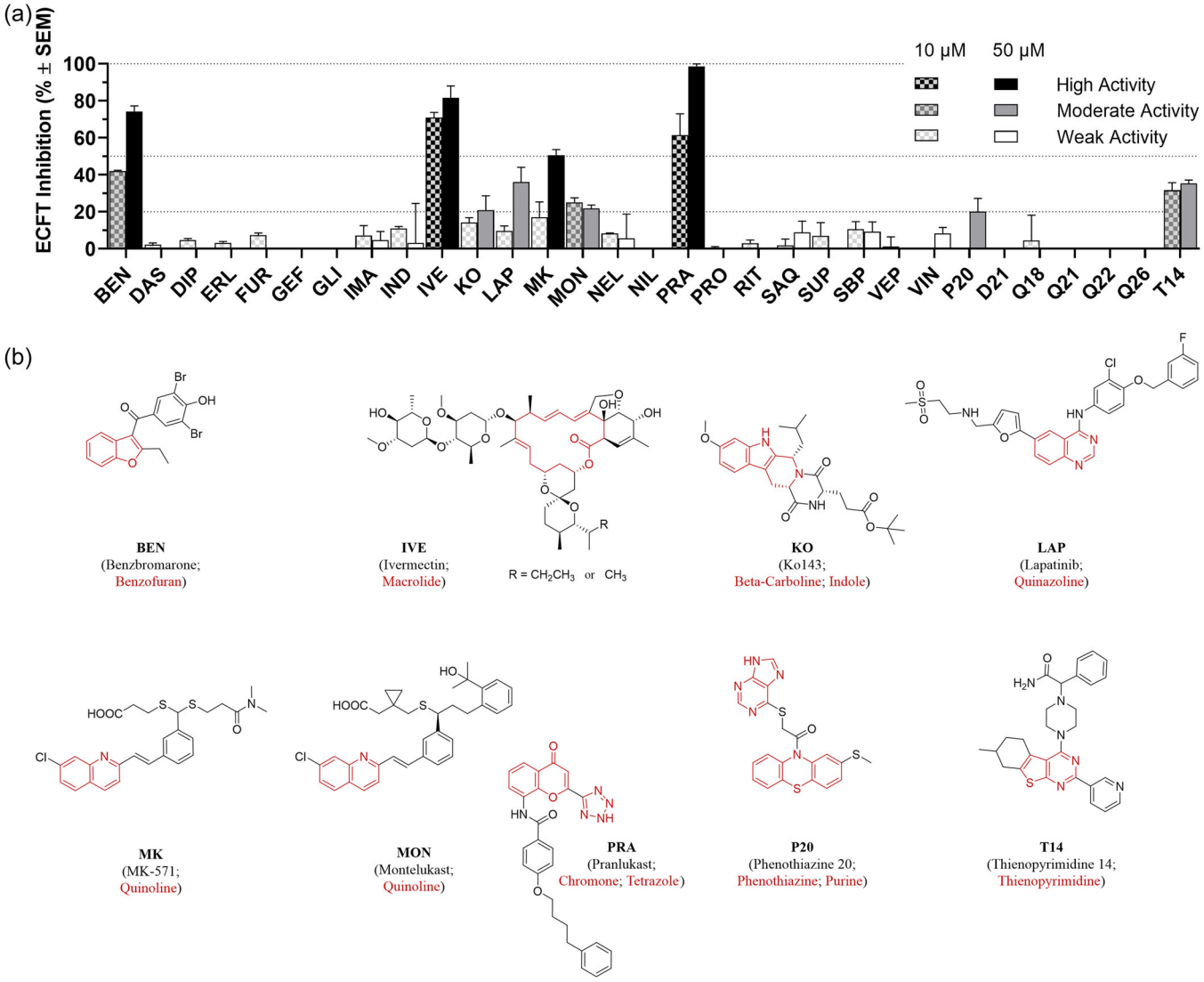

FIGURE 3 Screening results applying 31 selected drugs and drug-like compounds in a whole-cell functional bacterial uptake assay in *Lactobacillus casei*.[18] (a) Effect values of the 31 drugs and drug-like compounds at 10 µM (patterned bars) and 50 µM (filled bars); highly (black), moderately (gray), and weakly/not (white) active compounds are annotated. Shown are the mean ± standard error of the mean (SEM) values of at least two independent experiments. (b) Molecular formulae of the nine hit compounds with annotated (red) scaffolds representing molecular-structural elements not associated with energy-coupling factor transporter (ECFT) inhibition until today.

