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A graphite furnace-atomic absorption spectrometry-based rubidium efflux assay for screening activators of the K_V7.2/3 channel

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Abstract

For the characterization of K_V7.2/3 channel activators, several analytical methods are available that vary in effort and cost. In addition to the technically elaborate patch-clamp method, which serves as a reference method, there exist several medium to high-throughput screening methods including a rubidium efflux flame-atomic absorption spectrometry (F-AAS) assay and a commercial thallium uptake fluorescence-based assay. In this study, the general suitability of a graphite furnace atomic absorption spectrometry (GF-AAS)-based rubidium efflux assay as a screening method for K_V7.2/3 channel activators was demonstrated. With flupirtine serving as a reference compound, 16 newly synthesizedcompounds and the known K_V7.2/3 activator retigabine were first classified as either active or inactive by using the GF-AAS-based rubidium (Rb) efflux assay. Then, the results were compared with a thallium (Tl) uptake fluorescence-based fluorometric imaging plate reader (FLIPR) potassium assay. Overall, 16 of 17 compounds were classified by the GF-AAS-based assay in agreement with their channel-activating properties determined by the more expensive Tl uptake, fluorescence-based assay. Thus, the performance of the GF-AAS-based Rb assay for primary drug screening of K_V7.2/3-activating compounds was clearly demonstrated, as documented by the calculated Z'-factor of the GF-AAS-based method. Moreover, method development included optimization of the coating of the microtiter plates and the washing procedure, which extended the range of this assay to poorly adherent cells such as the HEK293 cells used in this study.

KEYWORDS
drug screening, graphite furnace atomic absorption spectrometry, HEK293, KCNQ, K_V7.2/3 channel activators

1 | INTRODUCTION

The voltage-gated homotetrameric $K_v7.2$ (KCNQ2) and $K_v7.3$ (KCNQ3) channels as well as their heterotetrameric type $K_v7.2/3$ (KCQN2/3) are representatives of the K_v7 potassium channel family. They are also counted among the so-called M channels because they are sensitive to muscarine and are inactivated upon activation of the muscarinic acetylcholine receptor M_1.[1] Potassium channels can be slowly activated by depolarizing the cell membrane. Through the influx of potassium into the cells, they exert a hyperpolarizing effect and can thus attenuate neuronal excitation.[2]

Several clinical pathologies, such as benign neonatal seizures and various pain afflictions, have been associated with K_v7 channels or their mutations.[3] Therefore, these channels generally represent a promising target for the treatment of such disorders.[4] The analgesic drug flupirtine and the anticonvulsant retigabine (Figure 1) stabilize the open state of the $K_v7.2/3$ channel. However, due to side effects such as drug-induced liver injury and tissue discoloration, respectively, approval for both drugs was withdrawn.[5] As a result, an auspicious target based on its broad spectrum of physiological and pathophysiological significance remains underutilized. Nevertheless, researchers such as ourselves are exploring strategies to design safer openers of K_v7 channels.[6]

The process of developing new K_v7 openers also includes studying their effect on channel activity. Several techniques already exist for determining channel activity, with the electrophysiological patch-clamp method being considered the gold standard.[7] Here, the ion current is measured directly at the cell membrane with a tiny microelectrode. Due to limitations,[8] the method has been further developed; for example, the transition from manual to the so-called loose patch clamp, which, however, is also fraught with disadvantages.[9] The high cost and complex implementation of this technique are major drawbacks, so its application is often not feasible, especially for drug screening purposes.[10]

In contrast to electrophysiological methods, fluorescence-based approaches only indirectly determine ion currents. Ion flux through membrane channels leads to changes in membrane potential and concentration of the transported ion. A dye sensitive to the ion of interest (ionophore) forms a complex having fluorescent properties, the concentration of which can be easily measured. Various assay kits based on this concept are commercially available. Often the flux of thallium (Tl) ions through the channels is exploited as an alternative to potassium.[11] The performance and optimization of these assays are less complex and better suited for high-throughput screening compared with patch-clamp methods. To obtain reliable results in the screening of new drug candidates, it is necessary that no adverse interaction of the drug with the ionophore takes place.[12] However, the fluorogenic ionophore used in commercial assays is usually subject to trade secrets, and thus the structure is largely unknown. In addition, some ionophores themselves are thought to have biological activities.[13] Moreover, test compounds may also show fluorescence similar to the Tl-bound ionophore. This could ultimately have an undesirable effect on channel activity and distort the results.

