Anti-hepatocellular carcinoma activity using HepG2 cells and hepatotoxicity of 6-substituted methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate derivatives: *in vitro* evaluation, cell cycle analysis and QSAR studies

Rui M. V. Abreu,[a,b] Isabel C. F. R. Ferreira,[a] Ricardo C. Calhelha,[a,c] Raquel T. Lima,[d,e] M. Helena Vasconcelos[d,f] Filomena Adega,[b] Raquel Chaves,[b] Maria-João R. P. Queiroz[c*]

[a]*CIMO-ESA, Instituto Politécnico de Bragança, Campus de Sta Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.*
[b]*Instituto de Biotecnologia e Bioengenharia, Centro de Genómica e Biotecnologia, Universidade de Trás-os-Montes e Alto-Douro (CGB-UTAD/IBB), Apartado 1013, 5001-801, Vila Real, Portugal.*
[c]*Centro/Departamento de Química, Universidade do Minho, Campus de Gualtar 4710-057 Braga,Portugal.*
[d]*Cancer Drug Resistance Group, IPATIMUP–Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal.*
[e]*Centro de Química Medicinal da Universidade do Porto (CEQUIMED-UP), Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal.*
[f]*Laboratório de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha 164, 4050-047 Porto, Portugal*

*Corresponding author: E-mail address: mjrpq@quimica.uminho.pt (M.-J.R.P. Queiroz)
Tel.: +351-253604378; fax: +351-253604382

Abstract

Hepatocellular carcinoma (HCC) is a highly complex cancer, resistant to commonly used treatments and new therapeutic agents are urgently needed. A total of thirty-two thieno[3,2-*b*]pyridine derivatives of two series: methyl 3-amino-6-(hetero)arylthieno[3,2-*b*]pyridine-2-carboxylates (**1a-1t**) and methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates (**2a-2n**), previously prepared by some of us, were evaluated as new potential anti-HCC agents by studying their *in vitro* cell growth inhibition on human HepG2 cells and hepatotoxicity using a porcine liver primary cell culture (PLP1). The presence of amino groups linked to a benzene moiety emerges as the key element for the anti-HCC activity. The methyl 3-amino-6-[(3-aminophenyl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylate (**2f**) is the most potent compound presenting GI_{50} values on HepG2 cells of 1.2 μM compared to 2.9 μM of the positive control ellipticine, with no observed hepatotoxicity (PLP1 GI_{50}>125 μM against 3.3 μM of ellipticine). Moreover this compound changes the cell cycle profile of the HepG2 cells, causing a decrease in the % of cells in the S phase and a cell cycle arrest in the G2/M phase. QSAR studies were also performed and the correlations obtained using molecular and 1D descriptors revealed the importance of the presence of amino groups and hydrogen bond donors for anti-HCC activity, and hydrogen bond acceptors for hepatotoxicity. The best correlations were obtained with 3D descriptors belonging to different subcategories for anti-HCC activity and hepatotoxicity, respectively. These results point to different molecular mechanisms of action of the compounds in anti-HCC activity and hepatotoxicity. This work presents some promising thieno[3,2-*b*]pyridine derivatives for potential use in the therapy of HCC. These compounds can also be used as scaffolds for further synthesis of more potent analogues.

Keywords: Thieno[3,2-*b*]pyridines; Anti-hepatocellular carcinoma activity; Hepatotoxicity; Cell cycle, QSAR studies

1. Introduction

Hepatocellular carcinoma (HCC) is a major health problem with more than 660,000 new cases per year worldwide [1]. HCC is a rapid fatal disease with a life expectancy of about 6 months from the time of diagnostics, and has the third highest mortality rate among all cancers [2]. The highest prevalence for HCC occurs in Asia and sub-Saharan Africa countries, mainly due to endemic hepatitis B and C and food contaminated by Aflatoxin B. In Western countries and Japan, the incidence has increased dramatically over the past decades, mainly due to the rise in hepatitis C virus (HCV) infection, cirrhosis related to type II diabetes, and non-alcoholic steatohepatitis [3,4].

Despite the available surgical treatment options, the majority of HCC patients have inoperable disease with very poor prognosis [5,6]. Also, the frequent presence of recurrence, metastasis or the development of new primary tumors, results in survival rates of 25–50% 5-year after surgery [7]. Alternative or palliative treatment options are very limited due to resistance to conventional chemotherapy and radiotherapy [8]. Because of the lack of survival benefits of treatment with conventional drugs, there is a need for more therapies with pharmacological agents, to help improve the prognosis of patients with HCC.

