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Bioactivity of Ionic Liquids Based on Valproate in SH-SY5Y Human Neuroblastoma Cell Line

Ana Rita Dias ¹, Ricardo Ferraz ^{1,2,*} , João Costa-Rodrigues ^{1,3,4}, Andreia F. M. Santos ⁵ , Manuel L. Jacinto ⁵, Cristina Prudêncio ^{1,4} , João Paulo Noronha ⁵ , Luis C. Branco ^{5,*}  and Željko Petrovski ^{5,*} 

¹ CISA, Chemical and Biomolecular Sciences, School of Health, Polytechnic Institute of Porto, 4200-072 Porto, Portugal

² LAQV-REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal

³ School of Health, Polytechnic Institute of Viana do Castelo, 4900-347 Viana do Castelo, Portugal

⁴ i3S, Institute for Innovation and Health Research, University of Porto, 4200-135 Porto, Portugal

⁵ LAQV-REQUIMTE, Department of Chemistry, NOVA School of Science and Technology, NOVA University of Lisbon, 2829-516 Caparica, Portugal

* Correspondence: ricardoferraz@eu.ipp.pt (R.F.); l.branco@fct.unl.pt (L.C.B.); z.petrovski@fct.unl.pt (Ž.P.)

Abstract: The search for alternative and effective therapies to fight cancer is one of the main goals of the pharmaceutical industry. Recently, ionic liquids (ILs) have emerged as potential therapeutic agents with antitumor properties. The goal of this study was to synthesize and evaluate the bioactivity of different ILs coupled with the active pharmaceutical ingredient (API) valproate (VPA) as an antitumor agent. The toxicity of the prepared ionic liquids was evaluated by the MTT cell metabolic assay in human neuroblastoma SH-SY5Y and human primary Gingival Fibroblast (GF) cell lines, in which they showed inhibitory effects during the study period. In addition, low cytotoxicity against GF cell lines was observed, suggesting that these compounds are not toxic to human cell lines. [C₂OHDMiM][VPA] demonstrated an outstanding antitumor activity against SH-SY5Y and lower activity against the non-neoplastic GF line. The herein assessed compounds played an important role in the modulation of the signaling pathways involved in the cellular behavior. This work also highlights the potential of these ILs-API as possible antitumor agents.

Keywords: ionic liquids; valproic acid; active pharmaceutical ingredients; antitumor agents; neuroblastoma; signaling pathways



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1. Introduction

In the last years, an increasing interest in ionic liquids (ILs) has been observed due to their potential applications in biological and pharmaceutical sciences [1,2]. Typically, ILs are organic salts constituted by large and asymmetric ions, in which their properties can be tuned by wise pairing of their cations and anions [2–6]. In this context, these compounds have attracted the interest of researchers and been applied in several fields, such as engineering processes, new advanced lubricants, reaction media, extraction and separation processes, electrochemistry, biotechnology and nanotechnology [7]. They are also used to overcome solubilization problems of some pharmaceutical drugs [1,8,9] and, more recently, have been exploited as alternative solvents, cosolvents and/or reagents to synthesize active pharmaceutical ingredients (APIs) [10–12].

Most commercially available drugs used in medicine are solid substances, exhibiting disadvantageous characteristics addressed to their physical state that affect their bioavailability: polymorphism, low solubility and the need for stabilizing the amorphous forms, which commonly tend to crystallize [2,13,14]. Moreover, the search for ways of improving the therapeutical potential of pharmaceutical drugs has emerged [15], introducing ionic liquids as a promising approach to develop new formulations through simple neutralization reactions [13,16], as most drugs are either acidic or basic [13,16–24]. In fact, it has

been reported that formulations based on ILs-API successfully eliminate the drawbacks of the drug, i.e., mainly the existence of polymorphs and the low solubility in water [12,25], contributing to enhance the drug delivery [26] and allowing, in some cases, the addition of a second complementary function to the API [12,27,28].

