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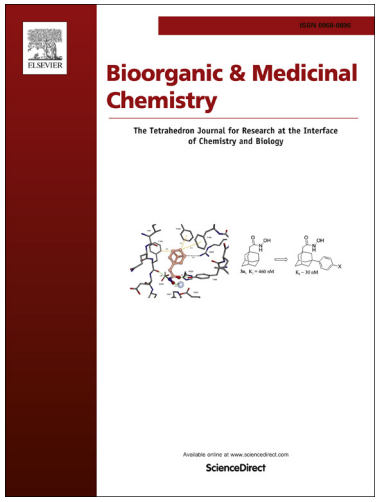

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Latvian Institute of Organic Synthesis, 21 Aizkraukles St., Riga LV-1006, Latvia

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ABSTRACT

To identify new potent multidrug resistance modulators , we have synthesized a series of novel thieno[2,3-*b*]pyridines and furo[2,3-*b*]pyridines, and examined their stucture-activity relationships. All synthesized compounds were tested to determine BCRP1, P-gp, and MRP1 inhibitor activity, and most potent MDR modulators were also screened for their toxicity, cytotoxicity and Ca^{2+} channel antagonist activity. Among these compounds, thieno[2,3-*b*]pyridine (6r) was found to exhibit a potent P-gp inhibitory action with EC_{50} = 0.3 ± 0.2 μM, MRP1 inhibitory action with EC_{50} = 1.1 ± 0.1 μM and BCRP1 inhibitory action with EC_{50} = 0.2 ± 0.05 μM and may represent suitable candidate for further pharmacological studies.

1. Introduction

Chemotherapy is one of the most frequently used forms of cancer therapy and has found clinical application in the treatment of almost every type of cancer. One of the major problems in cancer chemotherapy is the development of resistance to cytotoxic drugs. Patients who did not respond to a first course of chemotherapy are in even more serious condition because tumor cells develop resistance against chemotherapeutic agents or resistance to cytotoxic agents used in a previous treatment. A tumor may also manifest resistance to a cytotoxic agent to which it has not been previously exposed.

Multidrug resistance (MDR) in tumor cells has a significant impact on the efficacy of cancer chemotherapy and appears as a major obstacle in the modern cancer treatment. MDR is mainly related to the expression of the adenosine triphosphate (ATP)-binding cassette (ABC) transporters. P-glycoprotein (P-gp) (the best studied target for reverting MDR), multidrug resistance-associated protein (MRP1) and the breast cancer resistance protein (BCRP1) as major MDR proteins actively transport a wide variety of structurally different substrates out of the tumor cells, thereby decreasing their intracellular concentrations. Many actual chemotherapeutic agents are considered as potential P-gp, MRP1 and BCRP1 substrates.[1,2]

P-gp, a member of the ABCB subfamily, confers the strongest resistance to the wide variety of compounds. P-gp transports vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes. P-gp is normally expressed in epithelium of the liver, kidney and gastrointestinal tract at pharmacological barrier sites, in stem cells and cells of immune system.[2,3,4]

MRP1 is a member of ABCC subfamily and confers resistance to several hydrophobic compounds that are also P-gp substrates. But MRP1 can export glutathione, glucuronate or sulfate conjugates of organic anions. MRP1 is expressed in wide range of tissues, tumors and cancer cell lines.[4]

BCRP1 is a member of ABCG subfamily. The substrate specificity of BCRP1 overlaps considerably with that of P-gp. BCRP1 is involved in the mechanism of resistance to a topoisomerase I inhibitor (topotectan) or topoisomerase II inhibitor (mitoxantrone).[5,6] BCRP1 does not act on Paclitaxel or Vincristine transport, which are excreted by P-gp. BCRP1 is involved in excretion of a camptothecin derivative, which is barely transported by P-gp.[2,7] BCRP1 is expressed in many normal tissues, including liver, placenta, brain, hematopoietic stem cells and other types of stem cells.

From all numerous efforts to overcome MDR like transcription control of P-gp expression the most promising approach has been the development of MDR modulators, which are able to increase the intracellular drug levels in co-application with MDR substrates by the effect of efflux pump inhibition. Substances of different groups have been used as P-gp inhibitors. Ca^{2+} channel blocker Verapamil is the most investigated and often used as reference compound, but unfortunately in combination with actual anticancer drugs cardio toxicity is observed.[8]

* Corresponding author. Tel.: +(371)67014870; fax: +(371)67550338; e-mail: krauze@osi.lv

Extensive computational models, based on pharmacophore modeling and molecular docking techniques, have been developed to predict P-gp inhibitors or substrates. Unfortunately only limited *in silico* models can give satisfactory predictions. The improvement of prediction accuracy still remains a significant challenge.[9]

In recent years intensive efforts have been taken to develop small-molecule inhibitors with a favorable resorption in contrast to complex natural compounds with high and critical molecular weights which are unfavorable also because the synthetic access to such structures is difficult and expensive.[10,11]

There are no literature data when multidrug resistance modulating properties are searched in a series of thieno[2,3-*b*]pyridines which in last decade are characterized by a broad spectrum of biological activities. Some of them possess cytotoxic activity,[12-16] antiinflammatory,[16,17] antiviral[18] or antibacterial[19,20] activity. Others are useful as hypolipoproteinemic and antiatherosclerotic agents[21] and inhibitors of Mitogen-activated protein kinase enzymes, and are accordingly of benefit as pharmaceutical agents, especially in the treatment of adverse inflammatory, autoimmune, cardiovascular, proliferative and nociceptive conditions.[22]

Rational approach of drug design – structural analogy with known medicines – was used to develop more efficacious MDR modulators on the basis of thieno[2,3-*b*]pyridines. As part of our research interest towards bioactive *N*,*S*-containing heterocyclic compounds, we postulated that thieno[2,3-*b*]pyridine scaffold might be suitable for the linked pharmacophore approach. Model was created assuming one part of Verapamil as linker and methoxyphenyl groups as essential for pharmacophore (Fig. 1). „Linked pharmacophore" model was used in development of such P-gp ligands as anthranilamide derivatives (tariquidar),[23-25] 2,3-dehydrosilybin derivatives,[26] naphthalenyl derivatives,[27] tetrahydroisoquinoline derivatives[28] and others.

As Ca^{2+} channel blocker Verapamil in combination with anticancer drugs reveals cardio toxicity,[8] influence of obtained thieno[2,3-*b*]pyridines on cardiovascular system as well as their cytotoxicity were tested. Compounds possessing insignificant calcium antagonist properties as side effect and LD_{50} >2000 mg/kg were tested as potential MDR modulators. To reveal structure-activity relationship in these series, three compounds with LD_{50} <2000 mg/kg were tested

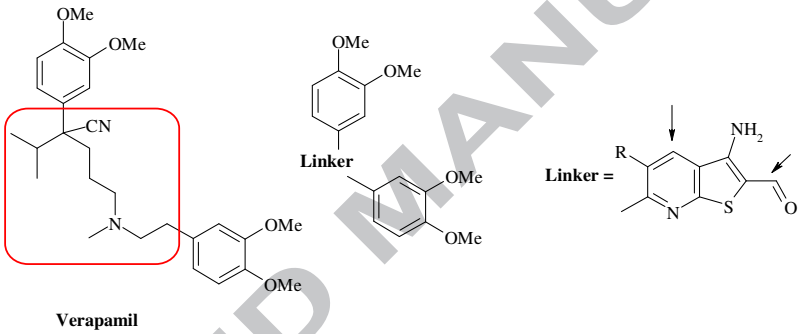
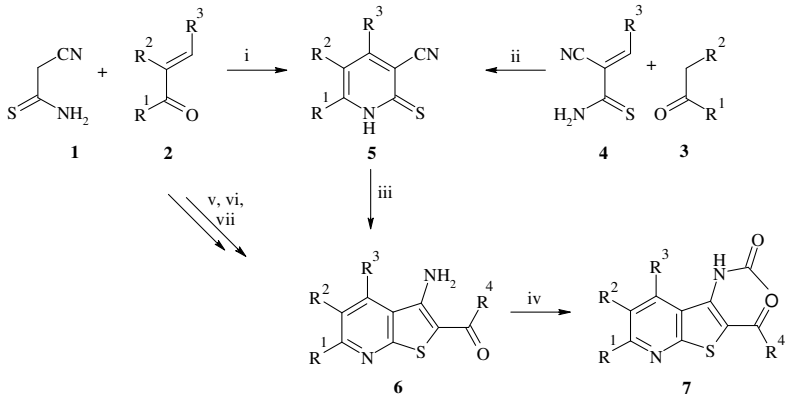


Figure 1. Pharmacophore approach with modified linker

2. Results and discussions

2.1. Chemistry

The synthesis of thieno[2,3-*b*]pyridines is very straightforward and demonstrated in Scheme 1. The key intermediates, 1,6-dihydro-6-thioxopyridines **5a-r**, were synthesized according to slightly modified synthesis protocols described in the literature.[29-32] 2-Cyanothioacetamide **1** was reacted with *α*,*β*-unsaturated ketones **1** in presence of catalytic amount of triethylamine (method A) to give thioxopyridines **5** in 42-80 % yield. As alternative method for synthesis of intermediates **5** was used condensation of ketones **3** with 3-aryl-2-cyanothioacrylamides **4** catalyzed by piperidine (method B), and intermediates were obtained in 41-60 % yield.