Sorafenib, a multikinase inhibitor, is the only approved drug for HCC treatment, although it is effective in a wide range of tumor models [9]. *In vivo* and *in vitro* studies have shown that sorafenib inhibits tumor growth and disrupts tumor microvasculature through antiproliferative, antiangiogenic, and/or pro-apoptotic effects. Sorafenib has shown antitumor activity in phase II/III trials involving patients with advanced renal cell carcinoma and hepatocellular carcinoma. The multiple molecular targets of sorafenib, the serine/threonine kinase Raf and VEGFR (the proangiogenic vascular endothelial growth

factor receptor) and PDGFR (platelet-derived growth factor receptor) tyrosine kinases, may explain its broad preclinical and clinical activity [10]. The relative success of Sorafenib, which was found to prolong about 3 months the average survival time of patients with advanced HCC, suggest that the small-molecule targeted chemotherapy can be a promising strategy to combat this cancer [11]. In fact several compounds are already under preclinical investigation, and accumulating evidence suggests that combination therapy targeting different pathways will potentiate anti-cancer effects and will become the future therapeutic approach [8].

HepG2, Hep3B, Huh7.5 and SK-Hep1 are commonly used human HCC cell lines [12]. Nevertheless, HepG2 is the most widely used cell line and generally regarded as a good HCC model [13-15]. In this study, thirty two methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate derivatives, synthesized in our group [16-18], were evaluated as potential new anti-HCC agents by studying their *in vitro* cell growth inhibition activity using HepG2 cells. Furthermore, an initial assessment of possible compound toxicity was performed by studying their *in vitro cell* growth inhibition activity in a porcine liver primary cell culture (PLP1). The cell culture methodology to establish PLP1 as a good initial model for *in vitro* hepatotoxicity is described. The cell profile of the most promising compound (more active and less toxic) was analysed. QSAR (Quantitative Structure-Activity Relationships) studies were also performed for the anti-HCC activity and hepatotoxicity in order to provide some insights on the molecular interactions of the compounds studied with proteins involved in signaling pathways.

2. Results and Discussion

2.1. Establishment of a porcine liver primary cell culture for hepatotoxicity studies

In order to perform a preliminary study of *in vitro* toxicity in normal liver cells a porcine liver primary cell culture (PLP1) was established. These primary cells (also referred as normal early passage cells) have undergone very few population doublings and are therefore more representative of the main functional component of the tissue from which they are derived in comparison to continuous (tumor or artificially immortalized) cell lines [19].

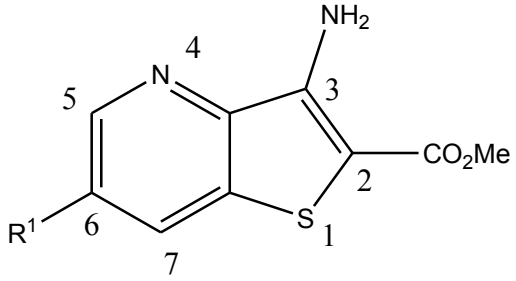
This assay is very important since mammalian hepatocytes still represent an obligatory step in the evaluation of toxic compounds that lead to the production of various metabolites, which are the ultimate cause of toxicity. We used porcine liver as an *in vitro* cytotoxicity model because it is known, in terms of cellular and physiological functioning, to be very similar to human [20]. The use of non-tumor liver cells is even more important in this work, as we are studying the effect of the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylates derivatives in liver cancer (hepatocellular carcinoma). Although a human primary cell culture or even fresh isolated human hepatocytes would be preferred, there are still a number of ethical concerns to consider when using fresh human tissue for cell lines establishment. The use of porcine primary cells allows for a preliminary toxicity screening of the compounds in normal liver cells. This screening narrows the number of compounds that will then be further screened, using *in vitro* and/or *in vivo* human liver for hepatotoxicity.

Although the use of the PLP1 culture will not give us a definite answer, no hepatotoxicity (no *in vitro* screening will do that) it is still a cheaper, faster and ethically defensible, to perform preliminary hepatotoxicity studies.

2.2. Anti-hepatocellular carcinoma (HCC) activity using HepG2 cells and hepatotoxicity: SAR analysis

In this study, a total of thirty-two methyl 3-amino-thieno[3,2-*b*]pyridine-2-carboxylates derivatives, belonging to two different series: the methyl 3-amino-6-(hetero)arylthieno[3,2-*b*]pyridine-2-carboxylates derivatives (**1a-1t**) [17] and the methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates derivatives (**2a-2n**) [18], were evaluated for the *in vitro* anti-HCC (Hepatocellular carcinoma) activity and hepatotoxicity. Anti-HCC activity was studied by performing *in vitro* HepG2 cell growth inhibition assays. The HepG2 is a HCC derived cell line commonly used as a good model for *in vitro* anti-HCC studies. To investigate the possible hepatotoxicity of the thirty-two compounds, an *in vitro* cell growth inhibition assay was performed using PLP1. The GI_{50} values for both anti-HCC and hepatotoxicity studies are presented in tables 1 and 2.