chromone, indole, macrolide, phenothiazine, purine, quinazoline, quinoline, tetrazole, and thienopyrimidine; Figure 3b). These hits are therefore excellent starting points for further investigations. Table 1 provides the IC_{50} values resultant from in-depth analysis taking concentration-effect curves into account. Although Ko143 (KO), lapatinib (LAP), montelukast (MON), phenothiazine/purine 20 (P20), and thienopyrimidine 14 (T14) demonstrated inhibitory activities in the triple-digit micromolar concentration range, identifying them as rather weak inhibitors, these compounds still belong to the 63 only known ECFT inhibitors (including the compounds discovered within this work).[15–20] BEN, IVE, MK, and PRA, on the other hand, belong to the 36 most potent ECFT inhibitors identified until today, with IVE as one of the four (IC_{50} <5 μM) as well as IVE and PRA as two of the seven (IC_{50} <10 μM) most potent reported representatives, which is directly comparable as the until today most potent inhibitors of ECFTs (i.e., ureidothiophenes) have been evaluated in the same assessment platform as used within this study.[17] To elucidate how well the inhibitory activity in vitro correlated with whole-cell activity, we evaluated the antibacterial activity of BEN, IVE, MK, and PRA against ECFT-expressing *Streptococcus pneumoniae (S. pneumoniae)*.

2.3 | Analysis of antibacterial activity of selected compounds against *S. pneumoniae*

S. pneumoniae is a pathogen long known to contribute to AMR[6–8] and was found to develop resistance against several first-line antibiotics, e.g., cotrimoxazole, oxacillin, and penicillin G,[4] and was associated with death by infection due to resistance against carbapenems, third generation cephalosporines, fluoroquinolones, β-lactamase inhibitors, and macrolides.[10] *S. pneumoniae* is listed by the WHO as a priority for the development of novel antimicrobial agents.[12] As *S. Pneumoniae* expresses many ECFTs and has only a very limited capability to synthesize vitamins, heavily relying on vitamin import,[14] we considered it as a suitable model system to assess whether selected hit compounds were able to translate their ECFT inhibition into antibacterial activity. Table 1 summarizes the minimum inhibitory concentration (MIC_{95}) values of BEN, IVE, MK, and PRA, which is the minimum concentration of no (<5%) bacterial growth. Here, BEN and IVE showed marked antibacterial activity (MIC_{95}: 40.5 and 22.8 μM, respectively), while the MIC_{95} values of MK and PRA could not be defined (>50 μM).

To obtain more insights into the mode of action of the biological hit molecules, we assessed the bacterial growth inhibition in a concentration-dependent manner. Figure 4 provides the resultant growth inhibition curves for BEN, IVE, MK, and PRA against *S. pneumoniae*, and Table 1 outlines the half-maximal growth inhibition concentration (MIC_{50}) values of these compounds. All tested compounds were able to impact bacterial growth concentration-dependently, although the effect of MK was rather weak (MIC_{50}: 52.5 μM). IVE showed the largest impact with an MIC_{50} value of 5.58 μM, which is a rather low value compared with ECFTs-related literature.[18,20] Interestingly, both the MIC_{95} and MIC_{50} values correlated quite well (Table 1).

2.4 | Analysis of avermectins and derivatives

As it turned out, the avermectin derivative IVE, which is a mixture of 22,23-dihydroavermectins B1a and B1b, showed both strongest ECFT inhibition (IC_{50}) as well as strongest bacterial growth inhibition (MIC_{95} and MIC_{50}). This prompted us to analyze other avermectins and avermectin derivatives (i.e., milbemycins), which were commercially available and affordable. Figure 5 shows the molecular formulae of the analyzed molecules (the avermectin derivatives abamectin [ABA; mixture of avermectin B1a and B1b], doramectin [DOR], emamectin [EMA], and eprinomectin [EPR; mixture of acyl amino-

TABLE 1 Bioactivity (IC_{50}) and antibacterial activity (MIC_{95} and MIC_{50}) values of the nine hit compounds (Figure 3a) as determined in a whole-cell functional bacterial uptake assay in *Lactobacillus casei*[18] as well as in a bacterial growth inhibition assay using *Streptococcus pneumoniae*[20] as reported previously.