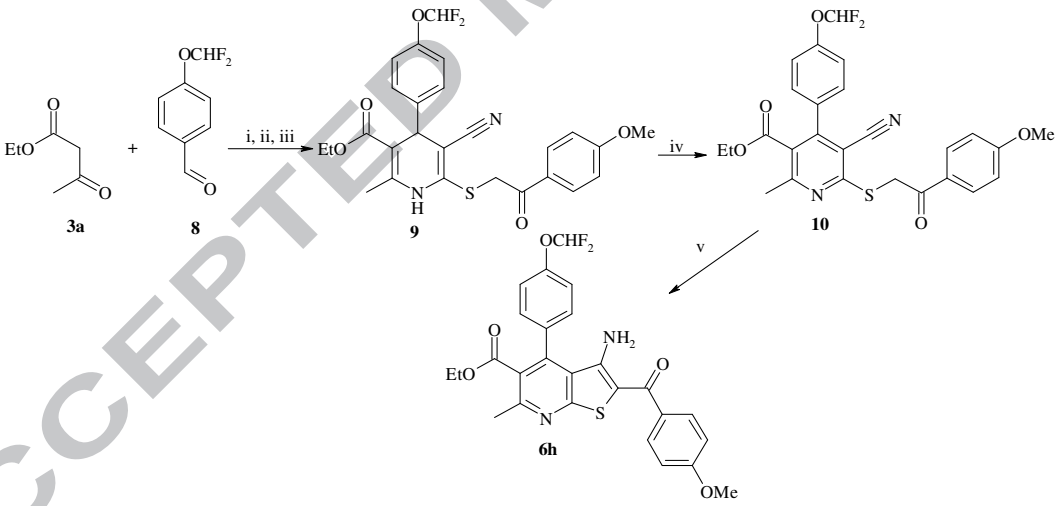
Scheme 1. Reagents and conditions: (i) 0.07 equiv Et_3N , EtOH, reflux, 14 h; (ii) 0.1 equiv piperidine, EtOH 15 h; (iii) 1.0 equiv bromoacetophenone, 2.4 equiv NaOH/H_2O, EtOH, short reflux, rt, 30 min; (iv) ClCOMe, 2.0 equiv NaOH, reflux, 1 h; (v) 1.1 equiv piperidine, EtOH, rt, 12 h; (vi) 1.0 equiv bromoacetophenone, EtOH, rt, 3h (vii) 1.0 equiv 3M NaOH/H_2O, EtOH, rt, 30 min

Table 1. Structure and yields of thioxodihydropyridines **5a-r**

Compound	R^1	R^2	R^3	Yield, %	
				A	B
5a	Me	COMe	3,4,5-$(OMe)_3C_6H_2$	42	
5b	Me	COOMe	4-$OMeC_6H_4$		53
5c	Me	COOMe	3,4-$(OMe)_2C_6H_3$	42	
5d	Me	COOMe	3,4,5-$(OMe)_3C_6H_2$	45	
5e	Me	COOEt	Ph	46[29]	
5f	Me	COOEt	4-$OMeC_6H_4$		60[30]
5g	Me	COOEt	3,4-$(OMe)_2C_6H_3$	43[31]	
5h	Me	COOEt	3,4,5-$(OMe)_3C_6H_2$	45[32]	
5i	Me	COOEt	4-$OEtC_6H_4$		54
5j	Me	COOEt	4-*n*-$OBuC_6H_4$		59
5k	Me	COO*n*-Bu	3,4,5-$(OMe)_3C_6H_2$		46
5l	Me	$COOC_2H_4OMe$	4-$OMeC_6H_4$		53
5m	Me	$COOC_2H_4OMe$	3,4,5-$(OMe)_3C_6H_2$	42	
5n	Me	$COOC_2H_4O$*n*-Pr	3,4,5-$(OMe)_3C_6H_2$	57	
5o	3,4,5-$(OMe)_3C_6H_2$	COOEt	3,4,5-$(OMe)_3C_6H_2$		44
5p	NH_2	CN	3,4,5-$(OMe)_3C_6H_2$		41
5q	Me	H	Me	67[33]	
5r	Ph	H	3-pyridyl	80[34]	

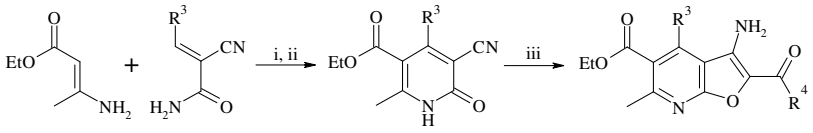
The synthesis of the corresponding thieno[2,3-*b*]pyridines was performed, using slightly modified synthesis protocol described in the literature.[35] First alkylation takes place by treatment of 1,6-dihydro-6-thioxopyridines **5a-r** with corresponding bromoacetophenones in presence of excess of sodium hydroxide. The formed intermediates 2-alkylsulfanyl-3-cyanopyridines easy undergo Thorpe-Ziegler cyclization giving thieno[2,3-*b*]pyridines **6a-g, i-x, z** in high yields.

N-Acetylated derivative **7** was prepared, by treatment of thieno[2,3-*b*]pyridine **6r** with acetylchloride in presence of sodium hydroxide. 5-Unsubstituted thieno[2,3-*b*]pyridine **6y** was prepared by treatment of chalcone **1** (R^1 = Me, R^2 = H, R^3 = 3,4,5-$(OMe)_3C_6H_2$)[36] with 2-cyanothioacetamide **2** in presence of piperidine, *in situ* alkylation with bromoacetophenone and sequential base catalyzed cyclization.



Scheme 2. Reagents and conditions: (i) 0.8 equiv piperidine, EtOH, rt, 30 min; (ii) 1.0 equiv 2-cyanothioacetamide, 0.32 equiv piperidine, EtOH, short reflux, then rt 3 h; (iii) 1.0 equiv 2-bromo-4'-methoxyacetophenone, EtOH, short reflux; (iv) 2.0 equiv $Mn(OAc)_3$ dihydrate, glacial AcOH, reflux, 2 h; (v) 1.0 equiv 3M $NaOH/H_2O$, EtOH, short reflux, then rt 30 min

In regard to synthesis of thieno[2,3-*b*]pyridine **6h** containing lipophilic $C_6H_4OCHF_2$ moiety in position 4, first 1,4-dihydropyridine **9** was built by four component condensation. Then 1,4-DHP **9** was oxidized with manganese(III) acetate dihydrate to pyridine **10** and transformed to the target product **6h** with sodium hydroxide (Scheme 2).



11 **12a,b** **13a,b** **14a,b**

a) R^3 = 3,4,5-$(OMe)_3C_6H_2$, R^4 = 4-$OMeC_6H_4$; b) R^3 = 4-$OMeC_6H_4$, R^4 = 3,4,5-$(OMe)_3C_6H_2$

Scheme 3. Reagents and conditions: (i) glacial acid/ethanol, reflux, 4h; (ii) HNO_3, ethanol, reflux, 48h; (iii) 4.0 equiv K_2CO_3, 1.0 equiv bromoacetophenone, DMF, 80 °C, 4h

In order to evaluate core structure, furo[2,3-*b*]pyridines as bioisosters of thieno[2,3-*b*]pyridines were investigated. The synthesis of furo[2,3-*b*]pyridine derivatives has been accomplished as described in Scheme 3. Aminocrotonic acid ethyl ester **11** was reacted with 3-cyano-4-(3,4,5-trimethoxyphenyl)- or 4-(4-methoxyphenil)acrylamide **12a,b** in mixture of absolute ethanol and glacial acetic acid. The formed intermediates 1,4,5,6-tetrahydro-6-oxopyridines undergo cycle oxidation in ethanol-nitric acid media to give the 1,6-dihydro-6-oxopyridines **13a** and **13b**[37]. In presence of potassium carbonate and bromoacetophenones, compounds **13a,b** consequently undergo *O*-alkylation and Thorpe-Ziegler cyclization reaction to give the corresponding furo[2,3-*b*]pyridines **14a,b**.

Table 2. Structure and yields of synthesized thieno- and furo[2,3-*b*]pyridines **6, 7** and **14** as MDR modulators.

Compound	R^1	R^2	R^3	R^4	Yield, %
6a	Me	COMe	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	78
6b	Me	COOMe	4-$OMeC_6H_4$	4-$OMeC_6H_4$	78
6c	Me	COOMe	3,4-$(OMe)_2C_6H_3$	4-$OMeC_6H_4$	84
6d	Me	COOMe	3,4-$(OMe)_2C_6H_3$	2,4-$(OMe)_2C_6H_3$	93
6e	Me	COOMe	3,4,5-$(OMe)_3C_6H_2$	3,4-$(OMe)_2C_6H_3NH$	97
6f	Me	COOEt	C_6H_5	4-$OMeC_6H_4$	94
6g	Me	COOEt	4-$OMeC_6H_4$	4-$OMeC_6H_4$	86
6h	Me	COOEt	4-$OCHF_2C_6H_4$	4-$OMeC_6H_4$	60
6i	Me	COOEt	3,4-$(OMe)_2C_6H_3$	Me	70
6j	Me	COOEt	3,4,5-$(OMe)_3C_6H_2$	C_6H_5	40[35]
6k	Me	COOEt	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	89
6l	Me	COOEt	3,4,5-$(OMe)_3C_6H_2$	2,4-$(OMe)_2C_6H_3$	96
6m	Me	COOEt	4-$OEtC_6H_4$	4-$OMeC_6H_4$	96
6n	Me	COOEt	4-OBu(n)C_6H_4	4-$OMeC_6H_4$	89
6o	Me	COOBu	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	93
6p	Me	$COOC_2H_4OMe$	4-$OMeC_6H_4$	4-$OMeC_6H_4$	90
6q	Me	$COOC_2H_4OMe$	3,4,5-$(OMe)_3C_6H_2$	Ph	90
6r	Me	$COOC_2H_4OMe$	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	82
6s	Me	$COOC_2H_4OMe$	3,4,5-$(OMe)_3C_6H_2$	2,4-$(OMe)_2C_6H_3$	80
6t	Me	$COOC_2H_4OMe$	3,4,5-$(OMe)_3C_6H_2$	3,4,5-$(OMe)_3C_6H_2$	81
6u	Me	$COOC_2H_4OPr$	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	86
6v	3,4,5-$(OMe)_3C_6H_2$	COOEt	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	95
6w	NH_2	CN	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	41
6x	Me	H	Me	2,4-$(OMe)_2C_6H_3$	70
6y	Me	H	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	89
6z	Ph	H	3-pyridyl	Ph	91[34]
7	Me	$COOC_2H_4OMe$	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	71
14a	Me	COOEt	3,4,5-$(OMe)_3C_6H_2$	4-$OMeC_6H_4$	19
14b	Me	COOEt	4-$OMeC_6H_4$	3,4,5-$(OMe)_3C_6H_2$	38

2.2. Biological evaluation, structure-activity relationships, P-gp, MRP1 and BCRP1 modulating activity

The tested compounds showed multidrug resistance modulating activities which are listed in Table 3. MK-571, Reversan, and Verapamil were used as reference inhibitors of P-gp, MRP1 and BCRP1. Besides the clinically important drugs, several fluorescent compounds are transported by P-gp, MRP1 and BCRP1 such as rhodamine 123 (P-gp), calcein (MRP1) and Hoechst 33342 (P-gp, BCRP1). These fluorescent compounds are used during studying ABC transporters in cell lines.