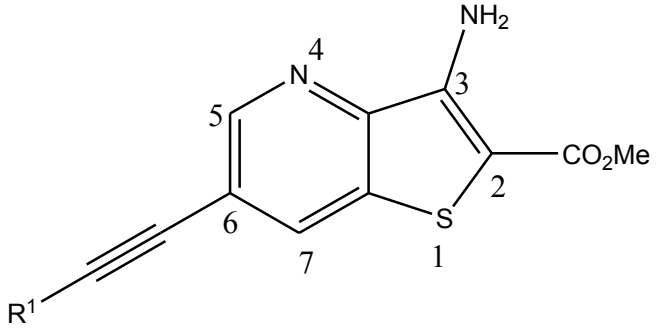
Table 1- Growth inhibition activity (GI_{50}) of compounds **1a-t** and ellipticine, against HepG2 and PLP1 cells.



Compound	R^1	HepG2 GI_{50} (µM)	PLP1 GI_{50} (µM)
1a	4-$MeOC_6H_4$	>125	>125
1b	3,5-di$MeOC_6H_3$	124.6 ± 3.7	25.0 ± 4.1
1c	4-$MeCOC_6H_4$	>125	>125
1d	2-$NH_2C_6H_4$	21.7 ± 2.0	>125
1e	4-CNC_6H_4	>125	96.7 ± 9.8
1f	4-$CF_3C_6H_4$	>125	>125
1g	Pyrid-3-yl	>125	>125
1h	6-MeOPyrid-3-yl-	>125	>125
1i	6-FPyrid-3-yl-	>125	47.0 ± 12.5
1j	2,4-diMeOPyrimid-5-yl-	23.8 ± 4.0	4.4 ± 0.8
1l	Fur-3-yl	>125	>125
1m	1-Me-1*H*-pyrazol-4-yl	>125	>125
1n	Thien-3-yl	>125	25.2 ± 5.4
1o	Naphth-2-yl	>125	101.7 ± 1.3
1p	Quinol-6-yl	>125	14.1 ± 2.1
1q	Quinol-3-yl	>125	32.5 ± 5.8
1r	Isoquinol-4-yl	>125	25.4 ± 1.9
1s	1-methyl-1*H*-indol-5-yl	117.2 ± 2.3	105.7 ± 3.9
1t	2,2'-Bithien-5-yl	70.6 ± 8.5	105.7 ± 12.3
Ellipticine	_____	2.9 ± 0.3	3.3 ± 0.7

Results are given in concentrations that were able to cause 50% of cell growth inhibition (GI_{50}) after a continuous exposure of 72 h and show means ± SEM of three-independent experiments.
Ellipticine was used as a control.

Table 2- Growth inhibition activity (GI_{50}) of the compounds **2a-n** and ellipticine, against HepG2 and PLP1 cells.



Compound	R^1	HepG2 GI_{50} (µM)	PLP1 GI_{50} (µM)
2a	Ph	>125	95.7 ± 10.0
2b	2-$MeOC_6H_4$	>125	121.2 ± 19.2
2c	3-$MeOC_6H_4$	>125	>125
2d	4-$MeOC_6H_4$	>125	76.5 ± 9.7
2e	2-$NH_2C_6H_4$	>125	>125
2f	3-$NH_2C_6H_4$	1.2 ± 0.2	>125
2g	4-$NH_2C_6H_4$	25.9 ± 1.9	>125
2h	2-FC_6H_4	>125	>125
2i	4-FC_6H_4	>125	>125
2j	4-$(Me)_2NC_6H_4$	67.6 ± 9.8	>125
2l	Thien-3-yl	30.0 ± 2.0	30.7 ± 5.6
2m	Pyrid-2-yl	75.7 ± 10.7	56.9 ± 12.0
2n	Pyrid-3-yl	>125	>125
Ellipticine	_____	2.9 ± 0.3	3.3 ± 0.7

Results are given in concentrations that were able to cause 50% of cell growth inhibition (GI_{50}) after a continuous exposure of 72 h and show means ± SEM of three-independent experiments. Ellipticine was used as a control.

To better rationalize the results, the five most potent compounds for anti-HCC activity are presented on Figure 1: **1d** and **1j** from the methyl 3-amino-6-(hetero)arylthieno[3,2-

b]pyridine-2-carboxylates series (Table 1); **2f**, **2g** and **2l** from the methyl 3-amino-6-[(heteroaryl)ethynyl]thieno[3,2-*b*]pyridine-2-carboxylates series (Table 2).

Figure 1. Compounds with best cell growth inhibition activity against HepG2 cells, showing different hepatotoxicity in PLP1.

The dose-response curves used to calculate GI_{50} values for the 5 compounds (and the positive control ellipticine) are presented for both anti-HCC activity (Figure 2a) and hepatotoxicity (Figure 2b).