Compound	Main scaffold	IC_{50} ECFT (μM)	MIC_{95} (μM)	MIC_{50} (μM)
BEN	Benzofuran	19.5 ± 0.7	40.5 ± 1.6	15.1 ± 0.3
IVE	Macrolide	4.27 ± 0.17	22.8 ± 1.7	5.58 ± 0.19
KO	β-Carboline, Indole	>200	n.d.	n.d.
LAP	Quinazoline	123 ± 4	n.d.	n.d.
MK	Quinoline	50.0 ± 1.9	>50	52.5 ± 3.6
MON	Quinoline	>200	n.d.	n.d.
PRA	Chromone, Tetrazole	9.82 ± 0.65	>50	15.8 ± 3.5
P20	Phenothiazine, Purine	>200	n.d.	n.d.
T14	Thienopyrimidine	>200	n.d.	n.d.

Note: Shown are the mean ± SEM of at least two independent experiments.

Abbreviations: BEN, benzbromarone; IVE, ivermectin; KO, Ko143; LAP, lapatinib; MK, MK-571; MON, montelukast; n.d., not determined; PRA, pranlukast; P20, phenothiazine/purine 20; T14, thienopyrimidine 14.

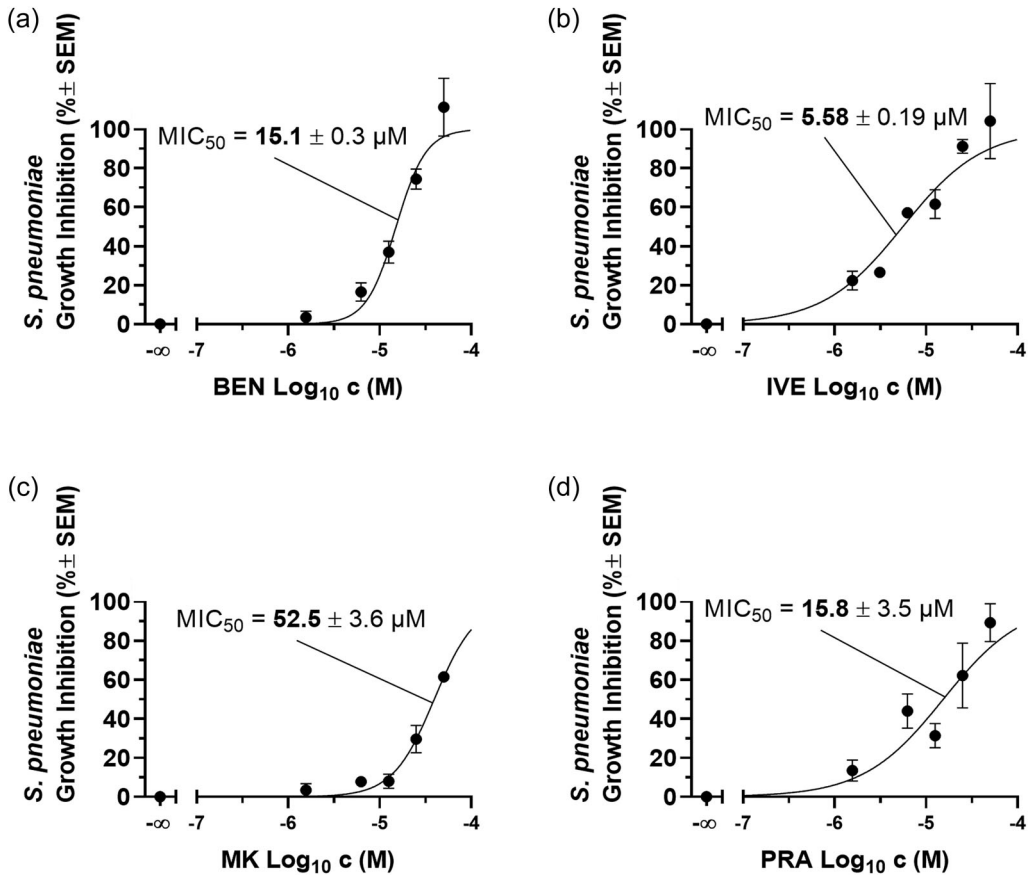

FIGURE 4 Assessment of concentration-dependent antibacterial activity of benzbromarone (BEN) (a), ivermectin (IVE) (b), MK-571 (MK) (c), and pranlukast (PRA) (d) against *Streptococcus pneumoniae*. Shown are the mean ± SEM of at least two independent experiments with duplicate measurements.

avermectin B1a and B1b], as well as the milbemycin derivative moxidectin [MOX]) with annotated structural differences.