Table 3. MDR modulating activity and calculated *log*P values of tested compounds

Compound	*log*P	MDR, EC_{50}, μM			Ca^{2+}, A7R5, IC_{50}, μM	LD_{50}, mg/kg
		P-gp	MRP1	BCRP1		
Verapamil		7.1 ± 2.0	27.8 ± 0.8	37.3 ± 7	0.3 ± 0.1	962
MK-571		n.e.	12.4 ± 2.2	-	n.e.	752
Reversan		18.4	9.8 ± 0.5	-	> 100	885
6a	4.10	3.8 ± 0.1	6.6 ± 1.0	2.6 ± 0.6	14.0 ± 0.9	>2000
6b	4.80	4.5 ± 0.2	n.e.	0.7 ± 0.1	15.4 ± 2.0	>2000
6c	4.70	5.6 ± 0.2	11.9 ± 1.3	3.6 ± 0.6	6.0 ± 0.8	2808
6d	4.58	10.3 ± 1.5	41.4	4.1 ± 0.9	5.6 ± 1.4	1045
6e	4.05	n.e.	n.e.	n.e.	-	-
6f	5.29	n.e.	n.e.	n.e.	-	-
6g	5.17	6.5 ± 0.9	n.e.	0.4 ± 0.1	100.0 ± 11.0	>2000
6h	5.88	n.e.	-	-	-	-
6i	3.27	n.e.	n.e.	-	-	-
6j	5.04	9.0 ± 0.5	5.2 ± 0.8	1.5 ± 0.0	14.0 ± 1.1	>2000
6k	4.92	0.3 ± 0.1	5.2 ± 0.6	0.7 ± 0.3	19.0 ± 3.0	>2000
6l	4.79	6.4 ± 0.6	12.4 ± 0.4	2.6 ± 0.3	46.0 ± 1.4	>2000
6m	5.51	4.2 ± 0.7	n.e.	1.3 ± 0.2	>100.0	>2000
6n	6.41	n.e.	n.e.	n.e.	-	-
6o	5.82	1.5 ± 0.1	n.e.	0.4 ± 0.08	5.0 ± 0.7	>2000
6p	4.67	1.0 ± 0.1	n.e.	0.8 ± 0.1	21.0 ± 4.0	>2000
6q	4.55	1.4 ± 0.1	3.9 ± 0.6	1.3 ± 0.2	2.2 ± 0.3	>2000
6r	4.42	0.3 ± 0.2	1.1 ± 0.1	0.2 ± 0.05	3.1 ± 0.4	2097
6s	4.30	2.0± 0.0	7.0±1.0	2.5 ± 0.5	9.0 ± 1	2983
6t	4.17	n.e.	n.e.	n.e.	-	-
6u	5.25	0.6 ± 0.1	n.e.	0.6 ± 0.1	>100.0	>2000
6v	5.93	22.0 ± 0.5	n.e.	3.0 ± 0.3	n.e.	-
6w	4.00	n.e.	n.e.	n.e.	-	-
6x	3.82	n.e.	n.e.	n.e.	-	959
6y	4.76	n.e.	n.e.	n.e.	-	-
6z	5.35	n.e.	-	-	-	-
7	4.13	n.e.	n.e.	n.e.	-	-
14a	3.55	n.e.	n.e.	3.4 ± 0.7	1.0 ± 0.1	1770
14b	3.55	n.e.	n.e.	n.e.	-	>2000

n.e. - No effect
A7R5 - rat aorta smooth muscle

As shown in Table 3, some 3-amino-thieno[2,3-*b*]pyridines displayed similar or more potent P-gp and MRP1 modulating activities in comparison with control inhibitors (Verapamil, MK-571, Reversan), and our target compounds significantly exceeded BRCP1 inhibitor activity of reference compound Verapamil.

Aroyl group (substituent R^4) in position 2 of thieno[2,3-*b*]pyridine ring is essential for MDR modulating activity, its substitution with acetyl and aromatic amide function (compounds **6i** and **6e**) results in loss of activity. Results show that benzoylderivative **6j** is relatively potent, introduction of one electron-donating methoxy group in *para*-position of the aromatic ring increases activity, while introducing additional methoxy groups to the *ortho*-position or two methoxygroups in *meta*-position leads to decrease in potency. Acylation of amino group in position 3 of thieno[2,3-*b*]pyridine ring (compound **7**) resulted in loss of activity and these results suggest that the free amino group as hydrogen bond donor contributes largely to the biological activity.

Only thieno[2,3-*b*]pyridines possessing at least one 4-methoxyphenyl group in position 4 (substituent R^3) and free amino group at position 3 exhibit a potent P-gp, MRP1 and BCRP1 inhibitory action. In case of 3,4-dimethoxyphenyl group potency decreases, but modulators containing 3,4,5-trimethoxyphenyl group have shown the most promising results. So, *para*-OMe substitution at one of the two aryl rings present in pharmacophore model is required for optimum MDR inhibition potency and selectivity. Introduction of two 3,4,5-trimethoxyphenyl rings is undesirable, and inhibitory action of compounds **6t**, **6v** has partially or completely disappeared. Compounds **6f** (R^3 = Ph), **6x** (R^3 = Me) and **6z** (R^3 = 3-pyridyl) are completely inactive.

As seen from Table 3, 4-(4-methoxyphenyl) group containing compounds **6b**, **6g** and **6p** selectively inhibit only P-gp and BCRP1. 4-(4-Ethoxyphenyl) group containing compound **6m** shows similar activity to corresponding **6g**, while butyloxyphenyl ether **6n** (calculated logP 6.41) has lost MDR modulating activity which indicate that optimum lipophylicity is required.

Introduction of lipophilic $C_6H_4OCHF_2$ moiety in position 4 (compound **6h**, calculated logP 5.88) confirms this requirement because activity is lost in this case, too.

Replacement of hydrogen at the position 5 of thieno[2,3-*b*]pyridine ring (substituent R^2) with acetyl or ester group results in significant increase of activity, reaching maximum in cases of ethyl or 2-methoxyethylester ester groups. As thienopyridine **6u** with lipophylic 2-propoxyethylester ester group (*log*P 5.25) is approximately 2 times less active in comparison with **6r** (*log*P 4.42), optimum lipophylicity has to be reached by modification of substituent R^2, too. Exchange of methyl group in position 6 with aryl or amino groups (substituent R^1) leads to diminution of activity.

Among furo[2,3-*b*]pyridines **14** only compound **14a** as bioisostere of thieno[2,3-*b*]pyridine **6k** has shown selective activity against BCRP1 and was completely inactive on P-gp and MRP1 lines. Nevertheless oxygen bioisostere **14a,** being ten times more active than verapamil, is five times less active than compound **6k** and, in addition, undesired Ca^{2+} channel blocking activity has revealed.

Lipophilicity is an important indicator of how target molecules can cross cell membranes. Predicted lipophilicity (*log*P) of the compounds was calculated with the computer program logP using ChemBioDraw® Ultra 12.0 suite**.** Calculated *log*P values are in range of 3.27 to 6.41 (Table 3). Most of potent compounds of thieno[2,3-*b*]pyridine series have *log*P approximately 4.4-5.8. Among tested compounds, those with lipophilicity lower than 4.0 and higher than 5.9 have shown significantly weaker or no effect of MDR modulating activity.

It is known that *H*-bond donor as well as acceptor functions play an important role in the binding possibilities of P-gp substrates. Association of the P-gp substrate (inhibitor) complex is determined by the number and strength of the hydrogen bonds formed between the substrate and the transporter.[38] The thieno[2,3-*b*]pyridine series bearing R^1 = Me, R^2 = COOAlk, R^3 = 3,4,5-$(OMe)_3C_6H_2$ and R^4 = 4-$OMeC_6H_4CO$ are the most potent MDR modulators. Amino group in position 3 serves as hydrogen bond donor, but OMe groups in different positions as hydrogen bond acceptors.

Thieno[2,3-*b*]pyridines **6a, 6g, 6n, 6o**, **6p** and **6u** selectively inhibited P-gp and BCRP1, but compounds **6a, 6c, 6k, 6l**, **6q**, **6r** and **6s** were potent and efficacious as they inhibited three MDR transport proteins: P-gp, MRP1 and BCRP1. In particular, compound **6r** was found to exhibit a potent P-gp inhibitory action with EC_{50} = 0.3 ± 0.2 μM, MRP1 inhibitory action with EC_{50} = 1.1 ± 0.1 μM and BCRP1 inhibitory action with EC_{50} = 0.20 ± 0.05 μM and may be promising leads for development of MDR-reversal drugs.

Thieno[2,3-*b*]pyridines not only are significantly potent MDR-blockers in comparison with reference compounds, but in addition reveal more than ten times lower Ca^{2+} antagonist effect than Verapamil (less cardiovascular side effects expected) and are non-toxic (LD_{50} > 2000 mg/kg). They are potential agents to overcome MDR problem in clinic and increase the effectiveness of chemotherapy in cancer treatment.

3. Conclusion

A new class of multidrug resistance (MDR) modulators, possessing a 3-amino-thieno[2,3-*b*]pyridine scaffold has been discovered. Pharmacophore model was created assuming thieno[2,3-*b*]pyridine scaffold as linker and methoxyphenyl groups as essential for potential MDR-reversal drug. Decoration of 3-amino-thieno[2,3-*b*]pyridine scaffold with hydrophobic aryl groups in positions 2 and 4, ester groups in position 5 (reaching optimum lipophilicity - *log*P range 4.4 - 5.8), amino group in position 3 (hydrogen bond donor) and methoxyphenyl groups (bearing appropriate amount of hydrogen bond acceptors) has led to potent P-glycoprotein, multidrug resistance-associated protein and the breast cancer resistance protein inhibitors which significantly exceeded activity of Verapamil, MK-571, Reversan. Significantly cheaper furo[2,3-*b*]pyridines as bioisosteres of thieno[2,3-*b*]pyridines have shown no activity on P-gp and MRP1 inhibition, but have found to be selective on BCRP1 inhibition.

SAR data in thieno[2,3-*b*]pyridine series indicate necessity of both hydrogen bond donor (acylation of amino group in position 3 resulted in loss of activity) and hydrogen bond acceptors (four to five OMe groups in different positions) to reach optimum activity.

4. Experimental

4.1. Chemistry

Unless noted otherwise, all reagents and solvents were used as purchased without further purification. Melting points were determined on OptiMelt MPA100 apparatus and are uncorrected. Analytical TLC was performed using silica gel 60 F_{254} plates (Merck). ^{1}H NMR spectra were recorded on a Varian Mercury BB 400 MHz spectrometer, and ^{13}C NMR spectra were recorded on Varian Mercury BB (100 MHz) spectrometer using $CDCl_3$ or DMSO-d_6 as the solvents. The chemical shifts of the atoms are reported in parts per million (ppm) relatively to the residual signals of the solvent: $CDCl_3$ (d:7.26) or DMSO-d_6 (2.50) for ^{1}H NMR spectra and $CDCl_3$ (d:77.16) or DMSO-d_6 (39.52) for ^{13}C NMR spectra. Multiplicities are abbreviated as: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, qui = quintet, m = multiplet. The coupling constants are expressed in Hz. The IR spectra have been recorded on Shimadzu IR Prestige-21 spectrometer in nujol or KBr tablet and peak positions ν_{max} are expressed in cm^{-1}. Compounds **5e**,[29] **5r**,[34] **6j**,[34] **6z**[34] have been described in our previous work. Compounds **5f**,[30] **5g**,[31] **5h**,[32] **5r**[33] and **13b**[37] are known.

4.1.1. General procedure for the preparation of 6-thioxo-1,6-dihydropyridines from chalcones (method A)

A mixture of corresponding chalcone 1 (5 mmol), 2-cyanothioacetamide 2 (5 mmol) and triethylamine (0.35 mmol) in ethanol was refluxed for 15 h. The precipitated crystals were separated by filtration and purified by washing with ethanol and water during the filtration.