Valproic acid, a derivative form of valeric acid, was firstly synthesized by Burton in 1882 and initially classified as an organic solvent. Nowadays, it is a drug used for neurologic pathologies, epilepsy and bipolar disorder due to its neuro-protection action [29–32]. According to Jentink et al. [33], valproic acid has been considered teratogenic. In addition to its teratogenicity, the effect of this pharmaceutical drug on cell cultures has aroused the interest of this compound for therapeutic purposes in cancer [34]. Recent studies conducted in vivo and in vitro have demonstrated the antitumor activity of valproic acid on the modulation of numerous signaling pathways involved in the increase in apoptosis and immunogenicity, inducing the differentiation and inhibition of cell proliferation, while decreasing the angiogenic potential [34,35]. Moreover, due to its properties, valproic acid is effective against several types of cancer, including neuroblastoma [36,37], since it impairs growth and induces differentiation in human neuroblastoma cells in harmless concentrations [35,37]. Therefore, there is currently a great interest in evaluating the physicochemical interactions of ionic liquids and valproate [38–42]. However, the biological activity and toxicity of these mixtures have never been investigated.

Neuroblastoma (NB) is the most common extracranial solid tumor in infants and children. It is classified as a neuroendocrine cancer, commonly located in the adrenal medulla, although it can arise in any place where sympathetic neural tissue is found [43]. In fact, half of these tumors occur in infants, accounting for approximately 15% of pediatric cancer deaths. Patients classified as high risk, according to their histological and biological characteristics, comprise about 50% of the new NB cases each year. These patients usually require an aggressive therapeutic strategy, which includes radiotherapy, surgery, high doses of chemotherapy with stem cell transplantation and other therapeutical approaches to increase survival rates [44]. It is known that the neuroblastoma tumorigenesis and malignant transformations are associated to the expression of certain signaling pathways' precursors and their dominance, which, in addition to the decrease in normal cellular senescence or apoptosis, are related to cell survival [45]. Moreover, the main signaling pathways involved in NB include transcription factors, kinases and cell-cycle regulators. Therefore, the manipulation of these signaling pathways can positively contribute to the reduction of the NB's malignant potential [43] and the search for new promising alternatives to treat this disease is essential.

The potential of ILs-API to act as antitumor agents in certain cancers has recently been described [46–50]. Indeed, tumor heterogeneity, one of the major challenges in cancer treatment, can be surpassed with the development of suitable nanocarriers [51], in which ionic liquids can be involved by promoting the transport of APIs [26]. In addition, ILs-API can also contribute in cancer-combination therapy [52]. Previous studies on the antimicrobial activity against resistant bacteria have shown that these compounds may also promote a specific interaction in combination therapy by achieving a complementary function [20,53]. Thus, the scope of this work is focused on the development of new ionic liquids based on valproate (VPA), which is the anion form of valproic acid (2-propylpentanoic acid), and aims to describe their biological properties in brain metabolism. Several formulations based on the following organic cations were synthesized and fully characterized (Figure 1): (2-hydroxyethyl)trimethylammonium 2-propylpentanoate or cholinium valproate, [Ch][VPA]; 1-hexadecylpyridinium 2-propylpentanoate or cetylpyridinium valproate, [C₁₆Pyr][VPA]; 1-ethyl-3-methylimidazolium 2-propylpentanoate, [EMiM][VPA]; 1-(2-hydroxyethyl)-3-methylimidazolium 2-propylpentanoate, [C₂OHMiM][VPA]; 1-(2-hydroxyethyl)-2,3-dimethylimidazolium 2-propylpentanoate, [C₂OHDMiM][VPA]; and 1-(2-methoxyethyl)-3-methylimidazolium 2-propylpentanoate, [C₃OMiM][VPA]. Further bioactivity studies on neuroblastoma were conducted with four inhibitors: U0126, PDTC, SP600125 and Sb202190, being the inhibitors of the MEK, NFκB, JNK and p38 pathways,

respectively. It is worth noting that this approach would only slightly modulate valproate properties without changing its core chemical structure, which is crucial since chemically modified derivatives can have different activity profiles [34].