For the reasons mentioned above, commercial, fluorescence-based assays are not always best suited in the context of screening yet poorly characterized compounds. The omission of ionophores is possible for assays that quantify ion currents by atomic absorption spectrometry (AAS). It is quite common to study the activity of potassium channels with a rubidium (Rb) efflux assay based on flame-AAS (F-AAS).[14–16] However, the use of F-AAS can pose hazards to laboratories due to the use of highly flammable gases. Since potassium ions are abundant in cells, the particular measurement of potassium flux by AAS is hardly feasible due to high background levels. Instead, rubidium(I) serves as a surrogate ion and, in addition, the toxic concerns of thallium are avoided. Although this subtechnique shows a higher precision,[17] it usually has a poorer limit of quantification.[18] Furthermore, F-AAS suffers from lower sensitivity compared with graphite furnace AAS (GF-AAS). This is due to the less efficient sample introduction into the atomizing unit, that is, flame versus graphite tube. In addition, it is possible to obtain higher temperatures for atomization in the electrothermal approach than in the flame. Depending on the element, an insufficiently high temperature may cause incomplete atomization. The consistency of atomization with F-AAS is not as good as using a graphite tube.[19] By using electrothermal atomization in terms of GF-AAS, these disadvantages mentioned for F-AAS can be circumvented.[20]

To our knowledge, GF-AAS has not yet been evaluated for an Rb efflux assay. However, given the better sensitivity and lower sample volumes of the GF-AAS subtechnique, this is a reasonable approach, especially when measuring trace amounts of metal ions in microtiter plate volumes, as in the proposed Rb efflux assay.

Therefore, in the current work, an Rb efflux assay based on GF-AAS was developed. The general suitability of this assay for application in drug screening was investigated with 16 potential $K_v7.2/3$ openers as well as the known channel openers flupirtine and

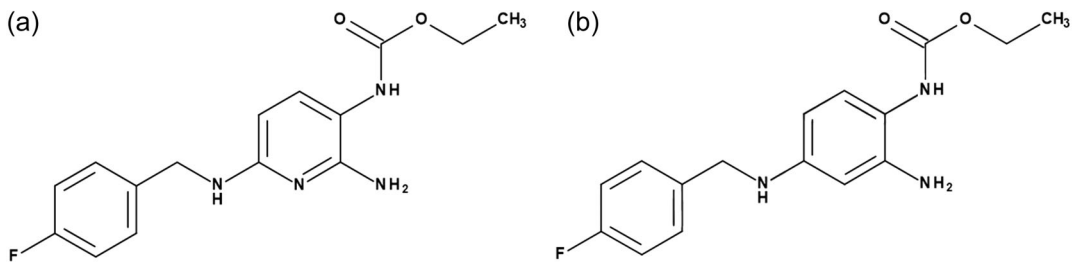

FIGURE 1 Chemical structures of flupirtine (a) and retigabine (b).

retigabine. The results were compared with those obtained with a commercial Tl fluorescence-based assay to demonstrate the utility of the GF-AAS-based assay and its impact on the future development of new drugs to activate K_v7 channels.

2 | RESULTS AND DISCUSSION

2.1 | Culturing and treatment of the cell line

A crucial aspect in the development of the GF-AAS-based Rb efflux assay was to ensure that the hKCNQ2/3-transfected HEK293 cells would adhere to the microtiter plates throughout the assay. If this were not the case, higher efflux values could result erroneously due to the detachment of cells from the plates during the workup. In this context, expensive poly-D-lysine-coated microplates commonly used with HEK293 cells were replaced by standard 96-well microtiter plates coated with a 0.1% gelatin solution.

Due to the generally poor adhesion of transfected HEK293 cells to plastic surfaces, cells can be lost by mechanical jolts, such as during washing steps. To prevent this, a more gentle washing procedure was developed and applied during the experiments. We initially diluted the medium or buffer three times in each well and removed it completely only once per wash.

In addition, a medium without antibiotic additives was used when seeding the cells into the microtiter plates. The absence of antimicrobial supplements was intended to avoid further stressing of the cells. The implementation of all these interventions resulted in an intact cell monolayer until lysis of the cells (Figure 2). This allowed for better reproducibility and avoided false high efflux values due to cell loss, especially before lysis.

2.2 | Primary and secondary screening for active compounds

To verify the practical applicability of the Rb efflux assay for the identification of $K_v7.2/3$-activating drugs, 16 compounds recently synthesized in a drug-development program in our laboratories were evaluated and finally classified with respect to their activity profile. The efflux values were determined by quantifying the Rb content of the supernatant and the lysate, respectively, by GF-AAS. One sample of each of the compounds to be tested was analyzed at 1.0 and 10 µM. Three samples of the reference compound flupirtine (10 µM) were determined and a total of six samples of the solvent control. To achieve a reasonable level of reproducibility, the entire procedure was repeated once.

To classify the effect of the compounds on the activity of $K_v7.2/3$ channels, the difference between the efflux values of the test compounds and the reference flupirtine (each concentration 10 µM) was determined independently in duplicate and finally averaged. An efflux difference above 0 indicates an active compound and a value between −10 and 0 a slightly active compound, while an efflux difference below −10 classifies the compound as inactive. If the efflux difference of the test compound was above 0 even at the lower test concentration of 1 µM, the compound was considered highly active.

Additionally, the compounds were analyzed with a commercially available fluorescence-based fluorometric imagining plate reader (FLIPR) potassium assay. For this purpose, a dilution series of each compound was tested three times, followed by the calculation of EC_{50} (50% effective concentration) and E_{max} (% maximal effect) values in relation to the maximum effect of flupirtine.