4.1.1.1. 5-Acetyl-6-methyl-2-thioxo-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3-carbonitrile (5a)

Yellow crystals; yield 42 %; mp 244 - 246 °C. ^{1}H NMR ($CDCl_3$) δ 1.83 (3H, s); 2.50 (3H, s); 3.87 (3H, s) 3.92 (6H, s); 6.62 (2H, s); 12.16 (1H, br.s). IR (KBr) ν 1685, 2224, 3233. Anal Calcd. for $C_{18}H_{18}N_2O_4S$: C, 60.32; H, 5.06; N, 7.82. Found: C, 60.03; H, 5.08; N, 7.76.

4.1.1.2. 5-Cyano-4-(3,4-dimethoxyphenyl)-2-methyl-6-thioxo-1,6-dihydropyridine-3-carboxylic acid methyl ester (5c)

Yellow crystals; yield 42 %; mp 230 - 232 °C. ^{1}H NMR ($CDCl_3$) δ 2.58 (3H, s); 3.54 (3H, s); 3.90 (3H, s); 3.93 (3H, s); 6.90-6.98 (3H, m); 12.50 (1H, br.s). IR (KBr) ν 1721, 2226, 3241. Anal

Calcd. for $C_{17}H_{16}N_2O_4S$: C, 59.29; H, 4.68; N, 8.13. Found: C, 59.20; H, 4.52; N, 7.98.

4.1.1.3. 5-Cyano-2-methyl-6-thioxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyridine-3-carboxylic acid methyl ester (5d)

Yellow crystals; yield 45 %; mp 233 - 234 °C. ^{1}H NMR ($CDCl_3$) δ 2.58 (3H, s); 3.54 (3H, s); 3.87 (6H, s); 3.90 (3H, s); 6.60 (2H, s); 12.48 (1H, br.s). IR (KBr) ν 1733, 2227, 3070, 3177. Anal Calcd. for $C_{18}H_{18}N_2O_5S$: C, 57.74; H, 4.85; N, 7.48. Found: C, 57.10; H, 4.61; N, 7.76.

4.1.1.4. 5-Cyano-2-methyl-6-thioxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyridine-3-carboxylic acid 2-methoxyethyl ester (5m)

Yellow crystals; yield 42 %; mp 192 - 194 °C. ^{1}H NMR ($CDCl_3$) δ 2.58 (3H, s); 3.21-3.23(5H, m); 3.86 (6H, s); 3.89 (3H, s); 4.07 (2H, t, J = 4.7 Hz); 6.59 (2H, s); 12.57 (1H, s). IR (KBr) ν 1738, 2232, 3178. Anal Calcd. for $C_{20}H_{22}N_2O_6S$: C, 57.40; H, 5.30; N, 6.69. Found: C, 57.08; H, 5.33; N, 6.75.

4.1.1.5. 5-Cyano-2-methyl-6-thioxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyridine-3-carboxylic acid 2-propoxyethyl ester (5n)

Yellow crystals; yield 57 %; mp 140 - 141 °C. 1H NMR ($CDCl_3$) δ 0.87 (3H, t, J = 7.0 Hz); 1.51 (2H, se, J = 7.0 Hz), 2.60 (3H, s); 3.25-3.30 (4H, m) 3.87 (6H, s); 3.90 (3H, s); 4.10 (2H, t, J = 4.7 Hz); 6.61 (2H, s); 12.71 (1H, br.s). IR (KBr) ν 1730, 2228, 3178. Anal Calcd. for $C_{22}H_{26}N_2O_6S$: C, 59.18; H, 5.87; N, 6.27. Found: C, 59.10; H, 5.78; N, 6.21.

4.1.2. General procedure for the preparation of 6-thioxo-1,6-dihydropyridines from thioacrylamides (method B)

A mixture of corresponding thioacrylamide **4** (5 mmol), acetoacetate **3** (5 mmol) and piperidine (0.5 mmol) in ethanol was refluxed for 15 h. The precipitated crystals were separated by filtration and purified by washing with ethanol and water during the filtration.

4.1.2.1. 5-Cyano-4-(4-methoxyphenyl)-2-methyl-6-thioxo-1,6-dihydropyridine-3-carboxylic acid methyl ester (5b)

Yellow crystals; yield 53 %; mp 183 - 185 °C. ^{1}H NMR ($CDCl_3$) δ 2.58 (3H, s); 3.53 (3H, s); 3.86 (3H, s); 6.99 (2H, d, J = 8.6 Hz); 7.32 (2H, d, J = 8.6 Hz); 12.65 (1H, br.s). IR (KBr) ν 1722, 2214, 2949, 3000. Anal Calcd. for $C_{16}H_{14}N_2O_3S$: C, 61.13; H, 4.49; N, 8,91. Found: C, 60.99; H, 4.22; N, 7.49.

4.1.2.2. 5-Cyano-4-(4-ethoxyphenyl)-2-methyl-6-thioxo-1,6-dihydropyridine-3-carboxylic acid ethyl ester (5i)

Yellow crystals; yield 54 %; mp 184 - 185 °C. ^{1}H NMR ($CDCl_3$) δ 0.91 (3H, t, J = 7.0 Hz); 1.44 (3H, t, J = 7.0 Hz); 2.57 (3H, s); 3.98 (2H, q, J = 7.0 Hz); 4.07 (2H, q, J = 7.0 Hz); 6.97 (2H, d, J = 8.6 Hz); 7.31 (2H, d, J = 8.6 Hz)); 12.06 (1H, br.s). IR (KBr) ν 1709, 2230, 3034, 3160. Anal Calcd. for $C_{18}H_{18}N_2O_3S$: C, 63.14; H, 5.30; N, 8.18. Found: C, 63.05; H, 5.25; N, 8.18.

4.1.2.3. 4-(4-Butoxyphenyl)-5-cyano-2-methyl-6-thioxo-1,6-dihydropyridine-3-carboxylic acid ethyl ester (5j)

Yellow crystals; yield 59 %; mp 175 - 176 °C. ^{1}H NMR ($CDCl_3$) δ 0.92 (3H, t, J = 7.0 Hz), 0.99 (2H, se, J = 7.0 Hz), 1.50 (2H, qui, J = 6.3 Hz); 1.79 (2H, t, J = 6.3 Hz); 2.58 (3H, s); 3.88 (2H, q, J = 7.0 Hz); 4.00 (2H, t, J = 7.0 Hz); 6.96 (2H, d, J = 9.0 Hz); 7.31 (2H, d, J = 9.0 Hz); 12.41 (1H, br.s). IR (KBr) ν 1710, 2231, 3159. Anal Calcd. for $C_{20}H_{22}N_2O_3S$: C, 64.84; H, 5.99; N, 7.56. Found: C, 64.48; H, 6.14; N, 7.50.

4.1.2.4. 5-Cyano-2-methyl-6-thioxo-4-(3,4,5-trimethoxyphenyl-1,6-dihydropyridine-3-carboxylic acid n-butyl ester (5k)

Yellow crystals; yield 46 %; mp 197 - 198 °C. ^{1}H NMR ($CDCl_3$) δ 0.79 (3H, t, J = 7.0 Hz); 1.05 (2H, se, J = 7.0 Hz); 1.25 (2H, qui, J = 6.3 Hz); 2.59 (3H, s); 3.87 (3H, s); 3.90 (6H, s); 3.92 (2H, t, J = 6.3 Hz); 6.61 (2H, s); 12.40 (1H, br.s). IR (KBr) ν 1705, 2228, 3303. Anal Calcd. for $C_{21}H_{24}N_2O_5S$: C, 60.56; H, 5.81; N, 6.73. Found: C, 60.02; H, 5.78; N, 6.55.

4.1.2.5. 5-Cyano-2-methyl-6-thioxo-4-(4-methoxyphenyl)-1,6-dihydropyridine-3-carboxylic acid 2-methoxyethyl ester (5l)

Yellow crystals; yield 53 %; mp 210 - 211 °C. ^{1}H NMR ($CDCl_3$) δ 2.57 (3H, s); 3.24-3.28 (5H, m); 3.86 (3H, s); 4.09 (2H, t, J = 4.7 Hz); 6.99 (2H, d, J = 8.6 Hz); 7.34 (2H, d, J = 8.6 Hz). IR (KBr) ν 1726, 2226, 3167, 3479, 3579. Anal Calcd. for $C_{18}H_{18}N_2O_4S$: C, 60.32; H, 5.06; N, 7.82. Found: C, 59.78; H, 5.13; N, 7.78.

4.1.2.6. 5-Cyano-6-thioxo-2,4-bis-(3,4,5-trimethoxyphenyl)-1,6-dihydropyridine-3-carboxylic acid ethyl ester (5o)

Yellow crystals; yield 44 %; mp 178 - 179 °C. ^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, J = 7.0 Hz); 3.86 (9H, s); 3.90 (9H, s); 4.00 (2H, q, J = 7.0 Hz); 6.57 (2H, s); 6.88 (2H, s); 8.67 (1H, s). IR (KBr) ν 1727, 2223, 3148. Anal Calcd. for $C_{27}H_{28}N_2O_8S$: C 59.99; H 5.22; N 5.18. Found: C 60.12; H 5.11; N 5.28.

4.1.2.7. 6-Amino-2-thioxo-4-(3,4,5-trimethoxyphenyl)-1,2-dihydropyridine-3,5-dicarbonitrile (5p)

Yellow crystals; yield 41 %; mp 197 - 198 °C. ^{1}H NMR (DMSO-d_6) δ 3.70 (6H, s) and 3.77 (3H, s); 6.84 (2H, s); 8.11 (2H, s); 13.25 (1H, s). IR (KBr) ν 2212, 3189, 3341. Anal Calcd. for $C_{16}H_{14}N_4O_3S$: C 56.13; H 4.12; N 16.36. Found: C 56.00; H 4.22; N 16.28.

4.1.3. General procedure for the preparation of thieno[2,3-b]pyridines

To a solution of corresponding 6-thioxo-1,6-dihydropyridine **5** (1 mmol) in ethanol 3M sodium hydroxide water solution (2.4 mmol) was added. The reaction mixture was shortly refluxed to form thiolate. Then corresponding 2-bromoacetophenone (1 mmol) was added, the reaction mixture was refluxed for 5 min and stirred at room temperature for 30 min. The precipitated crystals were separated by filtration and purified by washing with ethanol and water during the filtration.

4.1.3.1. 5-Acetyl-3-amino-2-(4-methoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine (6a)

Yellow crystals; yield 78 %; mp 162 - 164 °C. ^{1}H NMR ($CDCl_3$) δ 2.06 (3H, s); 2.60 (3H, s); 3.86 (6H, s); 3.88 (3H, s); 3.95 (3H, s); 6.59 (2H, s); 6.79 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.87

(2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 23.3, 32.3, 55.4, 56.4, 61.2, 105.2, 106.4, 113.6, 119.0, 128.5, 130.1, 133.4, 134.1, 139.0, 142.9, 150.1, 153.7, 155.3, 162.1, 189.6, 204.9. IR (nujol) v 1699, 3302, 3468. Anal Calcd. for $C_{27}H_{26}N_2O_6S$: C, 64.02; H, 5.17; N, 5.53. Found: C, 64.07; H, 5.18; N, 5.46.