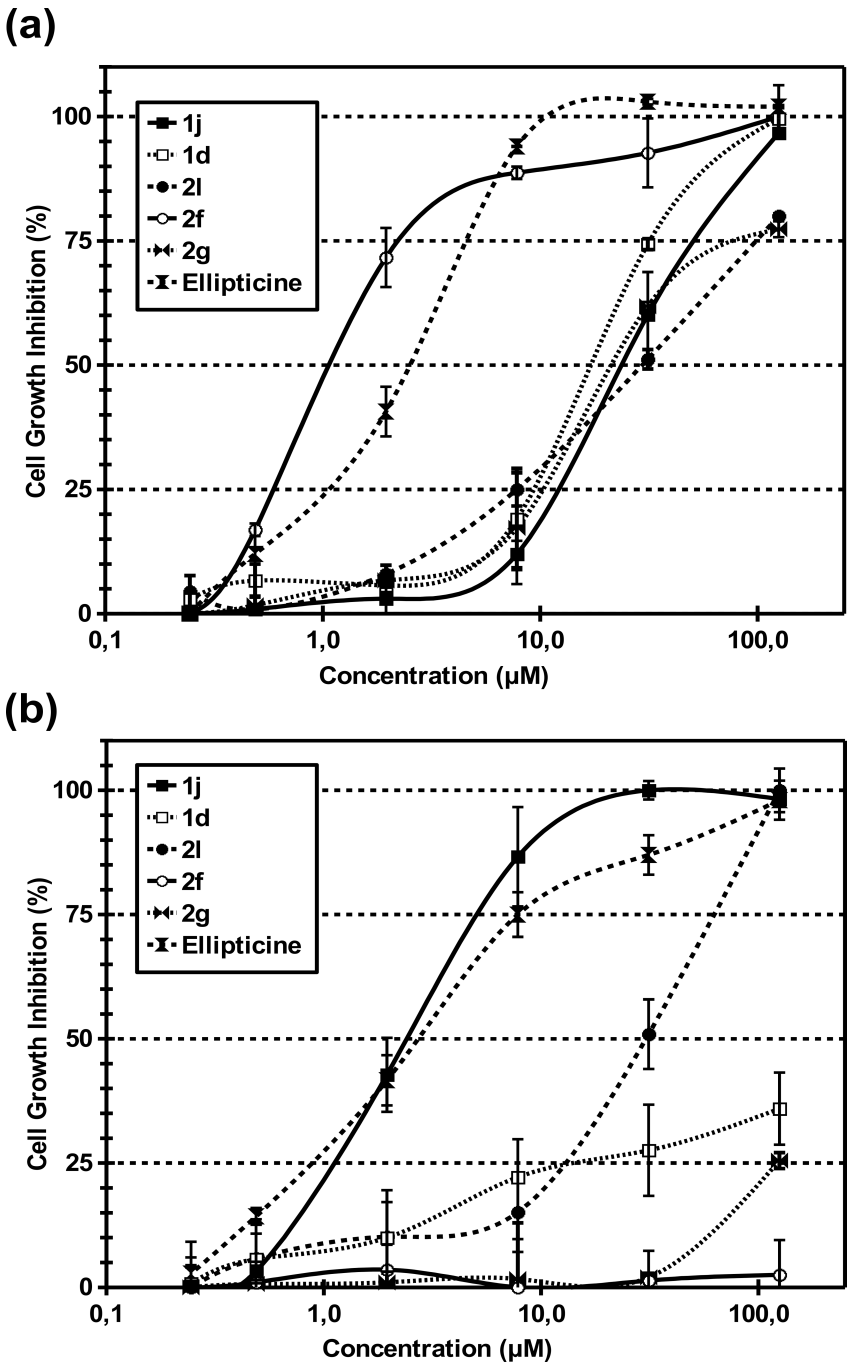


Figure 2. Dose–response analysis of cell growth inhibition activity for compounds **1j**, **1d**, **2l**, **2f, 2g** and ellipticine (positive control) against: **(a)** HepG2 cells and **(b)** PLP1 cells.