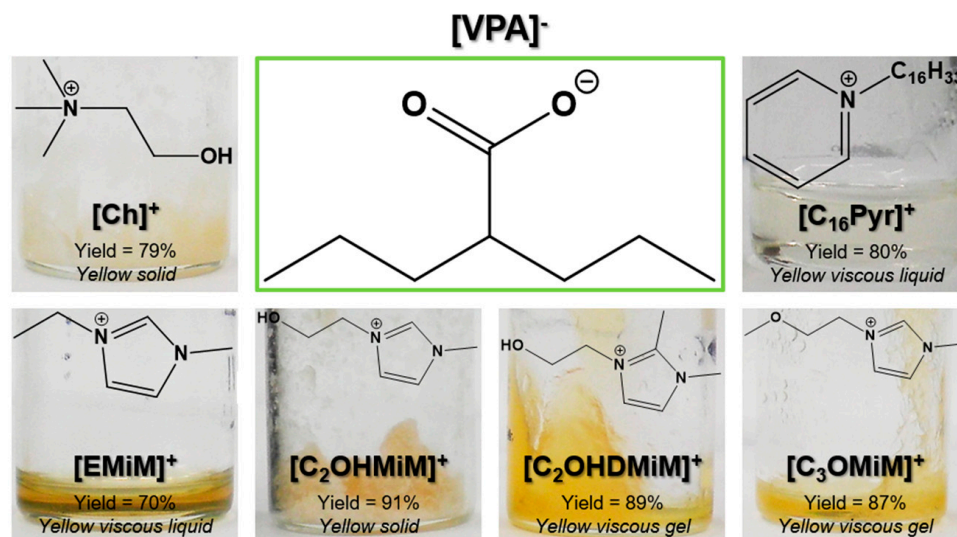
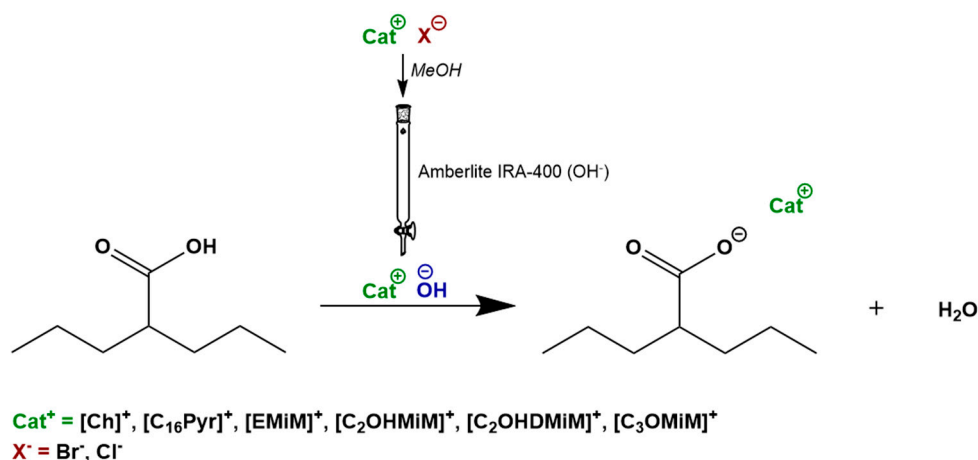


Figure 1. Structure, yield and physical state of the synthesized ionic liquids based on VPA.

2. Materials and Methods

2.1. Synthesis of ILs-API

In this work, several ionic liquids based on VPA with different cations were synthesized by the neutralization method, as previously reported [51], following the reaction Scheme 1. Each of the commercially available bromide or chloride organic salts were firstly converted into hydroxide, through an Amberlyst anion-exchange resin (A26-OH), to then be neutralized with an equimolar amount of VPA [16,54]. This synthetic method allowed the preparation of the ILs-API in high quantitative yields and purity levels, as confirmed further by elemental analysis, ¹H and ¹³C-NMR. In addition, ¹H-NMR provided information related to the complete deprotonation of VPA and the expected cation/anion ratio (1:1). Further experimental and characterization details are available in Supplementary Materials (Section 1, Figures S1–S18 for NMR and IR spectra).



Scheme 1. General synthetic procedure of the ILs-API.

2.2. Cell Culture Studies at Therapeutic Dosage

In order to compare the cell viability obtained for ILs-API on the SH-SY5Y and GF cell lines with the plasma valproate, MTT assays were performed at concentrations suitable and

harmless to patients (Table 1). These tests, which are widely used to assess that parameter, rely on the activity of mitochondrial enzymes and only viable cells originate a positive result. For the experimental procedure, see Supplementary Materials (Section 2).