The compounds were assessed in the whole-cell-based ECFT inhibition assay as described above.[18] Interestingly, all compounds (ABA, DOR, EMA, EPR, and MOX) displayed inhibitory activity against an ECFT in the low double-digit to low-single-digit micromolar concentration ranges (Table 2). Taking the results of both Tables 1 and 2 into account, the discovered compounds (apart from EMA) belong to the 21 most potent ECFT inhibitors (IC_{50} <20 µM).[17] Moreover, DOR and MOX belong to the five and eight most potent ECFT inhibitors with IC_{50} values below 5 and 10 µM, respectively.[17] Strikingly, MOX bears an inhibitory activity (IC_{50} = 2.23 µM; Figure 6a) similar to the one from the up to know most potent ECFT inhibitor, ureidothiophene 15 (IC_{50} = 2.35 µM), which was evaluated in the same assessment platform as the herein investigated compounds, allowing for a direct comparison.[17] Analysis of its capability to inhibit bacterial growth of *S. pneumoniae* revealed an MIC_{95} of 2.91 µM, while the MIC_{95} of DOR could not be specified (>50 µM; Table 2). Furthermore, MOX had a MIC_{50} value of 2.10 µM (Figure 6b)—which perfectly matches with its IC_{50} of ECFT inhibition. As a side note, MOX showed toxicity against HepG2 cells with an half-maximal growth inhibition value of 11.1 ± 0.2 µM, which needs consideration in future lead optimization studies.

2.5 | Preliminary SAR and suggested mode of action

It could be observed that larger substituents at the spiro part of the avermectin/milbemycin scaffolds as present in DOR and MOX are favored over shorter aliphatic chains (e.g., ABA and EMA), particularly either cyclic aliphatic rings (i.e., cyclohexyl; DOR) or unsaturated hydrocarbon side chains (i.e., 2-[[*E*]-4-methylpent-2-ene]; MOX). Additionally, nitrogen introduction at position C4 of the sugar moiety as present in EMA (methyl amino) and EPR (acyl amino) are less tolerated, however, well accepted at the spiro part (i.e., methoxim; MOX). Finally, the investigations revealed that the presence of sugars (i.e., AMA, DOR, EMA, and EPR) are not necessary for bioactivity against the ECFT and bacterial cell growth, and that their absence (i.e., MOX) rather promotes bioactivity. This finding suggests other aglycon avermectin derivatives, such as milbemycin D and other milbemycins, nemadectin, or milbemectins as potential antibacterial agents, which warrants further investigation.