Analyzing the five compounds for anti-HCC activity it is observed that compound **2f,** with the substituent 3-aminophenylethynyl, is the most potent compound presenting the lowest GI_{50} value (1.2 μM compared to 2.9 μM value of the positive control ellipticine), while **1d, 1j, 2g** and **2l** present GI_{50} values from 20 to 30 μM. Analyzing the same compounds for potential hepatotoxicity (using the PLP1 cell culture) it is observed that: compounds **1d**, **2f** and **2g** present low to none PLP1 cell growth inhibition activity, up to the concentration tested, while compounds **1j** and **2l** present high PLP1 cell growth inhibition activity, with **1j** being the most hepatotoxic of this study (GI_{50} = 4.4 μM). Taken together, these results clearly indicate that the presence of a 2-aminophenyl substituent in **1d** and a 3 or 4-aminophenylethynyl substituents in **2f** and **2g**, linked to position 6 of the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate scaffold, is the key factor for anti-HCC activity and low hepatotoxicity (Fig. 1). Compounds **1j** with a 2,4-dimethoxypyrimid-5-yl and **2l** with a thien-2-yl, respectively, linked to position 6 of the thieno[3,2-*b*]pyridine scaffold, although showing anti-HCC activity are not viable options as anti-HCC agents, due to their hepatotoxicity. Furthermore, the position of the amino group seems to be important, as compound **2f**, with the amino group in the *meta* position of the phenylethynyl substituent, is the most potent anti-HCC compound (GI_{50} = 1.2 μM), while the less potent compounds **1d** and **2g** (GI_{50} values 21.7 and 25.9 μM, respectively) have amino groups in the *ortho* and *para* positions on the phenyl and phenylethynyl substituents. A careful analysis of figure 2(b) shows that, although it was not possible to calculate GI_{50} values for compounds **1d** and **2g**, due to low hepatotoxicity, they still presented a small percentage of PLP1 cell growth inhibition at the highest concentration evaluated of 125 μM. This is even more interesting when it was observed that the most potent anti-HCC compound **2f** presents no hepatotoxicity, even at the highest concentrations evaluated.

In general we observe that, the presence of several substituents linked to position 6 of the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylate moiety contributed to hepatotoxicity including: a dimethoxyphenyl in **1b** and a methoxyphenylethynyl in **2d**, a 6-fluoro-3-pyrid-3-yl in **1i** and a pyrid-2-ylethynyl in **2m**, a thien-3-yl in **1n** and a thien-2-ylethynyl in **2l** and notably quinolines (**1p, 1q, 1r**). These compounds are not suitable scaffolds for pursuing synthesis of compounds with more efficient anti-HCC activity and low hepatotoxicity.

A recent study performed by Zeng *et al.* [13] showed a number of thieno[2,3-*b*]pyridine derivatives with strong cell growth inhibition activity against HepG2 cells. Remarkably some of the compounds studied presented HCC-specific anticancer activity. Although using a different thienopyridine scaffold (thieno[2,3-*b*]pyridine instead of our thieno[3,2-*b*]pyridine), this is exciting evidence that corroborate the anti-HCC potential of the compounds presented in our study. However, the compounds presented in that study were not studied for hepatotoxicity and, based on the SAR analysis performed above, it is predicted that some of the compounds will probably present some degree of hepatotoxicity. Taken together, our findings show that the most potent compounds presented in this work, above all **2f**, are good candidates for chemotherapy against HCC, with minimal potential hepatotoxicity effects.

Therefore we further analyzed the cell cycle profile of exponentially growing HepG2 cells treated for 72h with compound **2f**. The results are presented in Figure 3. Since the cells were growing exponentially during the entire course of the experiment, the blank cells (treated with complete medium only) and control cells (treated with DMSO) present a higher % of cells in S phase than that found in cells reaching confluence (data not shown). Results from cells treated with compound **2f** show that this compound causes a significant

decrease in the % of cells in the S phase and a significant cell cycle arrest (increase in the % of cells) in the G2/M phase of the cell cycle.

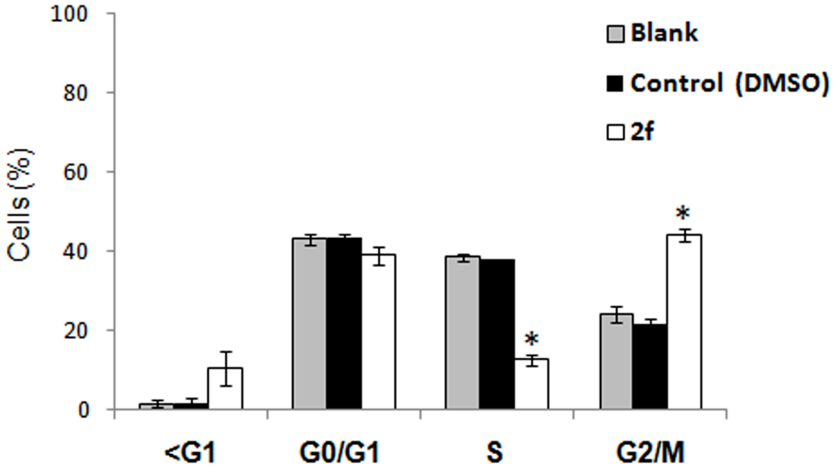


Figure 3 Effect of the compound **2f** in HepG2 cell cycle profile. Blank cells (treated with medium only) and Control cells (treated with the vehicle, DMSO) were also included. Results are the mean±SEM of three independent experiments. *Represents $p<0.05$.