Table 1. Therapeutic dosage of valproate drug.

Cell Line	Tested Concentrations (mol dm ⁻³)	Tested Concentrations (mg L ⁻¹)	Therapeutic Dosage (mg L ⁻¹)	Reference
SH-SY5Y	10 ⁻⁷ –10 ⁻²	0.01442–1442	50–100	[55]
GF	10 ⁻⁵ –10 ⁻¹	1.442–14,421		

The therapeutic dosage represents the tested concentration range of valproate in plasma. Concentrations lower than the therapeutic level were also tested, aiming to mimic the drug concentrations in the tumor environment. In general, the results discussed here reveal that the studied ILs-API interact with the cellular behavior, even for dosages below the concentration found in the plasma of patients treated with valproate.

3. Results and Discussion

3.1. Viability and Cellular Proliferation: ILs-API Bioactivity in Neoplastic Human Tumor Cell Line SH-SY5Y and Non-Neoplastic Gingival Fibroblasts (GF)

To gather information about the possibility of the prepared ILs-API act as antitumor agents, MTT metabolic assays were performed, allowing to determine the IC₅₀ and EC₅₀, i.e., the drug's concentration needed to reduce by half the cellular metabolic activity and cell viability, in the cell line SH-SY5Y and in Gingival Fibroblasts (GF). The results collected on days 1 and 3 are displayed in Table 2.

Table 2. Antitumor activity (IC₅₀ and EC₅₀) of the prepared formulations in the neoplastic human tumor cell line SH-SY5Y on days 1 and 3, as well as non-neoplastic Gingival Fibroblasts on day 1.

Compound	IC ₅₀ (μM)			EC ₅₀ (μM)		
	GF	SH-SY5Y		GF	SH-SY5Y	
	Day 1	Day 1	Day 3	Day 1	Day 1	Day 3
VPA ¹	294.2	0.633	n.d.	299.3	60.72	n.d.
[Ch][VPA]	n.d.	0.049	n.d.	n.d.	0.158	3528
[C ₁₆ Pyr][VPA]	75.54	1.408	1.411	76.07	4.358	1.681
[EMiM][VPA]	1058	1.038	n.d.	>1058	0.086	126.0
[C ₂ OHMiM][VPA]	23.66	31.09	3560	27.42	21.03	2.824
[C ₂ OHDMiM][VPA]	n.d.	0.263	227.8	n.d.	6.000	44.58
[C ₃ OMiM][VPA]	1374	0.646	n.d.	>1374	27.30	14.71

¹ VPA was used as control. n.d.—not determined in the concentration range tested.

Regarding the IC₅₀ obtained for the SH-SY5Y cell line, on day 1, both [Ch][VPA] and [C₂OHDMiM][VPA] exhibited the lowest values, indicating the highest toxicity, when compared with the control. Moreover, the remaining ionic liquids were classified according to their toxicity, in which the latter revealed the less promising result: [C₃OMiM][VPA] > [EMiM][VPA] > [C₁₆Pyr][VPA] > [C₂OHMiM][VPA]. On day 3, [C₁₆Pyr][VPA] presented higher toxicity, followed by [C₂OHDMiM][VPA]. Furthermore, with the exception of [C₂OHMiM][VPA], the other ILs-API, as well as the control, did not display any IC₅₀ in the tested concentration range (10⁻⁷ to 10⁻² M). For EC₅₀, the results are in accordance with the IC₅₀ assays on both days 1 and 3, being, in general, align to those already reported in literature [50,56]. In fact, the highest toxicity of [Ch][VPA] was unexpected as choline is considered an essential nutrient for normal cell metabolism. Therefore, low toxicity against the tumor cell line was foreseen and further studies need to be performed to understand its

mechanism. On the other hand, [C₂OHDMiM][VPA] presented strong antitumor activity, as described in previous works [57,58].

Concerning the values collected for Gingival Fibroblasts, it was possible to conclude that VPA is one of the most toxic compounds studied herein. However, the highest cytotoxicity values were exhibited for [C₁₆Pyr][VPA] and [C₂OHMiM][VPA]. On the contrary, [Ch][VPA] and [C₂OHDMiM][VPA] proved to be the least toxic ones. The last two were not anticipated since imidazolium and phosphonium derivatives have high toxicity, as previously reported [50,59].