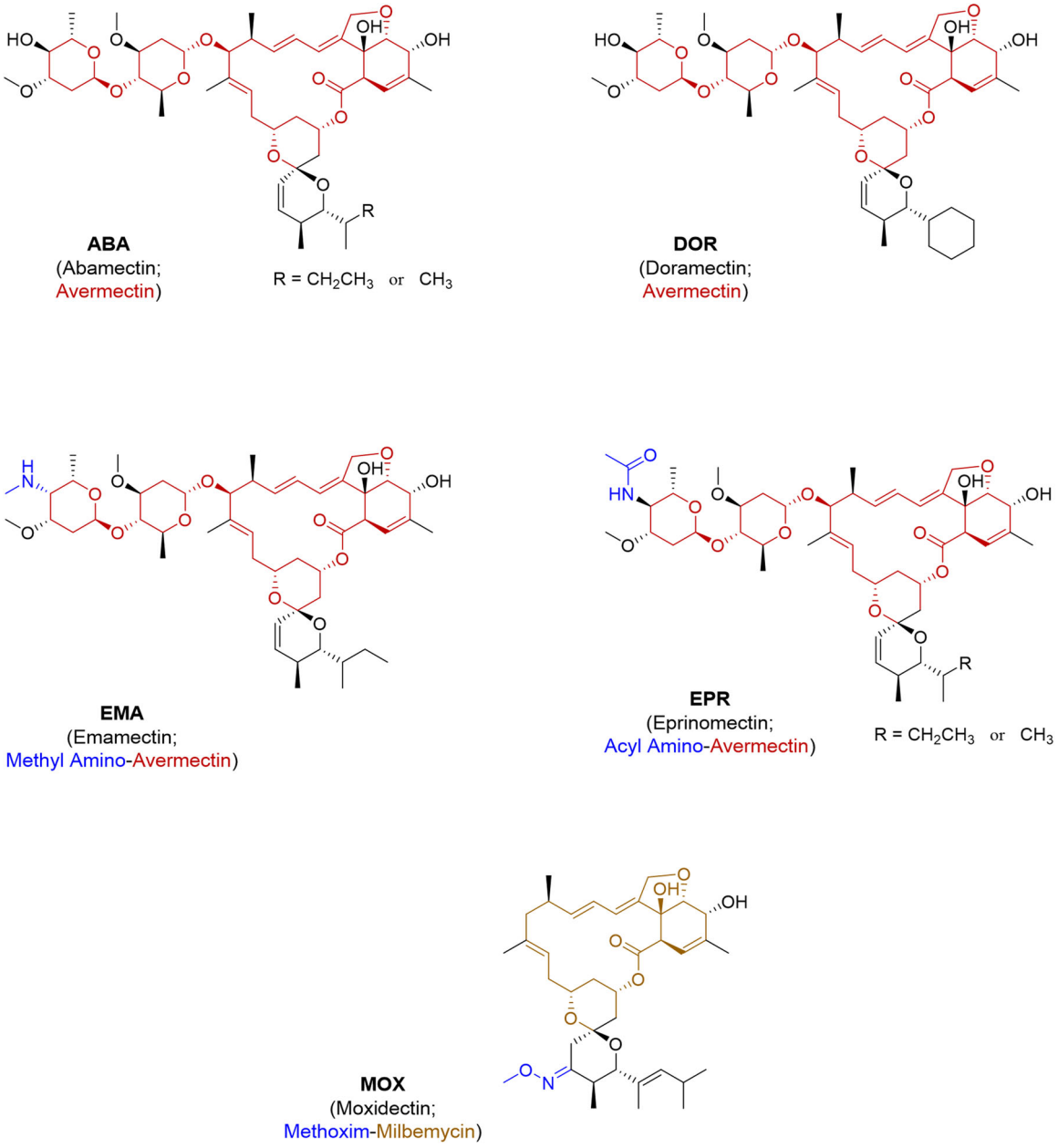

FIGURE 5 Molecular formulae of avermectins and derivatives studied within this work; red: common scaffold of avermectins; brown: common scaffold of milbemycins; blue: structural highlights of the investigated compounds.

TABLE 2 Bioactivity (IC_{50}) and antibacterial activity (MIC_{95} and MIC_{50}) values of avermectins and derivatives as determined in a whole-cell functional bacterial uptake assay in *Lactobacillus casei*[18] as well as in a bacterial growth inhibition assay using *Streptococcus pneumoniae*[20] as reported previously.

Compound	Structural class	IC_{50} ECFT (μM)	MIC_{95} (μM)	MIC_{50} (μM)
ABA	Avermectin	11.4 ± 0.4	n.d.	n.d.
DOR	Avermectin	4.97 ± 0.28	>50	>50
EMA	Avermectin	30.2 ± 3.1	n.d.	n.d.
EPR	Avermectin	16.2 ± 1.6	n.d.	n.d.
MOX	Milbemycin	2.23 ± 0.41	2.91 ± 0.19	2.10 ± 0.20

Note: Shown are the mean ± SEM of at least two independent experiments.

Abbreviations: ABA, abamectin; DOR, doramectin; EMA, emamectin; EPR, eprinomectin; MOX, moxidectin; n.d., not determined.