The cell cycle analysis allowed us to verify that following treatment of cells with compound **2f**, there was only a slight (not statistically significant) increase in the sub-G1 peak (which is suggestive of apoptosis) indicating that the mechanism of action of this compound does not probably involve apoptosis. An attempt was made to study apoptosis but, unfortunately, this compound presents fluorescence, interfering with the assays that we have currently implemented in our laboratory (Annexin V/PI labelling by flow cytometry and TUNEL).

2.3. QSAR studies for anti-HCC and hepatotoxicity activities

The anti-HCC activity and hepatotoxicity of the methyl 3-amino-thieno[3,2-*b*]pyridine-2-carboxylates derivatives was studied and a SAR analysis was performed. Still, this analysis

was purely based on chemical intuitions on the substituent effects of series of compounds. To better understand the studied biological activities in a quantitative manner, and to provide insights on the mechanism of action, we set out to perform QSAR studies. Compounds with no calculated GI_{50} values, due to lack of biological activity up to the 125 μM DMSO solubility limit, were not used for this (were left out of this) analysis, as they would distort this quantitative analysis. In the end QSAR studies were performed with: ten compounds with anti-HCC activity (**1b**, **1d**, **1j**, **1s**, **1t**, **2f**, **2g**, **2j**, **2l** and **2m**) and 16 with hepatotoxic activity (**1b**, **1e**, **1i**, **1j**, **1n**, **1o**, **1p**, **1q**, **1r**, **1s**, **1t**, **2a**, **2b**, **2d**, **2l** and **2n**). Then, a total of 1664 molecular descriptors were calculated for each of the selected compound, using the E-Dragon software [21,22]. The descriptors were separated in 4 categories: molecular properties (43), 1D (322), 2D (578) and 3D (721). This category separation is based on how they are calculated by E-Dragon thus providing different information. Then, the descriptors correlation with pGI_{50} values was calculated, for both anti-HCC and hepatotoxicity. Only correlation values above 0.80 were considered relevant and are presented on table 3. To better discuss the different descriptors with good correlations, they are separated in categories and the definition of each relevant descriptor is given on table 4.

From the molecular properties and 1D categories, only the Hy (hydrophilic factor), the nHDon (the number of hydrogen Donors) and nArNH2 (number of amino groups linked to aryl group) descriptors provided good correlations with anti-HCC activity. This information is in agreement with the SAR analysis made before, and demonstrates that amino groups linked to aryl (nArNH2) is a key factor on anti-HCC activity. In fact, the amino group was the only substituent used with hydrogen bond donating capability, thus explaining the nHDon descriptor correlation and consequently the Hy descriptor. We can assume that

probably other functional groups hydrogen bond donors, in the same positions as the amino groups, may yield similar or better anti-HCC activities.

Table 3

Correlations (above 0.80) between the calculated molecular descriptors and the pGI_{50} values of anti-HCC and hepatotoxicity.

Sample	Molecular Properties		1D		2D	3D	
	Descriptors	Corr.	Descriptors	Corr.		Descriptors	Corr.
Anti-HCC activity (10 compounds)	Hy	0.84	$nArNH_2$ nHDon	0.8385 0.8385	-	G1u G2s G1m G1e	0.9695 0.9339 0.9095 0.9095
Hepatotoxicity (16 compounds)	-	-	N-075 nPyrimidines nHAcc	0.8600 0.8228 0.8216	-	R8u+ R8e+ R6m R6v+	0.8381 0.8216 0.8100 0.8032

Table 4

Symbols for the descriptors used and their definition.

Category	Symbols	Descriptor definition
Molecular Properties	Hy	hydrophilic factor
	AlogP	Ghose-Crippen octanol-water partition coefficients (logP)
1D	$nArNH_2$	number of primary amines (aromatic)
	nHDon	number of donor atoms for H-bonds (N and O)
	N-075	R--N--Re / R--N--X
	nPyrimidines	number of Pyrimidines
	nHAcc	number of acceptor atoms for H-bonds (N, O, F)
3D GETWAY	G1u	1st component symmetry directional WHIM index / unweighted
	G2s	2st component symmetry directional WHIM index / weighted by atomic electrotopological states
	G1m	1st component symmetry directional WHIM index / weighted by atomic masses
	G1e	1st component symmetry directional WHIM index / weighted by atomic Sanderson electronegativities
	R8u+	R maximal autocorrelation of lag 8 / unweighted

R8e+	R maximal autocorrelation of lag 8 / weighted by atomic Sanderson electronegativities
R6m	R autocorrelation of lag 6 / weighted by atomic masses
R6v+	R maximal autocorrelation of lag 6 / weighted by atomic van der Waals volumes

However the best correlations with anti-HCC activity were obtained using 3D descriptors, while no correlation was obtained with 2D descriptors. The software E-Dragon calculates several categories of 3D descriptors based on the algorithms used and calculation approaches [23]. The 3D descriptors of the WHIM (weighted holistic invariant molecular) category were the only 3D correlated descriptors, with values well above 0.90. WHIM descriptors are built in such a way as to capture the relevant molecular 3-D information regarding molecular size, shape, symmetry, and atom distribution with respect to some invariant reference frame. Thus they usually perform better when the spatial configuration and the stereo-chemistry of the compounds are determinant factors to the biological activity studied, in this case the anti-HCC [23,24]. Therefore, the main mechanism of action of the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylates derivatives is probably as modulators of protein targets involved in signaling pathways that have been associated with HCC [25]. As more methyl 3-amino-thieno[3,2-*b*]pyridine-2-carboxylates derivatives will be synthesized, we expect to translate this information into predictive QSAR models, probably using the WHIM descriptors.