Table 1 shows the results of the primary screening with the GF-AAS-based Rb efflux assay as well as the EC_{50} and E_{max} values obtained by FLIPR potassium assay. In the listing, the compounds were sorted from the highest to the lowest E_{max} value.

All the compounds with an E_{max} value greater than 100%, that is, compounds **1–9** and retigabine, were classified as active compounds when their efflux differences were considered, with the exception of compound **10**. The latter compound would have to be classified as inactive based on its efflux difference of −15.4 at the concentration of 10 µM. In contrast, its maximum effect (E_{max} = 105%) obtained by FLIPR potassium assay was slightly higher than that of the reference flupirtine.

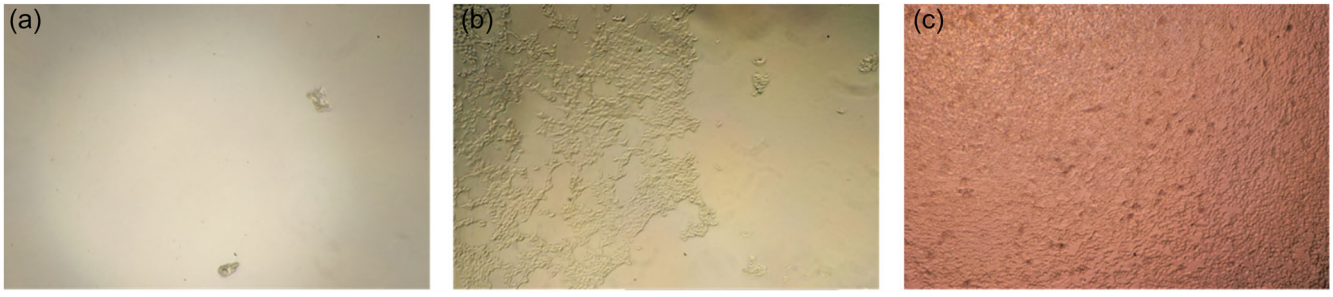

FIGURE 2 Cell monolayer after seeding 30,000 HEK293 cells per well into 96-well plates, followed by incubation for 72 h and before lysis. (a) Cells seeded on uncoated plates in a medium containing antimicrobial additives and washed six times. (b) Cells seeded on plates coated with 0.1% gelatine in a medium with antimicrobial additives and washed six times. (c) Cells seeded on plates coated with 0.1% gelatine in medium without an antimicrobial agent and washed with the optimized washing procedure.

TABLE 1 Results of primary screening with the Rb efflux assay compared to EC_{50} and E_{max} values obtained with the fluorescence-based FLIPR potassium assay. Data were ordered by descending E_{max} value (green: active compounds; red: inactive compounds according to Rb efflux assay).

Compound	Mean efflux difference at 10 µM	Mean efflux difference at 1 µM	Result primary screening	E_{max} [a] [%]	EC_{50} [µM]	Correctly predicted [b]
1	15.3	−15.6	active	176 ± 14	0.675 ± 0.276	☑
2	20.3	1.6	highly active	174 ± 12	0.339 ± 0.075	☑
3	10.3	−22.7	active	170 ± 14	3.78 ± 1.73	☑
4	12.7	−17.9	active	156 ± 14	0.036 ± 0.093	☑
5	−7.6	−29.8	slightly active	149 ± 25	2.25 ± 0.34	☑
6	14.8	1.4	highly active	144 ± 11	0.117 ± 0.029	☑
7	18.0	14.3	highly active	132 ± 13	0.017 ± 0.009	☑
Retigabine	17.7	−5.9	active	119 ± 7	0.249 ± 0.052	☑
8	7.0	6.7	highly active	117 ± 19	0.010 ± 0.006	☑
9	13.5	2.7	highly active	114 ± 19	0.126 ± 0.035	☑
10	−15.4	−27.4	not active	105 ± 12	0.310 ± 0.12	☒
Flupirtine	0	0		100	1.84 ± 0.84	-
11	−19.5	−24.8	not active	71 ± 9	7.86 ± 2.17	☑
12	−18.3	−25.5	not active	65 ± 9	3.52 ± 0.72	☑
13	−17.4	−31.5	not active	45 ± 4	1.18 ± 0.19	☑
14	−29.8	−28.4	not active	21 ± 18	2.05 ± 1.84	☑
15	−20.5	−22.6	not active	/	> 20	☑
16	−24.8	−27.1	not active	/	> 20	☑

Main efflux difference from two independent determinations with the Rb efflux assay, where an efflux difference at a test concentration of 10 µM above 0 classifies a compound as active, between - 10 and 0 as slightly active, and below −10 as inactive. Compounds with an efflux difference above 0 at a test concentration of 1 µM are classified as highly active. EC_{50} and E_{max} values were determined using the fluorescence-based FLIPR potassium assay and reported as mean ± SD (n ≥ 3); E_{max} values are relative to the maximum effect of flupirtine, which was set at 100%. [a] Efficacy related to flupirtine; [b] Based on the mean efflux difference at 10 µM compared to E_{max}.