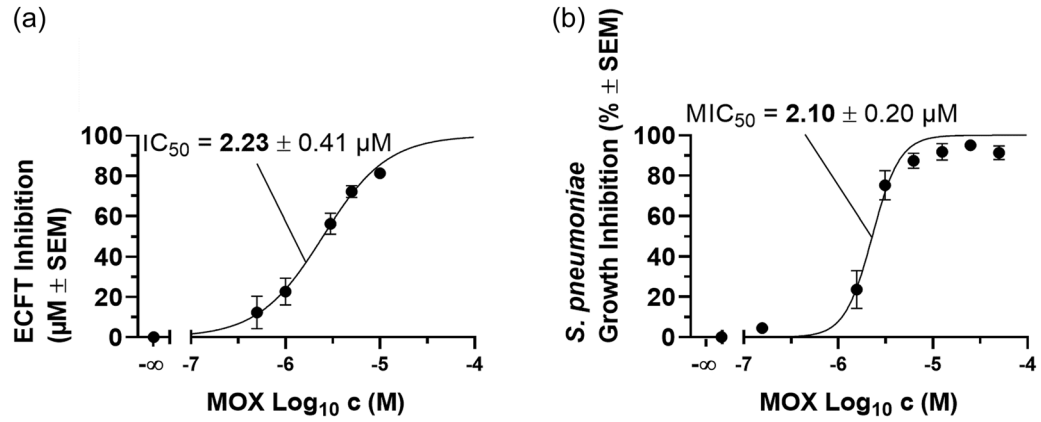

FIGURE 6 Biological assessment of moxidectin (MOX). (a) Evaluation of biological activity against an energy-coupling factor transporter (ECFT) in a whole-cell-based uptake assay using *Lactobacillus casei*.[18] (b) Investigation of the ability of MOX to impede cellular growth of ECFT-expressing *Streptococcus pneumoniae*.[20]

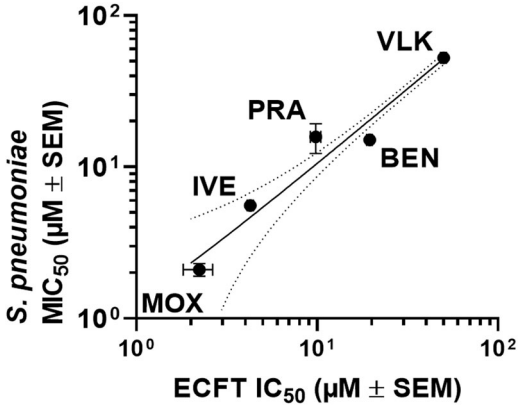

FIGURE 7 Scatter plot correlating IC_{50} and MIC_{50} values of benzbromarone (BEN), ivermectin (IVE), MK-571 (MK), pranlukast (PRA), and moxidectin (MOX). Significant correlation (p value: 0.0029; slope: 0.9820; R^2: 0.9643).

The IC_{50}/MIC_{50} value pairs correlated perfectly with slope and R^2 values of almost 1 (i.e., 0.9820 and 0.9643, respectively; Figure 7) with strong significance. Although further experimental evidence is warranted, this correlation suggests that the bacterial growth inhibition of *S. pneumoniae* may be (at least in part) caused by the inhibition of ECFTs. The other way around, Supporting Information S2: Figure S3 shows that ECFT inhibition by the most promising compounds (including MOX) is not due to inhibition of bacterial growth in the assay, excluding more general effects that may have interfered with the transporter assay.

Our whole-cell-based bacterial uptake assay as described previously[18] uses *L. casei* as a model organism which expresses an ECFT with the folate-specific S-component FolT. This is of interest as the antibacterial activity of the hit compounds was assessed against *S. pneumoniae*—a WHO-listed pathogen that does not express FolT but a panel of other S-components, for example, for riboflavin (vitamin B_2; RibU), niacin-(vitamin B_3; NiaX), pantothenic acid (vitamin B_5; PanT), and biotin (vitamin B_7; BioY), amongst others.[14] Considering the strong correlation as shown in Figure 7, a potential binding site of the hit compounds within or between ECFT components other than the (highly) specific "S-component" is suggested, although this is rather speculative considering the available experimental evidence. Additional experiments using *Enterococcus faecium* und *Enterococcus faecalis* (Supporting Information S2: Table S3) showed no antibacterial activity of the compounds. Further experiments are needed to elucidate the structure–activity relationships and exact mode(s)-of-action of the compounds.

3 | CONCLUSIONS

The "silent pandemic" of AMR is an escalating crisis that necessitates urgent action. Public health authorities around the globe are intensifying their awareness for the rational use of antibiotics and the collection of relevant data, but at the same time are in desperate need of novel drug targets and new antibiotics. ECFTs are new potential targets for anti-infective drug discovery. However, investigation and exploitation of ECFTs as therapeutic targets have just begun.