When we correlate descriptors with hepatotoxicity, we observe that only 1D and 3D descriptors have correlation values above 0.80. The best correlated 1D descriptor is N-075 (number of Nitrogen atoms linked though conjugated bond to two atoms), and, on this condition, nitrogens act as Hydrogen Bond Acceptors thus explaining the other correlated

descriptor nHAcc (number of hydrogen bond acceptors) observed. Furthermore, hepatotoxicity also correlates well with the nPyrimidines (number of Pyrimidines) descriptor, thus confirming the SAR analysis made before that pyrimidine groups are undesirable moieties on the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylates derivatives. Taken together, these informations confirm that substituted heteroaryl moieties, with hydrogen bond acceptor atoms, are undesirable characteristics for the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylates derivatives, as they confer greater hepatotoxicity.

The 3D descriptors that better correlate with hepatotoxicity belong to the GETAWAY (GEometry, Topology, and Atom-Weights AssemblY) category. These descriptors are based on matching 3D-molecular geometry with chemical information, and are based on well-known accepted algorithms and formula, possessing a track record of good predictive power in biological property modeling [23,26]. The fact that hepatotoxicity correlates with 3D descriptors from a different category, when compared to the anti-HCC activity, is also an indication that the mechanism of action in both biological activities is probably different. Although the PLP1 cell culture used is not a "pure" hepatocytes culture, biotransformation of the methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylates derivatives is also probably a factor in the observed hepatotoxicity, as the metabolites produced are maybe the cause of toxicity. This information is impossible to derive from the 3D descriptors and the hepatotoxicity mechanism will become more apparent as more studies, specially *in vivo* assays, are performed.

3. Conclusions

In this study, a total of thirty-two methyl 3-amino-thieno[3,2-*b*]pyridine-2-carboxylate derivatives, belonging to two different series: methyl 3-amino-6-(hetero)arylthieno[3,2-*b*]pyridine-2-carboxylates derivatives (**1a-1t**) and methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates derivatives (**2a-2n**) were evaluated for the *in vitro* anti-HCC activity and hepatotoxicity using HepG2 and PLP1 cell lines, respectively. Compounds **1d**, **2f** and **2g** revealed promising results, with strong anti-HCC activity and low hepatotoxicity. In common they have an amino substituted benzene moiety at the 6-position of the thieno[3,2-*b*]pyridine scaffold. Compound **2f** is the most potent anti-HCC compound (GI_{50} values of 1.2 μM compared to 2.9 μM of ellipticine) with no observed hepatotoxicity (GI_{50}>125 μM, compared to 3.3 μM of ellipticine). On the other hand, the presence of several moieties linked to the 6-position contributed to hepatotoxicity including: methoxy substituted benzene moieties (**1b, 2d**), pyridine moieties (**1i, 2m**), thiophene moieties (**1n, 2l**) and notably quinoline moieties (**1p, 1q, 1r**). These compounds are not suitable scaffolds for pursuing the synthesis of compounds with more efficient anti-HCC activity and low hepatotoxicity.

HepG2 cell cycle analysis of compound **2f** indicates that the mechanism of action of this compound is related with G2/M cell cycle arrest.

QSAR studies were also performed, and revealed that the 3D-WHIM descriptors were the ones that correlated best with anti-HCC activity, while the 3D-GETWAY descriptors correlated best with hepatotoxicity. All together, these findings indicate that the studied compounds promote anti-HCC and hepatotoxicity through different mechanisms, probably also as modulators of protein kinase targets such as growth factor receptors involved in different signaling pathways. These studies have also confirmed the importance of the

presence of the amino groups, and of hydrogen bond donors in general. In conclusion, several compounds present potent *in vitro* anti-HCC activity using HepG2 cells with no hepatotoxicity,. Furthermore, they might be used as scaffolds for synthesis of even more potent compounds.