A possible explanation for this discrepancy could be poor water solubility due to the lipophilic nature of compound **10**, as indicated by a Log$D_{7.4}$ value of 4.1. Only compound **12**, which was identified as inactive in both assays, showed a higher lipophilicity with a Log$D_{7.4}$ value of 4.7. For all other compounds tested, the Log$D_{7.4}$ ranged from value 2.3–3.8.[21–23] Consequently, poor water solubility could lead to the precipitation of compound **10** in the aqueous medium, so its channel-opening properties could not be properly determined in the Rb efflux assay. This assumption is also supported by the results from the FLIPR potassium assay. Indeed, a decreasing fluorescence signal was observed at assay concentrations of compound **10** higher than 2.5 µM. Nevertheless, the results of the Rb efflux assay of compounds **1–9** and retigabine in the primary screening allowed a classification into active compounds, which was also confirmed by the FLIPR assay. This clearly demonstrates that the Rb efflux assay can be used successfully for primary screening. This is also true for the inactive compounds **11–16**; these showed differences in efflux distinctly lower than −10 in the GF-AAS-based Rb-based assay and gave E_{max} values of 71% or even less in the FLIPR as well as EC_{50} up to 7.86 µM.

Interestingly, compounds that were classified as highly active (efflux difference > 0 at both 10 and 1 µM) by the Rb efflux assay had an EC_{50} value below 400 nM. Among the active representatives, the others were classified as active or slightly active based on the Rb efflux assay, and most of them also had considerably higher EC_{50} values (up to 3800 nM). Nevertheless, there are also some exceptions. For example, retigabine (E_{max} = 119% and EC_{50} = 249 nM) and compound **4** (E_{max} = 156% and EC_{50} = 36 nM) which indicated high potency by the FLIPR assay, were not classified as highly active in the Rb efflux assay, but simply as active. This implies that an exact agreement of the potency by the Rb efflux assay with the FLIPR assay is not possible. However, it is questionable whether this should really be required from an assay used for primary screening. The aim of this assay was to check for threshold activity, and according to the Rb efflux assay, retigabine and compound **4** were correctly classified in this respect, therefore not limiting the usefulness of the assay.

Thus, the GF-AAS-based Rb efflux assay appears to complement the more widely used but expensive FLIPR assay.

In addition, the concentration-dependent efflux of the drugs retigabine and flupirtine was investigated by the GF-AAS-based Rb efflux assay, and EC_{50} and E_{max} values were calculated from the data. These values were compared with those obtained by the fluorescence-based FLIPR potassium assay and are summarized in Table 2.

The dose–response curves with the Rb efflux assay showed a rightward shift compared with the curves by the fluorescence-based method (Figure 3). The EC_{50} value of flupirtine is greater when determined by the Rb efflux assay (6.36 µM) than that of the fluorescence-based assay (1.84 µM). The same is true for the EC_{50} value of retigabine with values of 0.249 µM (FLIPR assay) and 0.552 µM (Rb assay). However, the latter value is in good agreement with a previously reported EC_{50} value for retigabine of 0.5 µM, obtained with the Rb efflux assay.[15]

A similar finding of a rightward shift of the concentration-dependent response curves was also observed in studies determining the IC_{50} values of human-ether-a-go-go-related gene (hERG) channel inhibitors.[24] Here, the potency of the hERG inhibitors was underestimated with the Rb efflux assay compared with the potency obtained by patch clamp. As a cause for this, a conformational change of the channel pore by the rubidium as a conducting ion was suggested.[25]

However, it is not known whether a similar interaction could also lead to a shift of the curves to the right when examining $K_v7.2/3$ channels. Ion size could principally be assumed to be the cause, but seems rather unlikely since the ions transported through the channels in the Rb efflux assay as well as in the FLIPR potassium assay are larger than the physiologically occurring potassium ion, but have nearly identical ion radii. To support the suggestion that rubidium itself is responsible for the rightward shift of the curves, the GF-AAS method could be repeated in an upcoming study using thallium as the ion for cell saturation and detection instead of rubidium. However, the toxic properties of the thallium ion pose a potential concern, which should be explicitly circumvented by the use of rubidium in the current assay.

Furthermore, retigabine was found to have slightly higher intrinsic activity than flupirtine, as indicated by the E_{max} value of 108% calculated from the Rb efflux assay, while according to the fluorescence-based assay, retigabine had a higher E_{max} value of 119% (Table 2).

2.3 | Quality and performance of the Rb efflux assay

The *Z*′-factor is a characteristic parameter to evaluate the performance and quality of a screening assay. It can generally assume all values < 1, but practically is only significant in the range from 0 to 1. A value of 1 means an ideal assay without any deviation from the control. Desirable values are between 0.5 and 1 since in this interval a well-recognizable distinction of the signals of positive and negative control and a low variance can be assumed. Previous studies reported *Z*′-values of 0.73 and 0.81 for the Rb efflux assay using an automated F-AAS procedure.[15,16]

The *Z*′-factor was also determined for the present GF-AAS-based Rb efflux assay based on the data from 10 experiments (Figure 4a) performed during assay development and validation. Figure 4b shows the calculated *Z*′-factors.