Moreover, the comparison between the IC₅₀ values of both neoplastic (SH-SY5Y) and non-neoplastic (GF) cells lines was performed. In general, the results obtained for SH-SY5Y are lower than those collected for the GF cell line, with the exception of [C₂OHMiM][VPA].

Overall, the formulations assessed in this work showed low IC₅₀ and EC₅₀ values, inferring the potential of the prepared ILs-API as valid alternatives to cancer therapies.

3.2. Structural Activity Relationship of Human Tumor Cell Line SH-SY5Y

In this work, the bioactivity of several ionic liquids, as well as the original API, was studied, allowing to conclude that some structures are more toxic than others. The strength and stability associated to the interactions between the organic cations and valproate anion can be an explanation for the different antitumor activity values found on each prepared IL-API (Table 2).

In fact, it is known that long alkyl chains, as present in the cation [C₁₆Pyr][VPA], can be responsible for higher toxicity levels, being these cations referred as the most toxic to the cells [1,53,56,58,60–63]. Some authors justified this toxicity with a modification of the lipid membrane's physical properties and permeability, as the alkyl lengthening promotes the increasing of the compounds' lipophilic nature, as well as the interactions between the phospholipid bilayer and the hydrophobic domains of the membrane proteins, which might lead to the dissolution of the membrane's physiological functions and, ultimately, to cell death [64]. However, these results were only observed after 3 days. On day 1, both cations exhibited low antitumor activity against SH-SY5Y.

In addition, organic cations containing aromatic rings, such as imidazolium, also contribute to the increase of the compound toxicity [65]. This characteristic was registered on [C₂OHDMiM][VPA], which was characterized as one of the most toxic ionic liquids herein synthesized. The presence of hydroxyl groups (units that interact electrostatically with the valproate anion) and two methyl moieties in its structure, as well as the structural similarity between imidazolium-based ionic liquids and detergents, pesticides and antibiotics, are associated to the higher toxicity observed, as their mechanism of action can be responsible for the lipid membrane disruption [58,64,66]. Nonetheless, on the first day of supplementation, [C₂OHMiM][VPA] exhibited the lowest antitumor activity, an unexpected occurrence as its structure also allows for the formation of hydrogen bridges between the hydroxyl group and valproate. Finally, on the same day, the antitumor activity of the cation [C₃OMiM] was higher than [EMiM].

On the contrary, [Ch] was expected to be, among several cations, one of the least toxic because it is an essential nutrient, as previously mentioned. However, on day 1, this IL presented high cytotoxicity values, possibly, due to the established hydrogen bridges between the -OH group and the anion. Therefore, this result was not in agreement with the literature and further studies should be performed in order to understand this toxicity behavior.

3.3. Cell-Signaling Pathways in Cellular Behavior of Human Tumor Cell Line SH-SY5Y

Several cell viability tests were performed to study the influence of different inhibitors (U0126, PDTC, SP600125 and Sb202190) in four cell-signaling pathways (MEK, NFκB, JNK and p38) on the SH-SY5Y cell lines. Figure 2 plots the results obtained.