4.1.3.2. 3-Amino-2-(4-methoxybenzoyl)-4-(4-methoxyphenyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid methyl ester (6b)

Yellow crystals; yield 78 %; mp 179 - 180 °C. ^{1}H NMR ($CDCl_3$) δ 2.67 (3H, s); 3.58 (3H, s); 3.88 (3H, s); 3.90 (3H, s); 6.68 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.04 (2H, d, J = 8.6 Hz); 7.32 (2H, d, J = 8.6 Hz); 7.86 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 23.3, 52.3, 55.4, 105.0, 113.6, 114.3, 119.7, 125.7, 126.9, 129.7, 130.0, 133.4, 145.0, 150.3, 156.6, 160.4, 162.1, 162.6, 168.3, 189.5. IR (nujol) v 1606, 1730, 3302, 3474. Anal Calcd. for $C_{25}H_{22}N_2O_5S$: C, 64.92; H, 4.79; N, 6.06. Found: C, 64.55; H, 4.78; N, 5.94.

4.1.3.3. 3-Amino-4-(3,4-dimethoxyphenyl)-2-(4-methoxybenzoyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid methyl ester (6c)

Yellow crystals; yield 84 %; mp 192 - 194 °C. ^{1}H NMR ($CDCl_3$) δ 2.61 (3H, s); 3.54 (3H, s); 3.82 (3H,s); 3.83 (3H, s); 3.91 (3H, s); 6.67 (2H, br.s); 6.83-6.95 (3H, m); 6.91 (2H, d, J = 8.6 Hz); 7.80 (2H, d, J = 8.6Hz). ^{13}C NMR ($CDCl_3$) 23.3, 52.4, 55.4, 55.9, 56.1, 105.0, 111.1, 111.4, 113.6, 119.5, 120.9, 125.9, 126.8, 130.0, 133.4, 144.9, 149.0, 149.8, 150.2, 156.7, 162.1, 162.6, 168.4, 189.5. IR (nujol) v 1699, 3463. Anal Calcd. for $C_{26}H_{24}N_2O_6S$: C, 63.40; H, 4.91; N, 5.69. Found: C, 62.91; H, 4.79; N, 5.62.

4.1.3.4. 3-Amino-2-(2,4-dimethoxybenzoyl)-4-(3,4-dimethoxyphenyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid methyl ester (6d)

Yellow crystals; yield 93 %; mp 168 - 169 °C. ^{1}H NMR ($CDCl_3$) δ 2.58 (3H, s); 3.53 (3H, s); 3.75 (3H, s); 3,80 (3H, s); 3.83 (3H, s); 3.91 (3H, s); 6.45-6.48 (2H, m); 6.64 (2H, br.s); 6.82-6.94 (3H, m); 7.20 (1H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 23.3, 52.3, 55.4, 55.6, 98.9, 104.3, 111.0, 111.4, 119.6, 120.9, 123.6, 126.0, 126.6, 129.9, 144.9, 149.0, 149.4, 149.8, 156.4, 157.9, 162.5, 162.9, 168.4, 189.9. IR (nujol) v 1725, 3459. Anal Calcd. for $C_{27}H_{26}N_2O_7S$: C, 62.06; H, 5.01; N, 5.36. Found: C, 61.50; H, 4.97; N, 5.22.

4.1.3.5. 3-Amino-2-(3,4-dimethoxyphenylcarbamoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid methyl ester (6e)

Yellow crystals; yield 97 %; mp 203 - 204 °C. ^{1}H NMR (DMSO-d_6) δ 2.58 (3H, s); 3.58 (3H, s); 3.72 (3H, s); 3.73 (3H, s); 3.76 (3H, s); 3.77 (6H, s); 6.02 (2H, br. s); 6.70 (2H, s); 6.89 (1H, d, J = 8.9 Hz); 7.23 (1H, dd, J = 2.1 Hz, J = 8.5 Hz); 7.30 (1H, d, J = 2.1 Hz); 9.40 (1H, s). ^{13}C NMR ($CDCl_3$) δ 22.6, 52.3, 55.3, 55.6, 56.1, 60.2, 97.5, 106.1, 106.4, 111.6, 113.2, 119.7, 126.4, 128.6, 132.1, 137.9, 143.8, 145.1, 146.5, 148.2, 152.7, 154.3, 159.2, 163.4, 167.6. IR (nujol) v 1658, 1718, 3101, 3304. Anal Calcd. for $C_{28}H_{29}N_3O_8S$: C, 59.25; H, 5.15; N, 7.40. Found: C, 59.01; H, 4.90; N, 7.40.

4.1.3.6. 3-Amino-2-(4-methoxybenzoyl)-6-methyl-4-phenyl-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6f)

Yellow crystals; yield 94 %; mp 150 - 152 °C. ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, J = 7.0 Hz); 2.70 (3H, s); 3.88 (3H, s); 4.01 (2H, q, J = 7.0 Hz); 6.57 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz), 7.41-7.54 (5H, m); 7.86 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 23.4, 55.4, 61.5, 105.2, 107.8, 109.8, 113.6, 119.3, 128.5, 128.8, 129.5, 130.0, 133.4, 145.0, 147.9, 150.2, 162.1, 189.5. IR (nujol) v 1725, 3301, 3477. Anal Calcd. for $C_{25}H_{22}N_2O_4S$: C, 67.25; H, 4.97; N, 6.27. Found: C, 67.02; H, 4.82; N, 6.15.

4.1.3.7. 3-Amino-2-(4-methoxybenzoyl)-4-(4-methoxyphenyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6g)

Yellow crystals; yield 86 %; mp 150 - 151 °C. ^{1}H NMR ($CDCl_3$) δ 1.02 (3H, t, J = 7.0 Hz); 2.68 (3H, s); 3.88 (3H, s); 3.89 (3H, s); 4.06 (2H, q, J = 7.0 Hz); 6.67 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.04 (2H, d, J = 8.6 Hz), 7.33 (2H, d, J = 8.6 Hz); 7.86 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 13.8, 23.3, 55.4, 61.5, 105.6, 113.6, 114.2, 119.7, 125.8, 127.1, 129.8, 130.0, 133.5, 144.9, 150.4, 156.6, 160.4, 162.1, 162.5, 167.8, 189.5. IR (nujol) v 1606, 1723, 3299, 3474. Anal Calcd. for $C_{26}H_{24}N_2O_5S$: C, 65.53; H, 5.08; N, 5.88. Found: C, 65.62; H, 4.93; N, 5.57.

4.1.3.8. 2-Acetyl-3-amino-4-(3,4-dimethoxyphenyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6i)

Yellow crystals; yield 75 %; mp 191 - 193 °C. ^{1}H NMR ($CDCl_3$) δ 0.96 (3H, t, J = 7.0 Hz); 2.37 (3H, s); 2.62 (3H, s); 3.81 (3H, s); 3.89 (3H, s); 4.01 (2H, q, J = 7.0 Hz); 6.60 (2H, br.s); 6.80-6.93 (3H, m). ^{13}C NMR ($CDCl_3$) δ 13.9, 23.1, 29.2, 56.0, 56.1, 61.6, 106.2, 111.0, 111.5, 119.9, 120.9, 125.8, 127.1, 145.0, 148.2, 149.0, 149.9, 156.5, 161.0, 167.7, 193.0. IR (nujol) v . Anal Calcd. for $C_{21}H_{22}N_2O_5S$: C, 60.85; H, 5.35; N, 6.76. Found: C, 60.71; H, 5.11; N, 6.69.

4.1.3.9. 3-Amino-4-(3,4,5-trimethoxyphenyl)-6-methyl-2-(4-methoxybenzoyl)-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6k)

Yellow crystals; yield 89 %; mp 204 - 206 °C. ^{1}H NMR ($CDCl_3$) δ 0.96 (3H, t, J = 7.0 Hz); 2.63 (3H, s); 3.80 (6H, s); 3.83 (3H, s); 3.87 (3H, s,); 4.02 (2H, q, J = 7.0 Hz); 6.55 (2H, s); 6.70 (2H, s); 6.91 (2H, d, J = 8.6 Hz); 7.80 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 13.8, 22.5, 22.7, 55.4, 56.0, 56.1, 56.4, 61.1, 61.7, 105.3, 105.6, 111.1, 111.4, 113.7, 119.8, 120.9, 125.6, 126.8, 127.3, 128.8, 130.0, 133.2, 138.8, 145.6, 149.1, 149.8, 150.1, 153.5, 156.2, 162.2, 167.2, 167.3, 189.5. IR (nujol) v 1724, 3301, 3469. Anal Calcd. for $C_{28}H_{28}N_2O_7S$: C, 62.67; H, 5.26; N, 5.22. Found: C, 62.38; H, 5.06; N, 5.16.

4.1.3.10. 3-Amino-2-(2,4-dimethoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6l)

Yellow crystals; yield 96 %; mp 174 - 176 °C. ^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t, J = 7 Hz); 2.60 (3H, s); 3.75 (3H, s); 3.80 (9H, s); 3.86 (3H, s); 4.01 (2H, q, J = 7.0 Hz); 6.45-6.54 (2H, m); 7.29-7.33 (3H, m). ^{13}C NMR ($CDCl_3$) δ 13.8, 23.3, 55.5, 55.6, 56.3, 61.1, 61.5, 98.9, 104.3, 105.7, 108.1, 119.3, 123.6, 126.4, 129.2, 129.9, 138.6, 144.8, 149.2, 153.4, 156.6, 157.9, 162.5, 162.8, 167.8, 189.9. IR (nujol) v 1717, 3306, 3445. Anal Calcd. for $C_{29}H_{30}N_2O_8S$: C, 61.47; H, 5.34; N, 4.94. Found: C, 60.99; H, 5.36; N, 4.62.

4.1.3.11. 3-Amino-4-(4-ethoxyphenyl)-2-(4-methoxybenzoyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6m)

Yellow crystals; yield 96 %; mp 150 - 151 °C. ^{1}H NMR ($CDCl_3$) δ 1.02 (3H, t, J = 7.4 Hz); 1.48 (3H, t, J = 7.0 Hz); 2.68 (3H, s); 3.88 (3H, s); 4.06 (2H, q, J = 7.4 Hz); 4.11 (2H, q, J = 7.0 Hz); 6.66 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.02 (2H, d, J = 8.6 Hz); 7.31 (2H, d, J = 8.6 Hz); 7.86 (d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 13.8, 14.7, 23.3, 55.4, 61.5, 63.7, 105.0, 113.6, 114.7, 119.7, 125.6, 127.1, 129.8, 130.0, 133.5, 145.0, 150.4, 156.6, 159.8, 162.1, 167.8, 189.5. IR (nujol) v 1608, 1725, 3302, 3474. Anal Calcd. for $C_{27}H_{26}N_2O_5S$: C, 66.11; H, 5.34; N, 5.71. Found: C, 66.03; H, 5.34; N, 5.64.