The mean value of the *Z*′-factor was calculated to be 0.70, which can be considered excellent.[26] The strong deviation of the factors ranging from 0.52 to 0.90 is striking. The graphite tubes used in the GF technique are often subject to a kind of acclimatization phase until, after a few heating cycles, the tube is appropriately burned in to obtain maximal optical absorption values.[27] This could have an influence on any measurements, although the analysis of the reference flupirtine is also affected by this and therefore this effect may be compensated for, at least to a certain extent. However, further investigations in this regard were not performed as part of the present study.

Nevertheless, at no time was a value ≤ 0 reached, which always allows the distinction between the efflux after treatment with solvent (negative control) and the maximum efflux achieved by the addition of compound (positive control). The determined *Z*′-factor is only an indication of the assay's actual quality, particularly as its significance is reduced by the small number of experiments performed. However, the *Z*′-factor obtained here demonstrates the general suitability of a GF-AAS-based Rb efflux assay for use as a screening method and thus confirms the aim of this study.

3 | CONCLUSION

The aim of this study was to demonstrate the general suitability of the GF-AAS subtechnique for an Rb efflux assay. To the best of our knowledge, this has not yet been described in the literature. The GF-AAS method could provide valuable advantages compared with conventional F-AAS, especially when used in the primary screening of new drugs serving as potassium channel activators.

The described method combines the advantages of the GF-AAS subtechnique, such as easier handling in the laboratory as well as higher sensitivity, and smaller sample volumes, and is basically more

TABLE 2 Comparison of the EC_{50} and E_{max} values of flupirtine and retigabine from the GF-AAS-based Rb efflux assay and the fluorescence-based FLIPR potassium assay.

Compound	Rb efflux assay		FLIPR potassium assay	
	EC_{50} (µM)[a]	E_{max} (%)[b]	EC_{50} (µM)[a]	E_{max} (%)[c]
Flupirtine	6.36 ± 1.69	100	1.84 ± 0.84	100
Retigabine	0.552 ± 0.062	108 ± 8	0.249 ± 0.052	119 ± 7

[a]Mean value ± SD ($n \geq 3$).

[b]Relative to the maximum rel. efflux of flupirtine at 10 µM.

[c]Relative to the maximum flupirtine-induced fluorescence signal.

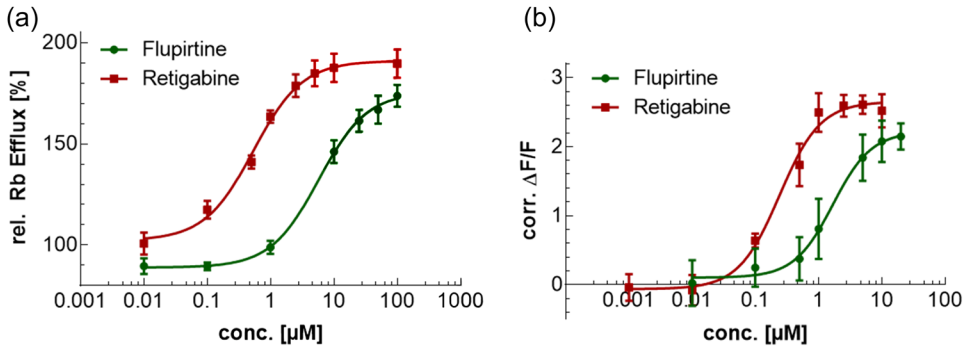

FIGURE 3 Concentration-dependent response curves of flupirtine and retigabine determined with graphite furnace atomic absorption spectrometry (GF-AAS)-based Rb efflux assay and the FLIPR potassium assay. (a) The Rb efflux of HEK293 KCNQ2/3 cells after incubation for 30 min with flupirtine and retigabine in concentrations ranging from 0.01 to 100 µM was expressed relative to the control efflux (Depolarization buffer with a dimethyl sulfoxide [DMSO] concentration of 1% [V/V] equal to treatment with compound). (b) Corr. $\Delta F/F$ values of HEK293 KCNQ2/3 cells after incubation for 30 min with flupirtine (0.01–20 µM) and retigabine (0.001–10 µM). The mean values ± SD ($n \geq 3$) for both compounds and assays are shown in Table 2.

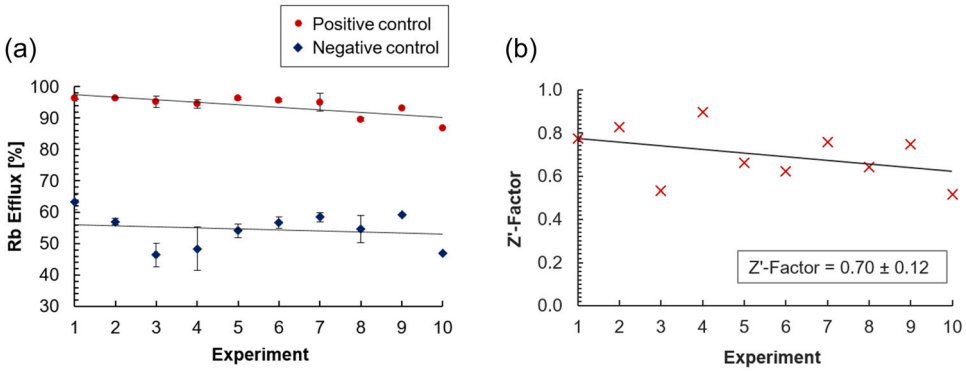

FIGURE 4 Evaluation of assay quality and suitability for screening purposes based on the *Z*'-factor. (a) Efflux values and their standard deviations of the positive control (sample with the highest efflux value obtained in the experiment) and the negative control (efflux value of the solvent control). (b) Respective *Z*'-factor determined from 10 experiments.