By applying a target repurposing strategy, we discovered nine structurally diverse, novel ECFT inhibitors. This rational strategy revealed from the very beginning highly potent hits, with IVE being one of the most potent ECFT inhibitors ever found. The strong correlation between target-based and antibacterial activities suggests that ECFTs may be the (major) molecular target of the hit compounds. Particularly as approved drug for human diseases, IVE represents an excellent candidate for drug repurposing strategies against medically relevant pathogens, such as *S. pneumoniae*, an ECFT-expressing pathogen[14] with strong associations with resistance to first-line antibiotics, treatment failure, and death.[4,6–8,10]

Further investigation of IVE analogs showed that both, the avermectin DOR, and the milbemycin MOX had similar and superior inhibitory activity against ECFT, respectively. Particularly MOX was

discovered as the most potent inhibitor known until today with good antibacterial activity against *S. pneumoniae* (MIC_{95}: 2.91 µM). Although MOX is in off-label use for human diseases (i.e., primary use in animal health),[39–41] much clinical experience in both human and animal diseases must have been accumulated, making this drug also suitable for (off-label) drug repurposing approaches tackling the challenge of AMR.

At this point, it is also worth mentioning that all of the herein assessed molecules, including the hit compounds against the ECFT, have additional, undesired off-targets, particularly other human ABC transporters. Thus—in mind that human ABC transporters exert physiologically critical functions in the human body—the therapeutic use of the compounds appears limited at first glance. However, two aspects need consideration: first, the aim of the study was not primarily to discover therapeutics but structurally novel ligands for ECFTs (which could be optimized toward selectivity in subsequent medicinal chemistry efforts), as the number of existing ligands is limited (four structural classes only) despite several years of investigation. In this sense, the strategy was very successful; and second, most of the hit molecules are approved drugs for humans (i.e., BEN, IVE, LAP, MON, and PRA) or animals (i.e., ABA, DOR, EMA, and EPR) despite their broad affinity to ABC transporters. Thus, their additional polypharmacological profiles are apparently of minor importance for their therapeutic use. Hence, their use as adjuvants in a drug repurposing strategy in combination with first- and second-line antibacterial agents is suggested.

In conclusion, our studies demonstrated that target repurposing is a valid strategy to gain structurally novel bioactive compounds with innovative modes-of-action and high originality (i.e., high structural diversity and novelty) and potency for under-studied drug targets of, for example, bacterial origin.

4 | EXPERIMENTAL

4.1 | Materials

The studied pan-ABC transporter modulators BEN (PubChem CID 2333; purity: 99.7%), DAS (PubChem CID 3062316; purity: 99.6%), DIP (PubChem CID 3108; purity: 99.6%), ERL (PubChem CID 176870; purity: 99.8%), FUR (PubChem CID 3440; 100%), GEF (PubChem CID 123631; purity: 99.8%), GLI (PubChem CID 3488; purity: 99.3%), IMA (PubChem CID 5291; purity: 99.9%), IND (PubChem CID 3715; 99.8%), IVE (PubChem CIDs 6321424 and 6321425; purity: 95.0%), KO (PubChem CID 10322450; purity: 99.9%), LAP (PubChem ID 208908; 99.6%), MK (PubChem CID 16760569; 97.1%), MON (PubChem CID 5281040; 99.0%), NEL (PubChem CID 64143; purity: 99.3%), NIL (PubChem CID 644241; purity: 99.6%), PRA (PubChem CID 4887; purity: 99.6%), PRO (PubChem CID 4911; purity: 100%), RIT (PubChem CID 392622; purity: 100%), SAQ (PbChem CID 441243; 99.8%), SUP (PubChem CID 5342; 100%), SBP (PubChem CID 5345), VEP (PubChem CID 2520; purity: 100%), VIN (PubChem CID 13342; purity: 99.3%), ABA (PubChem CID 6435890; purity: 98.3%), DOR (PubChem CID 9832750; purity: 98.1%); EMA (PubChem CID 11549937; purity: 97.8%), EPR (PubChem CIDs 6444397 and 20055319; purity: 94.9%), and MOX (PubChem CID 9832912; purity: 97.9%) were obtained from Sigma Aldrich, and the Certificate of Analysis sheets of all compounds are attached to the Supporting Information. Compounds P20[1] (PubChem CID 167827754; purity: 100%), D21[1] (PubChem CID: 166243554; purity: 100%), Q18[2] (PubChem CID: 154864357; purity: 100%), Q21[2] (PubChem CID 164621986; purity: 98.7%), Q22[2] (PubChem CID 154860935; purity: 100%), Q26[2] (PubChem ID: 164612681; purity: 100%), and T14[3] (PubChem CID 16618023; purity: 95.1%) were supplied by Enamine and the product specification sheets are attached to the Supporting Information. All test compounds were stored at −20°C as 10 mM stock solutions in dimethyl sulfoxide (DMSO).