4.1.3.12. 3-Amino-4-(4-butoxyphenyl)-2-(4-methoxybenzoyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6n)

Yellow crystals; yield 89 %; mp 105 - 106 °C. ^{1}H NMR ($CDCl_3$) δ 0.99-1.04 (6H, m); 1.49-1.59 (2H, m), 1.79-1.86 (2H, m); 2.68 (3H, s); 3.88 (3H, s); 4.02-4.09 (4H, m); 6.67 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.02 (2H, d, J = 8.6 Hz); 7.31 (2H, d, J = 8.6 Hz); 7.86 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 13.8, 19.2, 23.3, 31.2, 55.4, 61.5, 67.9, 105.0, 113.6, 114.7, 119.7, 125.6, 127.1, 129.7, 130.0, 133.5, 145.0, 150.4, 156.6, 160.0, 162.1, 162.5, 167.8, 189.5. IR (nujol) v 1608, 1726, 3296, 3474. Anal Calcd. for $C_{29}H_{30}N_2O_5S$: C, 67.16; H, 5.83; N, 5.40. Found: C, 67.00; H, 5.80; N, 5.36.

4.1.3.13. 3-Amino-4-(3,4,5-trimethoxyphenyl)-6-methyl-2-(4-methoxybenzoyl)-thieno[2,3-b]pyridine-5-carboxylic acid butyl ester (6o)

Yellow crystals; yield 93 %; mp 149 - 150 °C. ^{1}H NMR ($CDCl_3$) δ 0.85 (3H, t, J = 7.0 Hz), 1.20 (2H, se, J = 7.0 Hz), 1.38 (2H, qui, J = 6.7 Hz); 2.69 (3H, s); 3.86 (6H, s); 3.89 (3H, s); 3.94 (3H, s); 4.02 (2H, t, J = 6.7 Hz); 6.61 (2H, s); 6.77 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.87 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 13.5, 19.0, 23.3, 30.3, 55.4, 56.3, 61.1, 65.5, 105.1, 105.7, 113.6, 119.2, 126.7, 129.1, 130.0, 133.4, 138.6, 144.6, 150.1, 153.4, 156.7, 162.1, 162.5, 168.0, 189.5. IR (nujol) v 1582, 1723, 3305, 3468. Anal Calcd. for $C_{30}H_{32}N_2O_7S$: C, 63.81; H, 5.71; N, 4.96. Found: C, 63.62; H, 5.60; N, 5.02.

4.1.3.14. 3-Amino-2-(4-methoxybenzoyl)-4-(4-methoxyphenyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid 2-methoxyethyl ester (6p)

Yellow crystals; yield 90 %; mp 125 - 126 °C. ^{1}H NMR ($CDCl_3$) δ 2.69 (3H, s); 3.30 (3H, s); 3.36 (2H, t, J = 4.7 Hz); 3.88 (3H, s); 3.90 (3H, s); 4.15 (2H, t, J = 4.7 Hz); 6.64 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.04 (2H, d, J = 8.6 Hz); 7.34 (2H, d, J = 8.6 Hz); 7.86 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 23.3, 55.4, 58.8, 64.2, 70.0, 105.0, 113.6, 114.2, 119.6, 125.8, 126.8, 129.8, 130.0, 133.4, 145.0, 150.3, 156.7, 160.4, 162.1, 162.6, 167.8, 189.5. IR (nujol) v 1607, 1729, 3304, 3474. Anal Calcd. for $C_{27}H_{26}N_2O_6S$: C, 64.02; H, 5.17; N, 5.53. Found: C, 63.50; H, 5.15; N, 5.35.

4.1.3.15. 3-Amino-2-benzoyl-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid 2-methoxyethyl ester (6q)

Yellow crystals; yield 90 %; mp 100 - 101 °C. ^{1}H NMR ($CDCl_3$) δ 2.69 (3H, s); 3.28 (3H, s); 3.35 (2H, t, J = 4.7 Hz); 3.87 (6H, s); 3.95 (3H, s); 4.17 (2H, t, J = 4.7 Hz); 6.62 (2H, s); 6.82 (2H, br.s); 7.46-7.55(3H, m); 7.83 (2H, d, J = 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ 23.3, 56.3, 58.7, 61.1, 64.4, 70.0, 105.2, 105.6, 119.0, 126.4, 127.8, 128.4, 128.9, 130.0, 131.2, 138.6, 140.8, 145.0, 150.4, 153.5, 157.0, 162.8, 167.7, 190.6. IR (nujol) v 1745, 3466. Anal Calcd. for $C_{28}H_{28}N_2O_7S$: C, 62.67; H, 5.26; N, 5.22. Found: C, 62.08; H, 5.66; N, 5.00.

4.1.3.16. 3-Amino-2-(4-methoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid 2-methoxyethyl ester (6r)

Yellow crystals; yield 82 %; mp 154 - 155 °C. ^{1}H NMR ($CDCl_3$) δ 2.70 (3H, s), 3.28 (3H, s); 3.35 (2H, t, J = 4.7 Hz); 3.87 (6H, s); 3.89 (3H, s); 3.95 (3H, s); 4.17 (2H, t, J = 4.7 Hz); 6.62 (2H, s); 6.78 (2H, br.s); 6.97 (2H, d, J = 8.6 Hz); 7.87 (2H, d, J = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 23.3, 55.4, 56.3, 58.7, 61.1, 64.4, 70.0, 105.1, 113.6, 119.2, 126.3, 129.0, 130.0, 133.4, 138.6, 144.8, 150.1, 153.4, 156.8, 162.1, 162.6, 167.8, 189.5. IR (nujol) v 1727, 3304, 3467. Anal Calcd. for $C_{29}H_{30}N_2O_8S$: C, 61.47; H, 5.34; N, 4.94. Found: C, 61.06; H, 5.12; N, 5.09.

4.1.3.17. 3-Amino-2-(2,4-dimethoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid 2-methoxyethyl ester (6s)

Yellow crystals; yield 80 %; mp 129 - 131 °C. ^{1}H NMR ($CDCl_3$) δ 2.67 (3H, s); 3.28 (3H, s); 3.27 (2H, t, J = 4.7 Hz); 3.82 (3H, s); 3.87 (9H, s); 3.94 (3H, s); 4.16 (2H, t, J = 4.7 Hz); 6.52-6.55 (2H, m); 6.60 (2H, s); 6.72 (2H, br.s); 7.38 (1H, d, J = 8.2 Hz). ^{13}C NMR ($CDCl_3$) δ 23.3, 55.5, 55.6, 56.3, 58.7, 61.1, 64.3, 70.0, 99.0, 104.3, 105.6, 108.2, 119.3, 123.6, 126.1, 129.1, 130.0, 138.5, 144.9, 149.2, 153.4, 156.7, 162.5, 163.0, 167.8, 189.9. IR (nujol) v 1728, 3307, 3469. Anal Calcd. for $C_{30}H_{32}N_2O_9S$: C, 60.39; H, 5.41; N, 4.70. Found: C, 60.11; H, 5.32; N, 4.60.

4.1.3.18. 3-Amino-6-methyl-2-(3,4,5-trimethoxybenzoyl)-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid 2-methoxyethyl ester (6t)

Yellow crystals; yield 81 %; mp 172 - 173 °C. ^{1}H NMR ($CDCl_3$) δ 2.71 (3H, s); 3.29 (3H, s); 3.55 (2H, t, J = 4.7 Hz); 3.87 (6H, s); 3.92 (9H, s); 3.95 (3H, s); 4.18 (2H, t, J = 4.7 Hz); 6.62 (2H, s); 6.82 (2H, br.s); 7.15 (2H, s). ^{13}C NMR ($CDCl_3$) δ 23.4, 56.2, 56.3, 58.7, 61.0, 61.1, 64.4, 70.0, 104.7, 105.5, 105.6, 106.0 119.1, 126.5, 128.9, 135.8, 138.7, 140.7, 145.0, 150.7, 153.0, 153.5, 157.1, 162.7, 167.7, 189.3. IR (nujol) v 1582, 1728, 3464. Anal Calcd. for $C_{31}H_{24}N_2O_{10}S$: C, 59.41; H, 5.47; N, 4.47. Found: C, 59.37; H, 5.42; N, 4.39.

4.1.3.19. 3-Amino-2-(4-methoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid 2-propoxyethyl ester (6u)

Yellow crystals; yield 86 %; mp 79 - 80 °C. ^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, J = 7.0 Hz); 1.51-1.59 (2H, m); 2.70 (3H, s); 3.33 (2H, t, J = 6.7 Hz); 3.41 (2H, t, J = 4.7 Hz); 3.86 (6H, s); 3.89 (3H, s); 3.95 (3H, s); 4.17 (2H, t, J = 4.7 Hz); 6.62 (2H, s,); 6.78 (2H, br.s); 6.98 (2H, d, J = 9 Hz); 7.87 (2H, d, J = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 10.4, 22.7, 23.3, 55.4, 56.3, 61.1, 64.6, 68.1, 73.0, 105.1, 105.6, 113.6, 119.2, 126.4, 129.0, 130.0, 133.4, 138.6, 144.8, 150.1, 153.4, 156.8, 162.1, 162.6, 167.8, 189.5. IR (nujol) v 1582, 1729, 3303, 3467. Anal Calcd. for $C_{31}H_{34}N_2O_8S$: C, 62.61; H, 5.76; N, 4.71. Found: C, 61.99; H, 5.77; N, 4.60.

4.1.3.20. 3-Amino-2-(4-methoxybenzoyl)-4,6-bis-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6v)

Yellow crystals; yield 95%; mp 196 - 197 °C. ^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, J = 7.4 Hz); 3.86 (9H, s); 3.97 (9H, s); 3.89 (3H,

s); 3.94 (2H, q, *J* = 7.4 Hz); 6.65 (2H, s); 6.96 (2H, s); 6.98 (2H, d, *J* = 9.0 Hz); 7.87 (2H, d, *J* = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 13.7, 55.4, 56.1, 56.3, 60.9, 61.1, 61.6, 105.8, 105.9, 113.6, 119.9, 126.3, 128.7, 130.1, 133.3, 134.2, 138.7, 139.2, 145.6, 149.8, 153.2, 153.4, 156.7, 162.2, 162.5, 167.7, 189.5. IR (nujol) *v* 1583, 1728, 3300, 3466. Anal Calcd. for $C_{36}H_{36}N_2O_{10}S$: C, 62.78; H, 5.27; N, 4.07. Found: C, 62.69; H, 5.21; N, 4.05.