cost-effective and simpler than other modalities for measuring channel activities. This extends the practical application range of the assay.

In addition, the particular assay procedure was optimized with respect to poorly adhering cells, such as the K_v7.2/3-transfected HEK293 cells. By adding a 0.1% gelatin coating to the microtiter plates utilized for cell seeding and applying a gentle washing procedure, cell adhesion was maintained throughout the experiment. This ensured assay reproducibility without the purchase of significantly more expensive coated microplates.

The elaborated protocol was applied to the screening of 16 recently synthesized compounds as potential activators and retigabine as a known activator of the K_v7.2/3 channel. Predictions regarding the activity and nonactivity of the 17 studied compounds made with the Rb efflux assay were compared with the results from a commercial fluorescence-based FLIPR potassium assay. The results were confirmed for all the compounds, with just one exception (16 out of 17, i.e., about 95%). In addition to the close agreement of the GF-AAS-based screening with the activities of the compounds determined in the more cost-intensive FLIPR potassium assay, the calculated *Z*'-factor further highlights the suitability of the GF-AAS-based Rb efflux assay as a valid screening method for identifying potassium channel activators. Due to its cost-effectiveness and simplicity, this method is currently being used in the development of potassium channel activators.

A potential limitation of the assay is that precipitation of the test compound by the depolarization buffer must be avoided when preparing the samples. On the other hand, the strong dependence of assay performance on cell attachment was also revealed in this study. Therefore, in the case of transferring this assay for the investigation of other cells (expressing other potassium channels, such as the K_v7.2 or K_v7.3 homotetramers), it seems important that the (washing) procedure ensures sufficient cell adhesion. In this context, optimization by adjusting washing steps seems to be necessary depending on the individual cell line. With respect to the G-FAAS measurement, the time-temperature program could be optimized by using appropriate modifiers, if necessary. This is often accompanied by a preservation of the cuvette, extending its lifetime. Even if potential future developments still exist, this work shows

a further example of the general suitability of the GF-AAS technique for the modern screening of organic drugs.

4 | EXPERIMENTAL

4.1 | General materials

Rubidium chloride (RbCl, purity: 99%) and nitric acid suprapur® (HNO_3) were purchased from Sigma-Aldrich (Cat. No.: 55727-100 mL) and Merck (Cat. No.: 1.00441.0250), respectively. They were of analytical grade and therefore suitable for the AAS measurements. In addition, standard 96-well microtiter plates (Sarstedt; Cat. No.: 83.3924.005) were used for the Rb efflux assay. For the fluorescence-based FLIPR potassium assay, the commercially available FLIPR® Potassium Assay Kit from Molecular Devices® was employed as well as 96-well Vision Plate™ Black microtiter plates (Azenta; Cat. No.: 4ti-0221). A total of 16 compounds from the in-house library[21–23] were selected for investigation in the current study. Ultrapure water obtained from an ELGA Purelab flex system (Veolia) was used throughout the experiments. Flupirtine as a reference compound was used in the form of flupirtine maleate. All other chemicals, reagents, and solvents were of high quality and commercially available.

4.2 | Cell culture

The biological investigations were carried out with the transfected HEK293 hKCNQ2/3 cell line obtained from SB Drug Discovery. This cell line stably expressed $K_v7.2/3$ (KCNQ2/3) potassium channels. It was cultured in 75 cm^2 flasks in minimum essential medium (MEM) purchased from Thermo Fischer and supplemented with heat-inactivated fetal calf serum (10%, V/V), 2.00 mmol/L of L-glutamine, both purchased from Sigma-Aldrich, a ready-to-use mixture of penicillin and streptomycin (1%, V/V) from Pan Biotech, 0.78 mmol/L of geneticin disulfate solution from Carl Roth and 4.00 mmol/L of blasticidin S purchased from VWR at 37°C under a humidified atmosphere (5% CO_2 and 95% air). Cells were serially passaged once per week and routinely monitored for possible contamination with mycoplasma.

4.3 | Pharmacological/biological assays

4.3.1 | Rb efflux assay

To ensure sufficient adhesion of the cells to the microtiter plates throughout the assay, the plates were coated before seeding the cells. For impregnation, a sterile gelatine solution (0.1%) was added and incubated at 37°C for 30 min. After removing the gelatine solution, the plate was dried under a laminar flow cabinet for 10 min. Cells (HEK293 hKCNQ2/3) were seeded with the supplemented medium as described but without antibiotic supplements. To perform the Rb efflux assay, 3.0×10^4 cells were seeded in each well of the 96-well plate and incubated for 72 h. After 48 h of incubation, half of the cell culture medium was carefully replaced with fresh culture medium.