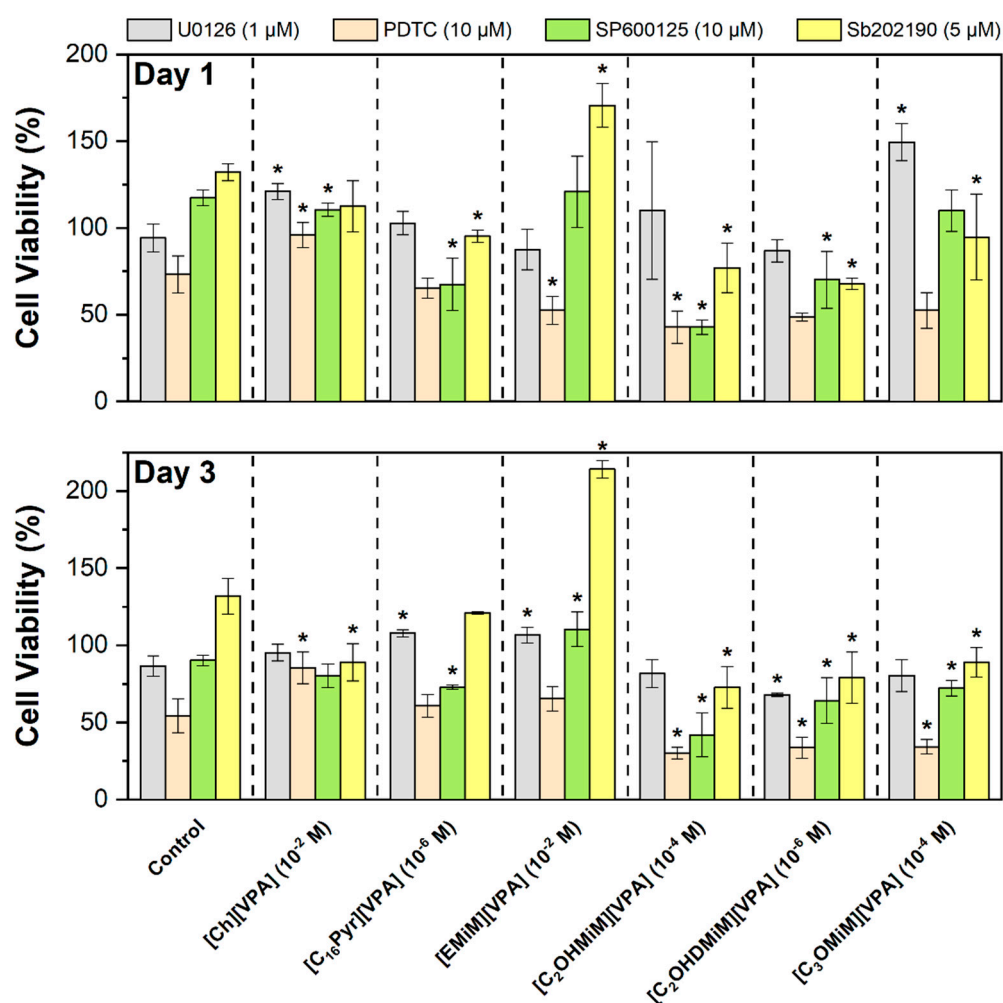


Figure 2. Cell viability in cultures of SH-SY5Y cells supplemented with different inhibitors of cell-signaling pathways: U0126, PDTC, SP600125 and Sb202190, which are the inhibitors of the MEK, NFκB, JNK and p38 pathways, respectively. The results are presented using as internal control the value obtained in the absence of any inhibitor for each IL-API experimental condition. Error bars are also provided, corresponding to the standard deviation of the three replicates, whose asterisk highlights the data significantly different from the control ($p < 0.05$).

Regarding the first inhibitor tested, U0126, it was observed that it did not promote any change on the MEK pathway, meaning that this pathway was not involved in the cellular response in any of the experimental conditions tested. This indicates that ILs-API did not significantly affect the MEK signaling pathway of NB SH-SY5Y cells.

On the other hand, the PDTC inhibitor led to a decrease in the cell viability in all conditions tested, except for the cultures supplemented with [Ch][VPA]. This effect was also visible for the cells treated with [C₁₆Pyr][VPA] on day 3 that revealed a similar behavior to the negative control. For the other ionic liquids, the reduction in the cellular viability was more accentuated. Therefore, this study allows to conclude that ILs-API do not interfere with the NFκB pathway, which plays a significant role in the cellular behavior.

Concerning the inhibitor SP600125, the imidazolium derivatives [C₂OHMiM][VPA], [C₃OMiM][VPA] and [C₂OHDMiM][VPA] influenced the JNK pathway on day 3 since a decrease in cellular viability was observed. For day 1, no significant differences between these compounds were registered.

Finally, the Sb202190 inhibitor exclusively modified the cell response in cultures supplemented with [EMiM][VPA], resulting in an increase on viability and contrary to what

was observed for the negative control. For this reason, the presence of this IL influenced the p38 pathway.