4.2 | ECFT folate uptake assay

A bacterial uptake assay using *L. casei* as a model microorganism and tritium-labeled folic acid (Moravek Biochemicals) as the substrate to be quantified was conducted as described previously by us.[18] The assay is consequently based on the ECF transporter produced naturally by this strain (without overexpression). By employing the substrate folic acid, the focus of this assay is on the ECF transporter with S-component FolT. The quantification of the ^{3}H-folic acid taken up in the bacteria was carried out as a single-point measurement after 30 min of reaction. The amounts detected after treatment with substance in corresponding concentrations (10 and 50 µM; for the most active representatives, lower concentrations were chosen to determine IC_{50} values) were set in relation to the DMSO control. Further details have been described previously.[18]

4.3 | Minimal inhibitory concentration (MIC_{95}) determination

MIC_{95} values for *S. pneumoniae* (DSM-20566), *Enterococcus faecium* (DSM-20477), and *E. faecalis* (DSM-20478) were determined for six selected compounds (BEN, IVE, MK, PRA, DOR, and MOX). As a bacteria start OD_{600} we used 0.03 in a total volume of 200 µL in Todd Hewitt Broth with 0.1% cholin (*Streptococcus*) or Trypticase Soy Yeast Extract Medium (Enterococci) containing the compounds predissolved in DMSO (maximal DMSO concentration in the experiment: 1%). Final compound concentrations prepared from serial dilutions ranged from 0.01 to 64 µM (double values for each concentration) depending on their antibacterial activity. The OD values were determined directly after the addition of the compounds plus after incubation for 18 h at 37°C without shaking in 96-well plates (Sarstedt) using a FLUOStar Omega (BMG labtech). Growth of *S. pneumoniae* took place in the presence of 5% CO_2. Given MIC_{95} and MIC_{50} values are means of at least three independent determinations and are defined as the (lowest) concentration of compounds that reduced OD_{600} by ≥95% and 50%, respectively.

4.4 | Cytotoxicity determination

To obtain information regarding the cytotoxicity of MOX, its impact on the viability of human cells was investigated. HepG2 cells (2×10^4 cells per well) were seeded in 96-well, flat-bottomed culture plates in 100 µL culture medium (DMEM containing 10% fetal calf serum, 1% penicillin-streptomycin). Twenty-four hours after seeding the cells, medium was removed and replaced by medium containing test compounds in a final DMSO concentration of 1%. Compounds were tested in duplicates at a single concentration or, for half-maximal growth inhibition determination, at eight concentrations that were prepared via twofold serial dilutions in 1% DMSO/medium. Epirubicin and doxorubicin were used as positive controls in serial dilutions starting from 10 µM; MOX was tested starting from 100 µM. Rifampicin was used as a negative control at 100 µM. The living cell mass was determined 48 h after treatment with compounds by adding 0.1 volumes of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in sterile phosphate-buffered saline [PBS]; Sigma) to the wells. After incubating the cells for 30 min at 37°C (atmosphere containing 5% CO_2), the medium was removed and MTT crystals were dissolved in 75 µL of a solution containing 10% sodium dodecyl sulfate (SDS) and 0.5% acetic acid in DMSO. The optical density (OD) of the samples was determined photometrically at 570 nm in a PHERAstar Omega plate reader (BMG labtech). To obtain percent viability for each sample, their ODs were related to those of DMSO controls. At least two independent measurements were performed for each compound. The calculation of half-maximal growth inhibition concentrations was performed using the nonlinear regression function of GraphPad Prism 10 (GraphPad Software).

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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