To preserve the cell layer, a gentle washing procedure was applied as follows: approximately 75% of the medium was removed and replaced with wash buffer (25 mmol/L 2-[4-(2-Hydroxyethyl) piperazin-1-yl]ethane-1sulfonic acid (HEPES), 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.8 mmol/L NaH_2PO_4, 2 mmol/L $CaCl_2$, pH adjusted to 7.4 with NaOH). This step was then repeated two more times, overall accompanied by an increase in the absolute amount of wash buffer. Finally, the medium was completely removed.

The procedure of the Rb efflux assay is summarized in Figure 5. Each well was loaded with 200 µL of loading buffer and incubated at 37 °C for 3 h to saturate the cells with rubidium. The loading buffer consisted of a wash buffer supplemented with 5.4 mmol/L of RbCl and 5.0 mmol/l of glucose. This pretreatment was followed by a washing step corresponding to the first washing process. The test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with depolarization buffer (DP10: 25 mmol/L HEPES, 140 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.8 mmol/L NaH_2PO_4, 2 mmol/L $CaCl_2$, 10 mmol/L KCl, pH adjusted to 7.4 with NaOH) to the desired concentration. Finally, 200 µL of the solutions prepared in this way were added to the wells containing the cells. The concentration of DMSO in each well was 1% (V/V). The reference consisted of depolarization buffer and DMSO at a concentration equal to that of the samples containing the compound.

The cells were incubated at room temperature for 30 min followed by the removal of 40 µL of depolarization buffer, which was transferred to another microtiter plate. The remaining depolarization buffer was carefully removed and discarded. Lysis buffer (Triton X-100 0.1% [V/V] dissolved in depolarization buffer) was then added to the remaining cell monolayer and lysed at 37 °C. The lysate fraction was diluted to the same volume as the depolarization buffer fraction.

Before AAS measurement, depending on the desired dilution (total dilution factor 100–500), the respective volume of buffer and lysate fraction was transferred to AAS sample tubes and diluted to a final volume of 1000 µL with 0.5% HNO_3. However, care was taken to ensure that the amount of depolarization buffer was consistently 4% (40 µL in 1000 µL). Depending on the preceding dilution step, a respective volume of depolarization buffer was therefore also added, if necessary, when filling up to 1000 µL.

The levels of Rb in the samples were measured with a UNICAM SOLAAR 989 QZ AAS spectrometer (Thermo Elemental) based on the GF technique. The measurement of the absorption of Rb was performed at its characteristic wavelength of 780.0 nm. The diffraction grating was set to a slit width of 0.2 nm and half height. As a measurement signal, the peak height of the absorption signal was chosen. The reason for this is that a greater sensitivity was implied compared with the use of the peak area, contrary to the so-called stabilized temperature platform furnace (STPF) concept. The Zeeman background correction was applied; the magnetic field was generated at the atomizer (inverse Zeeman) with a field strength of 0.85 T. For each measurement, 20 µL of the sample was injected

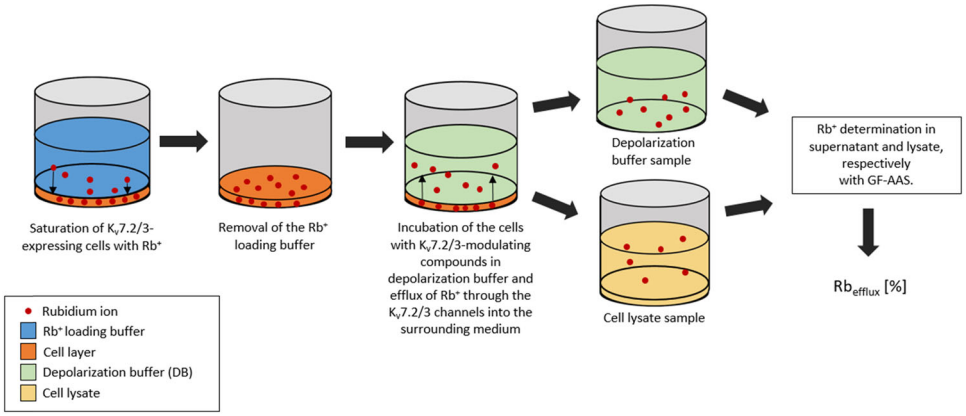

FIGURE 5 Schematic overview of the rubidium (Rb) efflux assay procedure.

TABLE 3 Time-temperature program applied for the determination of Rb with GF-AAS.