4.1.3.21. 3,6-Diamino-2-(4-methoxybenzoyl)-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carbonitrile (6w)

Yellow crystals; yield 41 %; mp 241 - 242 °C. ^{1}H NMR (DMSO-d_6) δ 3.72 (3H, s); 3.78 (6H, s); 3.84 (3H, s); 6.89 (2H, s); 7.06 (2H, d, *J* = 9.0 Hz); 7.70 (2H, br.s); 7.89 (2H, br.s); 8.02 (2H, d, *J* = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 55.3, 56.2, 60.1, 90.8, 99.2, 105.4, 112.5, 113.6, 115.5, 128.3, 129.1, 133.3, 138.3, 151.0, 153.3, 153.6, 159.1, 161.2, 165.8, 186.6. IR (nujol) *v* 1709, 2237, 3290, 3453. Anal Calcd. for $C_{25}H_{22}N_4O_5S$: C, 61.21; H, 4.52; N, 11.42. Found: C, 61.05; H, 4.55; N, 11.21.

4.1.3.22. 3-Amino-4,6-dimethyl-2-(2,4-dimetoxybenzoyl)-thieno[2,3-b]pyridine (6x)

Yellow crystals; yield 70 %; mp 184 - 186 °C. ^{1}H NMR ($CDCl_3$) δ 2.51 (3H, s); 2.70 (3H, s); 3.75 (3H, s); 3.80 (3H, s); 6.46-6.48 (2H, m); 6.89 (1H, s); 7.14 (2H, br.s); 7.33 (1H, d, *J* = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 20.5, 55.5, 55.6, 98.9, 104.4, 108.5, 121.9, 123.5, 129.9, 150.1, 157.9, 162.5, 190.1. IR (nujol) *v* 1597, 3297, 3467. Anal Calcd. for $C_{18}H_{18}N_2O_3S$: C, 63.14; H, 5.30; N, 8.18. Found: C, 62.87; H, 5.17; N, 8.09.

4.1.4. Preparation of 3-amino-6-methyl-4-(3,4,5-trimethoxyphenyl)-2-(4-methoxybenzoyl)-thieno[2,3-b]pyridine (6y)

A mixture of 4-(3,4,5-trimethoxyphenyl)-3-buten-2-one (1 mmol), 2-cyanothioacetamide 2 (1 mmol) and piperidine (1.1 mmol) in 10 ml of ethanol was stirred at room temperature for 12 h. Then 2-bromo-1-(4-methoxyphenyl)ethanone (1 mmol) was added and reaction mixture was shortly heated until reflux and stirred for 3 h at room temperature. Then 3M sodium hydroxide water solution (1 mmol) was added. Reaction mixture was refluxed for 1 min and stirred at room temperature for 30 min. The precipitates was filtered and purified by washing with 1 ml of cold ethanol and water during the filtration. Yellow crystals; yield 89 %; mp 234 - 235 °C. ^{1}H NMR ($CDCl_3$) δ 2.67 (3H, s); 3.87 (3H, s); 3.88 (6H, s); 3.93 (3H, s); 6.63 (2H, s); 6.90 (2H, br.s); 6.96 (2H, d, *J* = 9.0 Hz); 6.98 (1H, s); 7.87 (2H, d, *J* = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 24.7, 55.4, 56.3, 61.1, 104.6, 105.5, 113.5, 119.3, 121.2, 130.1, 132.0, 133.6, 138.5, 147.8, 150.1, 153.5, 160.1, 162.0, 189.5. IR (nujol) *v* 3303, 3467. Anal Calcd. for $C_{25}H_{24}N_2O_5S$: C, 64.64, H, 5.21, N, 6.03. Found: C, 64.52, H, 5.14, N, 5.93.

4.1.5. Preparation of 3-acetylamino-2-(4-methoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-b]pyridine-5-carboxylic acid 2-methoxyethylester (7)

3-Amino-2-(4-methoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-thieno[2,3-*b*]pyridine-5-carboxylic acid 2-methoxyethyl ester (0.3 mmol) and sodium hydroxide (0.6 mmol) in 2 ml acethylchloride was refluxed for 2 h under argon. Reaction mixture was poured into ice water, neutralized, extracted with DCM, and dried over $MgSO_4$. Resulting oil was recrystallized from ethanol. Yellow crystals; yield 71%; mp 183 - 184 °C. ^{1}H NMR ($CDCl_3$) δ 1.51 (3H, s); 2.73 (3H, s); 3.28 (3H, s); 3.36 (2H, t, *J* = 4.7 Hz); 3.87 (6H, s); 3.88 (3H, s); 3.90 (3H, s); 4.20 (2H, t, *J* = 4.7 Hz); 6.60 (2H, s); 6.94 (2H, d, *J* = 8.6 Hz); 7.19 (1H, s); 7.94 (2H, d, *J* = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 22.9, 23.1, 55.5, 56.3, 58.7, 60.9, 64.4, 70.0, 106.3, 113.7, 114.6, 123.1, 127.4, 129.1, 129.2, 129.9, 130.2, 132.2, 138.1, 143.5, 153.0, 154.7, 159.8, 163.8, 167.7, 167.8, 188.0. IR (nujol) *v* 1732, 3272, 3467. Anal Calcd. for $C_{31}H_{32}N_2O_9S$: C, 61.17; H 5.30; N 4.60. Found: C, 61.25, H 5.22, N 4.66.

4.1.6. Preparation of 5-cyano-4-(4-difluoromethoxyphenyl)-6-[2-(4-methoxyphenyl)-2-oxo-ethylsulfanyl]-2-methyl-1,4-dihydropyridine-3-carboxylic acid ethyl ester (9)

4-Difluoromethoxybenzaldehyde (2.5 mmol) and ethyl 3-oxobutanoate (2.5 mmol) were dissolved in 10 ml of ethanol and piperidine was added (2.0 mmol). Mixture was stirred at room temperature for 30 min. Then 2-cyanothioacetamide (2.5 mmol) and piperidine (0.8 mmol) were added and reaction mixture was shortly refluxed for 30 seconds and stirred at room temperature for 3 hours. 2-Bromo-1-(4-methoxyphenyl)ethanone (2.5 mmol) was added and reaction mixture was shortly refluxed and stirred at room temperature for 30 min. The formed precipitate was filtered and purified by washing with 3 ml of cold ethanol and water during the filtration. White crystals; yield 85 %; mp 148 - 149 °C. ^{1}H NMR ($CDCl_3$) δ 1.13 (3H, t, *J* = 7.0 Hz); 2.41 (3H, s); 3.89 (3H, s); 4.03 (2H, q, *J* = 7.0 Hz); 4.66 (1H, s); 6.44 (1H, t, *J* = 73.2 Hz); 6.96 (2H, d, *J* = 9.0 Hz); 7.22 (2H, d, *J* = 9.0 Hz); 7.22 (2H, d, *J* = 8.6 Hz); 7.93 (2H, d, *J* = 8.6 Hz); 8.61 (1H, s). ^{13}C NMR ($CDCl_3$) δ 14.1, 19.5, 37.9, 42.0, 55.7, 60.1, 90.5, 101.6, 114.4, 118.7, 119.5, 127.5, 128.8, 131.5, 142.0, 144.9, 150.3, 165.1, 166.7, 195.8. IR (nujol) *v* 1668, 2199, 3282. Anal Calcd. for $C_{26}H_{24}N_2O_5S$: C, 60.69, H, 4.70, N, 5.44. Found: C, 60.59, H, 4.65, N, 5.77.

4.1.7. Preparation of 5-cyano-4-(4-difluoromethoxyphenyl)-6-[2-(4-methoxyphenyl)-2-oxo-ethylsulfanyl]-2-methyl-nicotinic acid ethyl ester (10)

5-Cyano-4-(4-difluoromethoxyphenyl)-6-[2-(4-methoxyphenyl)-2-oxo-ethylsulfanyl]-2-methyl-1,4-dihydropyridine-3-carboxylic acid ethyl ester (1 mmol) was dissolved in 8 ml glacial acetic acid and manganese (III) acetate dihydrate (2 mmol) was added. Reaction mixture was refluxed for 2 hours, then poured into ice water, neutralized, extracted with DCM, and dried over $MgSO_4$. Resulting oil was was recrystallized from ethanol. White crystals; yield 30 %; mp 126 - 127 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.0 Hz); 2.36 (3H, s); 3.89 (3H, s); 3.98 (2H, q, *J* = 7.0 Hz); 4.66 (2H, s); 6.55 (1H, t, *J* = 73.2 Hz); 6.97 (2H, d, *J* = 9.0 Hz); 7.18 (2H, *J* = 8.6 Hz); 7.32 (2H, d, *J* = 9.0 Hz); 8.05 (2H, d, *J* = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 13.5, 23.2, 37.2, 55.5, 61.8, 104.5, 112.9, 113.9, 114.3, 115.5, 118.1, 119.5, 125.2, 129.2, 129.9, 130.7, 131.6, 151.2, 152.1, 158.9, 162.3, 163.9, 166.5, 191.3. IR (nujol) *v* 1684, 1724, 2224**.** Anal Calcd. for $C_{26}H_{22}F_2N_2O_5S$: C, 60.93; H 4.33; N 5.47. Found: C, 60.25, H 4.22, N 5.66.

4.1.8. Preparation of 3-amino-4-(4-difluoromethoxyphenyl)-2-(4-methoxybenzoyl)-6-methyl-thieno[2,3-b]pyridine-5-carboxylic acid ethyl ester (6h)

5-Cyano-4-(4-difluoromethoxyphenyl)-6-[2-(4-methoxyphenyl)-2-oxo-ethylsulfanyl]-2-methyl-nicotinic acid ethyl ester (0.2 mmol) was dissolved in 3 ml of ethanol and 3M sodium hydroxide water solution (0.2 mmol) was added. Reaction mixture was shortly refluxed and stirred at room temperature for 1 hour. The precipitate was filtered and purified by washing with 1 ml of cold methanol and water during the filtration. Yellow crystals; yield 60 %; mp 140 - 141 °C. ^{1}H NMR ($CDCl_3$) δ 0.96 (3H, t, *J* = 7.0 Hz); 4.00 (2H, q, *J* = 7.0 Hz); 2.68 (3H, s); 3.87

(3H, s); 6.61 (1H, t, *J* = 73.2 Hz); 6.94 (2H, d, *J* = 9.0 Hz); 6.82 (2H, br.s); 7.27 (2H, d, *J* = 8.6 Hz); 7.41 (2H, d, *J* = 8.6 Hz); 7.85 (2H, d, *J* = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 13.7, 23.4, 55.4, 61.6, 113.6, 115.3, 119.2, 119.8, 130.1, 131.1, 133.3, 143.7, 149.8, 156.8, 162.2, 162.5, 167.5, 189.6. IR (nujol) *v* 1610, 1724, 3296, 3465. Anal Calcd. for $C_{26}H_{22}F_2N_2O_5S$: C, 60.93; H 4.33; N 5.47. Found: C, 60.86, H 4.22, N 5.43.