4. Experimental

4.1. Tested compounds

Thirty-two methyl 3-aminothieno[3,2-*b*]pyridine-2-carboxylates derivatives belonging to two different series: the methyl 3-amino-6-(heteroaryl)thieno[3,2-*b*]pyridine-2-carboxylates derivatives (**1a-1t**) and the methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates derivatives (**2a-2n**), were prepared. The synthesis was performed by our group as previously described [16-18]. Stock solutions were prepared in DMSO and kept at -80 ºC. Appropriate fourfold serial dilutions were freshly prepared in 10% (v/v) DMSO to allow for final well concentrations on the cell assays of: 125.0, 31.2, 7.8, 1.95, 0.49, 0.12 and 0.03 μM. The effect of the vehicle solvent (DMSO) on the growth of the cell lines was evaluated by exposing untreated control cells to the maximum concentration of DMSO used in the assay (0.25 %). No influence was found.

4.2. Reagents

Dulbecco's modified Eagle's medium, hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penincillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), all purchased from Gibco Invitrogen LifeScience (California, USA). Sulforhodamine B, trypan blue, acetic acid, dimethyl sulfoxide

(DMSO), ellipticine, ethylene diamine tetraacetic acid (EDTA), Trichloroacetic acid (TCA) and Tris were purchased from SigmaChemical Co. (Saint Louis, USA).

4.3. Cell Lines

In this work, two cell lines were used, the human hepatocellular liver carcinoma cell line HepG2 and a porcine liver primary cell culture (PLP1). The PLP1 cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U/mL penicillin, 100 ug/mL streptomycin (Gibco Invitrogen Life Technologies) and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin. Both cell lines were incubated at 37°C with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope.

Before confluence was reached, both HepG2 and PLP1 cells were subcultured and plated in 96-well plates at a density of 2.5×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin.

4.4. Cell Growth Inhibition Assay using HepG2 and PLP1 cells.

The tested compounds *in vitro* effect on HepG2 and PLP1 cell growth was studied, using the sulforhodamine B (SRB) colorimetric assay [27], according to Vishai *et al*., with some adaptations [28]. Briefly, exponentially growing cells were seeded in 96-well plates and

exposed to six serial dilutions of each tested compound, with final concentrations of 125.00, 31.25, 7.81, 1.95, 0.49 and 0.12 μM. After 72 h of exposure, cells were fixed by adding cold 50% (wt/vol) trichloroacetic acid (TCA, 25 μL) and incubated for 60 min at 4 ºC. Plates were then washed with deionized water and dried; SRB solution (0.1% wt/vol in 1% acetic acid, 50 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried and bound stain was solubilized with 100 μL of a 100 mM Tris base solution. Optical densities were read on an automated spectrophotometer plate reader at a single wavelength of 540 nm (Biotek Elx800). Dose-response curves were obtained for each tested compound and cell line, and the GI_{50} value, corresponding to the concentration of the compounds that inhibited 50 % of the net cell growth, was calculated as described elsewhere [28]. The GI_{50} values are expressed as mean ± SEM of three independent experiments.

4.5 Analysis of cell cycle profile

HepG2 cells were seeded in 6-well plates (8x 10^4 cells/well) and treated 24h later with 4.1 μM of compound 2f. Blank cells (treated with medium only) or Control cells (treated with the compound's vehicle, DMSO) were also included. Following 72 h treatment, cells were fixed overnight in ice-cold 70% ethanol and re-suspended in PBS containing 0.1 mg/mL RNase A and 5 μg/mL propidium iodide. The cellular DNA content was analysed by flow cytometry using an Epics XL-MCL Coulter Flow cytometer (Brea, CA, USA) plotting at least 5 000 events *per* sample, as previously described [17, 29]. The % of cells in G0/G1, S and G2/M phases of the cell cycle and the % of cells in the sub-G1 peak were determined

using the FlowJo 7.2 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion**.**

4.6. 3D structure preparation and molecular descriptors calculation

The 2D chemical structures of the tested compounds were drawn using the Symyx/Draw software [30]. Then, 2D to 3D structure conversion and geometry optimization calculations were performed for each compound, using the AMMP force field sp4 [31], developed on the basis of the UFF potential set [32] and available via the VegaZZ software [33]. Finally the 3D structure of each compound was recorded in mol2 file format also using the VegaZZ software. The E-Dragon computer software was then employed to calculate the molecular descriptors totaling 1664 descriptors belonging to different categories: molecular properties, OD, 1D, 2D and 3D [20,23]. pGI_{50} values used for molecular modeling were calculated according to the equation: $pGI_{50} = \log 1/GI_{50}$. Correlations were calculated using the OpenOffice.org spreadsheet software, version 3.2.

Acknowledgements

The authors are grateful to Foundation for Science and Technology (FCT, Portugal) through the financial support of the research centres and through the research project PTDC/QUI-QUI/111060/2009 also financed by FEDER/COMPETE/QREN/EU. R.M.V. Abreu and R.C. Calhelha thank to FCT, POPH-QREN and FSE for their SFRH/PROTEC/49450/2009 and SFRH/BPD/ 68344/2010 grants, respectively.

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