Through this section, it is possible to conclude that the inhibitors U0126 and SP600125 did not promote significant changes in the cell viability during the experiment period. However, PDTC decreased the cell viability between days 1 and 3 of the culture in all the studied compounds, whereas Sb202190 revealed an increase during the same period.

4. Conclusions

In this work, one tumor cell line of NB (SH-SY5Y) and GF were used as an in vitro study model to characterize the effect in cell metabolism of several ionic liquids based on valproate anion, allowing for the comparison in terms of toxicity between neoplastic cells and non-neoplastic cells. The latter was used as a control, which helped to understand if the results are tumor-specific or a consequence of general cytotoxicity. Moreover, this study also aimed to evaluate the influence of these compounds in different signaling pathways, which are important for the behavior of tumor cells. The cations assessed herein were selected due to their biocompatibility, being already applied in the pharmaceutical area. The signaling pathways were chosen according to the previous literature related to NB tumorigenesis.

The cellular viability tests revealed that some of the compounds possess higher cytotoxicity than VPA, demonstrating that the antitumor activity can be attributed to the presence of the organic cation. In addition, the prepared ionic liquids showed low cytotoxicity and high selectivity against the GF and SH-SY5Y cell lines, respectively, suggesting that the compounds are not toxic for human cell lines. [C₂OHDMiM][VPA] stood out as one of the most interesting formulations, exhibiting high antitumor activity and low toxicity to non-neoplastic cells during the overall treatment period. These results highlight this compound as a possible antitumor agent. Nonetheless, further studies are needed to fully understand the action mechanism of ILs-API since it is a crucial aspect for the development of novel antitumor agents, as well as for studying their effect on other tumor cell lines.

Regarding the influence on the four studied inhibitors, the ionic liquids synthesized herein revealed the ability to modulate the signaling pathways in SH-SY5Y cells, which enhances, once again, their potential as antitumor agents and opens new possibilities for novel therapies.

This work also highlights the importance of the wise combination between the cation and anion on the preparation of ILs with specific biological activity and cytotoxicity that are key steps when developing novel therapeutic drugs.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/futurepharmacol2030022/s1>: Figure S1. [Ch][VPA] ¹H NMR spectrum in CDCl₃. Figure S2. [Ch][VPA] ¹³C NMR spectrum in CDCl₃. Figure S3. [Ch][VPA] IR spectrum in KBr. Figure S4. [C₁₆Pyr][VPA] ¹H NMR spectrum in CD₃OD. Figure S5. [C₁₆Pyr][VPA] ¹³C NMR spectrum in CD₃OD. Figure S6. [C₁₆Pyr][VPA] IR spectrum in KBr. Figure S7. [EMiM][VPA] ¹H NMR spectrum in CDCl₃. Figure S8. [EMiM][VPA] ¹³C NMR spectrum in CDCl₃. Figure S9. [EMiM][VPA] IR spectrum in KBr. Figure S10. [C₂OHMiM][VPA] ¹H NMR spectrum in CDCl₃. Figure S11. [C₂OHMiM][VPA] ¹³C NMR spectrum in CDCl₃. Figure S12. [C₂OHMiM][VPA] IR spectrum in KBr. Figure S13. [C₂OHDMiM][VPA] ¹H NMR spectrum in CD₃OD. Figure S14. [C₂OHDMiM][VPA] ¹³C NMR spectrum in CD₃OD. Figure S15. [C₂OHDMiM][VPA] IR spectrum in KBr. Figure S16. [C₃OMiM][VPA] ¹H NMR spectrum in CD₃OD. Figure S17. [C₃OMiM][VPA] ¹³C NMR spectrum in CD₃OD. Figure S18. [C₃OMiM][VPA] IR spectrum in KBr. References [67–70] are cited in Supplementary Materials.

Author Contributions: Conceptualization, J.C.-R., L.C.B. and Ž.P.; methodology, A.R.D. and R.F.; validation, C.P., J.P.N., L.C.B. and Ž.P.; investigation, A.R.D., R.F., A.F.M.S. and M.L.J.; writing—original draft preparation, A.R.D. and R.F.; writing—review and editing, R.F., A.F.M.S. and M.L.J.; supervision, C.P., J.P.N., L.C.B. and Ž.P. All authors have read and agreed to the published version of the manuscript.

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