4.1.9. Preparation of 5-cyano-2-methyl-6-oxo-4-(3,4,5-trimethoxyphenyl)-1,6-dihydropyridine-3-carboxylic acid ethyl ester (13a)

A mixture of 3-cyano-4-(3,4,5-trimethoxyphenyl)acrylamide (2.62 g, 10 mmol), aminocrotonic acid ethyl ester (1.29g, 10 mmol), 10 ml of glacial acid and 10 ml of absolute ethanol was refluxed for 4h. Then reaction mixture was evaporated under reduced pressure and resulting slurry was washed with water. Then reaction mixture was diluted with 10 ml of ethanol, and 1 ml of concentrated nitric acid was added. The resulting mixture was refluxed for 8 h, then cooled to the room temperature, diluted with water and neutralized with 2M $NaHCO_3$ solution in water. The resulting crude product was recrystallized from ethanol. White crystals; yield 32 %; mp 223 - 224 °C. ^{1}H NMR ($CDCl_3$) δ 0.85 (3H, t, *J* = 7.0 Hz); 2.54 (3H, s); 3.81 (6H, s); 3.84 (3H, s); 3.93 (2H, q, *J* = 7.0 Hz); 6.54 (2H, s); 13.53 (1H, s). IR (nujol) *v* 1659, 1717, 2226, 3012, 3147. Anal Calcd. for $C_{19}H_{20}N_2O_6$: C, 61.28; H 5.41; N 7.52. Found: C, 60.73, H 5.27, N 7.21.

4.1.10. General procedure for the preparation of furo[2,3-b]pyridines

To a solution of corresponding 6-oxo-1,6-dihydropyridine (1 equiv) in *N*,*N*-dimethylformamide, potassium carbonate (4 equiv) was added and reaction mixture was stirred for 5 min at room temperature. Then corresponding bromoacetofenone (1 equiv) was added and reaction mixture was stirred at 80°C for 4h. Then reaction mixture was cooled to room temperature, diluted with water and neutralized with 2M HCl water solution. The precipitate was filtered and recrystallized from ethanol.

4.1.10.1. 3-Amino-2-(4-methoxybenzoyl)-6-methyl-4-(3,4,5-trimethoxyphenyl)-furo[2,3-b]pyridine-5-carboxylic acid ethyl ester (14a)

White crystals; yield 19 %; mp 174 - 176 °C. ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, *J* = 7.0 Hz); 2.40 (3H, s); 3.86 (6H, s); 3.89 (3H, s); 3.93 (3H, s); 4.00 (2H, q, *J* = 7.0 Hz); 5.72 (2H, s); 6.61 (2H, s); 6.98 (2H, d, *J* = 9.0 Hz); 7.92 (2H, d, *J* = 9.0 Hz). ^{13}C NMR ($CDCl_3$) δ 13.8, 23.4, 55.4, 56.3, 61.1, 61.7, 105.4, 108.2, 113.6, 125.7, 129.3, 129.9, 131.6, 133.4, 138.7, 140.6, 145.1, 153.5, 156.9, 159.4, 162.9, 168.0, 181.9. IR (nujol) *v* 1723, 3337, 3478. Anal Calcd. for $C_{28}H_{28}N_2O_8$: C, 64.61; H 5.42; N 5.38. Found: C, 64.23, H 5.17, N 5.18.

4.1.10.2. 3-Amino-4-(4-methoxyphenyl)-6-methyl-2-(3,4,5-trimethoxybenzoyl)-furo[2,3-b]pyridine-5-carboxylic acid ethyl ester (14b)

White crystals; yield 38 %; mp 188 - 190 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (3H, t, *J* = 7.0 Hz); 2.37 (3H, s); 3.78 (3H, s); 3.88 (9H, s); 3.95 (2H, q, *J* = 7.0 Hz); (2H, b.s); 6.91 (2H, d, *J* = 8.6 Hz); 7.18 (2H, s); 7.27 (2H, d, *J* = 8.6 Hz). ^{13}C NMR ($CDCl_3$) δ 13.6, 23.2, 55.3, 56.4, 61.0, 61.6, 68.6, 94.1, 105.4, 114.1, 114.4, 124.2, 127.0, 129.5, 129.6, 143.2, 153.3, 155.0, 158.5, 160.8, 162.5, 167.1, 191.6. IR (nujol) *v* 1617, 1719, 3481. Anal Calcd. for $C_{28}H_{28}N_2O_8$: C, 64.61; H 5.42; N 5.38. Found: C, 64.33, H 5.27, N 5.24.

4.2. Biology

4.2.1. Cell cultures

NIH3T3 (normal mouse fibroblasts), MES-SA (human uterine sarcoma), MES-SA/Dx5 (doxorubicin resistant, 300 ng/ml), MES-SA/MX2 (mitoxantrone resistant), H69 (human lung carcinoma), H69/AR (doxorubicin resistant) and A7R5 (rat aorta smooth muscle) cells were obtained from the ATCC cell collection.

The potency of P-glycoprotein-mediated MDR modulator *in vitro* was evaluated in the drug sensitive human sarcoma MES-SA cells and doxorubicin (DOX) resistant cells by accumulation of fluorescent substrate - rhodamine 123. Effects on MRP1-mediated drug efflux were observed in the DOX resistant human lung carcinoma H69AR cells using calcein AM assay. BCRP1-mediated drug efflux was also evaluated using fluorescent substrate Hoechst 33342 in the mitoxantrone resistant human sarcoma MES-SA/MX2 cells. The half maximal effective concentrations (EC_{50}) were calculated from dose response curve as the most effective method to compare MDR-modulating activities of compounds.[39,40]

4.2.2. Measurement of P-glycoprotein activity

About 50000 cells of the human uterine sarcoma cell line MES-SA and MES-SA/Dx5 were seeded into the 96-well plates and incubated for 24 h. The MDR modulators were incubated with the cells for 15 min. Then rhodamine-123 (2.6 μM) was added and the cells were incubated for 1h, 37 °C, 5% CO_2. The cells were washed twice with phosphate buffered saline (PBS) and fluorescence values were measured with excitation at 487 nm and emission at 557 nm by using the fluorescence reader Tecan infinite M1000. Verapamil, the known P-glycoprotein inhibitor was used as a positive control.[41] The EC_{50} values was calculated using the program Graph Pad Prism® 4.0. Verapamil was dissolved in water, while MK-571 and tested compounds in DMSO (0.5% in final concentration).

4.2.3. Measurement of MRP1 activity

About 60000 cells of the human lung carcinoma cell line H69 and H69AR were seeded into the 96-well plates and incubated for 24 h. The compounds were incubated with the cells for 15 min. Then Calcein AM (0.25 μM) was added and the cells were incubated for 30 min in a humidified atmosphere 5% CO_2 at 37 °C. The cells were washed twice with phosphate buffered saline (PBS) and fluorescence values were measured with excitation at 494 nm and emission at 517 nm by using the fluorescence reader Tecan infinite M1000. MK-571 was used as the known inhibitor of MRP1.[42] The EC50 values was calculated using the program Graph Pad Prism® 4.0.

4.2.4. Measurement of BCRP1 activity

About 50000 cells of the MES-SA and MES-SA/MX2 were seeded into the 96-well plates and incubated for 24 h. The MDR modulators were incubated with the cells for 15 min. Hoechst 33342 (5 μg/ml) was added and the cells were incubated for 90 min, 37 °C, 5% CO_2. The cells were washed twice with phosphate buffered saline (PBS) and fluorescence values were measured with excitation at 360 nm and emission at 450 nm. Verapamil was used as a positive control.[43] The EC_{50} values was calculated using the program Graph Pad Prism® 4.0.

4.2.5. Intracellular Ca^{2+} measurements

Changes in intracellular $[Ca^{2+}]_i$ concentration were studied using Fluo-4 NW Calcium Assay Kit ("Invitrogen", Sweden) accordingly to manufacturer's instructions. The A7R5 (rat aorta smooth muscle) cells were grown at 37 °C in a humidified atmosphere with 5% CO_2/95% air in DMEM medium containing 2 mM glutamine and supplemented with 10% FBS. The cells

were seeded into 96 well plate at 10000 cells per well and incubated for 72 hours. The cells were loaded with Fura-4NW for 45 min. The Fura-4NW loaded cells were pre-incubated in the dark for 15 minutes with tested compounds at concentrations from 0.8 to 100 μM. Then A7R5 cells were pre-treated with 1.5 mM $CaCl_2$ for 5 minutes and KCl (50 mM) was added to A7R5 cells to induce $[Ca^{2+}]_i$ increase. Amlodipine, the well-known calcium channel inhibitor, was used as the positive control. Changes in $[Ca^{2+}]_i$ were measured from the fluorescence emitted at 516 nm due to alternate excitation at 494 nm using the fluorescence spectrophotometer (Thermo Ascient, Finland). The IC_{50} values was calculated using the program Graph Pad Prism® 4.0.

4.2.6. Basal toxicity test

A low therapeutic index of chemotherapy agents is due to affecting not only cancer formation but also normal cells. Our compounds were tested on NIH 3T3 (normal mouse fibroblasts, "ATCC") cell line according the basal toxicity test.[44] Alternative LD_{50} values were calculated and non-toxic compounds were selected. 9000 NIH 3T3 cells/well were placed into 96-well plates for 24 h. Then exposed to the test compound over a range of eight concentrations (1-1000 μg/ml) for 24 h. Upon that, the cells were incubated with the neutral red dye for 4 h and then OD was determined at 540 nm. Alternative LD_{50} values were calculated according to the formula: log (LD_{50}[mmol/kg] = 0,435 x log (IC_{50}[mmol/l]) = 0,625. The IC_{50} values was calculated using the program Graph Pad Prism® 4.0.

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Supplementary Material

Supplementary data associated with this article can be found in the online version

Graphical Abstract

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Thieno[2,3-*b*]pyridines – a new class of multidrug resistance (MDR) modulators

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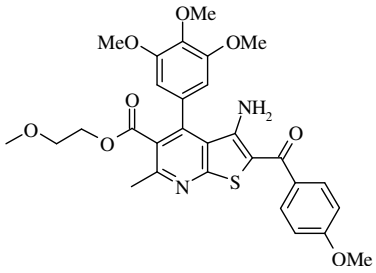
Aivars Krauze, Signe Grinberga, Laura Krasnova, Ilze Adlere, Elina Sokolova, Ilona Domracheva, Irina Shestakova, Zigmars Andzans, Gunars Duburs

Latvian Institute of Organic Synthesis, 21 Aizkraukles St., Riga, LV-1006, Latvia



P-gp inhibitor $EC_{50} = 0.3 \pm 0.2$ μM
MRP1 inhibitor $EC_{50} = 1.1 \pm 0.1$ μM
BCRP1 inhibitor $EC_{50} = 0.2 \pm 0.05$ μM