Operation	Temperature (°C)	Heating rate (°C/s)	Time (s)	Gas/rate
Drying	100	10	30	N_2/0.2 L/min
Pyrolysis	800	150	20	Ar/0.2 L/min
Atomization	1900	200	3	Stop
Cleaning	2200	200	3	N_2/0.2 L/min

Abbreviation: GF-AAS, graphite furnace atomic absorption spectrometry.

directly into the pyrographite-coated cuvettes with extended lifetime (Altmann Analytik; Cat. No.: 9423-393-95041-ASP) by using a FS90 plus furnace sampler before starting the furnace program. The time-temperature program is shown in Table 3. Depending on the lifetime of the graphite cuvette, the method used showed a limit of quantification between 2.5 and 5.0 ppb Rb, calculated as the mean plus 10 times the standard deviation from the successive measurement of 10 blanks (40 µL of depolarization buffer in 960 µL of 0.5% HNO_3). Each sample was determined in triplicate. A new calibration was performed before each series of measurements. Depending on the cuvette used and its lifetime, calibration concentrations ranged from 2.5 to 100 ppm. For this purpose, six to seven standards were prepared with the Rb standard solution in 0.5% HNO_3. To each standard, 40 µL of depolarization buffer (4% V/V) was added to provide the same matrix as the samples. The concentration of the depolarization buffer ensured that the same matrix of samples and calibration standards was achieved despite different dilutions of each sample.

The values of the Rb_{efflux} were calculated according to the following equation:

$$Rb_{efflux}\,[\%] = \frac{Rb_{sup}}{Rb_{sup} + Rb_{lys}} \cdot 100,$$

where Rb_{efflux} is the percent Rb efflux, Rb_{sup} represents the Rb concentration of the supernatant sample and Rb_{lys} reflects the concentration of the lysate sample.

The relative efflux *rel*. Rb_{efflux} is the quotient of the compound efflux and the solvent control efflux:

$$rel.\ Rb_{efflux}\,[\%] = \frac{Rb_{efflux}\ (compound)}{Rb_{efflux}\ (solvent\quad control)} \cdot 100.$$

4.3.2 | FLIPR potassium assay

In this study, the FLIPR® potassium assay kit from Molecular Devices® (Biberach an der Riss, Germany) was used. It deploys a Tl-sensitive dye that is taken up by cells in a preincubation phase and then generates a fluorescent signal after intracellular binding to thallium ions. In this process, the singly charged thallium ions serve as a surrogate for potassium ions. The intensity of the fluorescence signal allows conclusions to be drawn about the activity of the potassium channels and ultimately about the influence of the test compounds on the channels. The performance of the assay and the buffer preparation was made according to the kit manufacturer's instructions.

The same cell line (HEK293 hKCNQ2/3) as used for the Rb efflux assay was selected. To perform the FLIPR assay, 6.0×10^4 cells were seeded in each well of the 96-well plate and incubated for 24 h. After incubation, each well containing the cells was loaded with 100 µL of loading buffer (including 5 mmol/L probenecid) and incubated for 1 h at room temperature in the dark. The test compounds were then dissolved in DMSO, added to the wells at the desired concentration, and incubated for an additional 30 min under the same conditions. The control consisted of loading buffer and DMSO (1%, V/V) at a concentration equal to that of the samples containing the compound. Fluorescence was detected with an Infinite F200 Pro plate reader (Tecan) at excitation and emission wavelengths of 485 and 535 nm, respectively. After determining the background fluorescence (baseline) in each well for 20 s, a stimulus buffer (25 mmol/L K^+ and 15 mmol/L Tl^+) was added to each sample immediately before measurement.

To determine the dose–response curves, calculations were performed in the same manner as previously published.[21,28] First, the fluorescence intensity of the tested compounds was normalized with the baseline signal (F/F_0) at each time point of signal acquisition. Then, a value for the corrected negative control was determined by subtracting the value of 1 from the normalized value of the negative control. Next, the corrected negative control value was subtracted from the maximum value (corr. F/F_0) of a compound at a given concentration (corr. $\Delta F/F_0$) and then plotted against the logarithm of the concentration. The EC_{50} value was calculated as relative values with the software GraphPad Prism 6. The maximum response (E_{max}) indicates the intrinsic activity of a compound. This value was determined relative to the reference drug flupirtine. It is maximum corr. $\Delta F/F_0$ value was defined as 100%. The EC_{50} and E_{max} values of each compound represent the mean ± standard deviation (SD) of at least three independent experiments.

4.3.3 | Z'-factor

The so-called Z'-factor is used to assess the quality of an assay and its suitability for screening purposes.[29] It represents the dynamic assay range and data variability. Since the factor is dimensionless, it is ideal for comparing different assays. The Z'-factor was calculated according to the following equation:

$$Z' = \frac{3s_{c+} + 3s_{c-}}{|\bar{x}s_{c+} - \bar{x}s_{c-}|},$$

where s_{c+} and s_{c-} represent the standard deviation and $\bar{x}s_{c+}$ and $\bar{x}s_{c-}$ represent the mean values of positive and negative control, respectively. The efflux value of the most active compound (efflux values > 90%) served as the positive control, whereas the efflux of the negative control was determined by measuring the depolarization buffer containing DMSO at a concentration equal to that of the samples with the compound. The Z'-factor can generally take on all values <1 but is practically only significant in the limits from 0 to 1. A value of 1 means an ideal assay without any variance in the control signals.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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