INTRODUCTION

Voltage-gated potassium channels are a new potentially important molecular target in cancer therapy (1). One of main reasons of that importance is the fact that activity of these channels is necessary for cancer cell proliferation (1-3). It was shown that inhibition of potassium channels also inhibited the process of proliferation of cancer cells in various phases of this process (1-3). It is also known that expression of potassium channels is changed in many types of cancer cells in relation to corresponding healthy tissues (1). It may therefore be possible to block preferentially the channels in cancer cells.

One of the types of voltage-gated potassium channels, which may regulate cancer cell proliferation, are Shaker-related Kv1.3 channels (1-4). These channels, discovered in 1984 in T lymphocytes (TL), are expressed in many other tissues, both healthy (5) and cancer (6-13). It was shown that the expression of Kv1.3 channels is changed in case of prostate, colon and breast cancer...
Investigation performed on Kv1.3 channels expressed in TL showed that inhibition of these channels inhibited the cell proliferation in the G1 phase (1, 14-16). Therefore, specific blockers of Kv1.3 channels are promising candidates for a selective immunosuppression (16). It is also possible that inhibition of Kv1.3 channels may inhibit the proliferation of cancer cells over-expressing these channels. It was shown that inhibition of voltage-gated potassium channels, among them also Kv1.3 channels, inhibited the proliferation of T84 human colonic carcinoma cells (13).

Investigation on the influence of selected multidrug resistance inhibitors on the activity of Kv1.3 channels seems to be important for potential application of these compounds in cancer therapy. It may be related both to their ability to modulate the multidrug resistance, which would sensitize cancer cells to anti-cancer drugs, and inhibition of Kv1.3 channels, which may inhibit cell proliferation. Previously, a group of compounds was tested in our laboratory. To this group belonged phenothiazine derivatives: trifluoperazine and thioridazine, the anti-cancer drug applied in therapy of breast cancer: tamoxifen, and natural polyphenolic compounds such as the isoflavone: genistein, the flavanolignan: silibin, and the stilbene - resveratrol. Obtained results provide evidence that, except for silibin, all the compounds tested were effective inhibitors of Kv1.3 channel activity (17-21). It was also shown that the Kv1.3 channel inhibition was due to specific interactions of these compounds with the channel proteins (17-21). Inhibition of Kv1.3 channels by genistein occurred in the same concentration range as modulation of the multidrug resistance and inhibition of colon cancer cell proliferation by this substance (18). On the other hand, it was shown that silibin was not an inhibitor of Kv1.3 channel activity, although this substance effectively inhibited the multidrug resistance-associated protein MRP1 (19, 21). This allowed us to suggest that the ability to inhibit Kv1.3 channel activity was not a general property of all multidrug resistance modulators. Inhibition of Kv1.3 channels by some of them was probably not related to the inhibition of the multidrug transporters: P-gp or MRP1 by these compounds.

However, since the majority of the previously tested compounds appeared to be effective inhibitors of Kv1.3 channels, investigations on the influence of the multidrug resistance modulators on the channel activity were continued with application of other compounds. The tested substances were both plant-derived compounds and their derivatives (22). The first group of compounds tested were stilbenes: piceatannol (1) and its derivatives - 3,5,3',4'-tetramethoxy-piceatannol (2) and 3,5,3',4'-tetracetoxy-piceatannol (3), (Fig. 1). The studies were also performed using flavonoids: naringenin (4) and its derivatives 4',7-dimethylether (5), and 7-methylether (6) and aromadendrin (7), coumarins: esculetin (8) and scopoletin (9), and ent-abietane diterpenes: helioscopinolide B (10), its 3β-acetoxy derivative (11) and helioscopinolide E (12), (Figs. 2-4). In previous experiments it was shown that, except for compounds 8 and 9, the compounds listed above were inhibitors of the multidrug transporter MRP1 (22).

Results of these experiments provide evidence that methoxy-derivatives of piceatannol and naringenin (compounds 2, 5 and 6) applied at a concentration of 30 µM are effective inhibitors of Kv1.3 channels. Other tested compounds are ineffective at this concentration. A preliminary report was published in a form of an abstract (23).

**MATERIALS AND METHODS**

**Isolation of human T lymphocytes**

Since Kv1.3 channels are expressed endogenously in a large number in human T lymphocytes (TL) isolated from peripheral blood, these cells were used in our experiments as a model system. The cells were isolated at the Department of Biophysics, Wroclaw Medical University, from the peripheral blood of 5 healthy donors using a standard method of described elsewhere (24). The procedure of blood sample extraction is in accordance with the Good Medical Practice and it was approved by the Committee of Bioethics at the Wroclaw Medical University. After the isolation the cells were cultured for at least 24 hours in the standard medium RPMI-1640 (Sigma Co., St. Louis, MO, USA) supplemented with 5% vol/vol of fetal calf serum (Sigma Co., St. Louis, MO, USA). During the experiments the cells were suspended in the
extracellular solution containing (in mM): 150 NaCl, 4.5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, pH = 7.35 adjusted with NaOH, 300 mOsm. The pipette solution contained (in mM): 150 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 EGTA; pH=7.2 adjusted with KOH, 300 mOsm. The concentration of free calcium in the internal solution was below 100 nM, assuming the dissociation constant for EGTA at pH=7.2 of 10⁻⁷ M (25). Such a low calcium concentration was applied in order to prevent the activation of calcium-activated KCa3.1 channels (25). The reagents were provided by the Polish Chemical Company (POCH), except for HEPES and EGTA, which were purchased from Sigma Co., St. Louis, MO, USA.

**Tested compounds**

Compounds 1, 8 and 9 were isolated from the methanol extract of *Euphorbia lagascae* seeds (26), compounds 4, 7, 10 and 12 were isolated from the methanol extract of *Euphorbia tuckeyana* (27); compounds 2, 3, 5, 6 and 11 were obtained by methylation or acetylation of compounds 1, 4 and 10, respectively (22). The procedure of chemical synthesis and purification of the compounds was described in more detail elsewhere (22, 26, 27). The chemical structure of synthesized compounds was confirmed both by physical methods (Thin Layer Chromatography) and spectroscopic methods, such as UV, IR, ¹H NMR and ¹³C NMR spectroscopy (22). The final purity of the compounds assessed by High Pressure Liquid Chromatography (HPLC) was more than 98% (M-J. U. Ferreira - personal communication). All tested compounds were dissolved in DMSO. The DMSO stock solutions of tested compounds were diluted and applied at a final concentration of 30 µM except for piceatannol (1), which was applied at 40 µM concentration. These concentrations are similar to those applied previously in experiments on the influence of these compounds on the activity of membrane multidrug transporter MRP1 (22).

**Patch-clamp recording**

Dishes with cells were placed under an inverted Olympus IMT-2 microscope. Solutions containing tested compounds were applied using a perfusion system developed in our laboratory. Pipettes were pulled from a borosilicate glass (Hilgenberg, Germany) and fire-polished before the experiment. The pipette resistance was in the range of 3-5 MΩ.

Whole-cell potassium currents in TL were recorded applying the patch-clamp technique (28). The currents were recorded using an EPC-7 Amplifier (HEKA, Germany), low-pass filtered at 3 kHz, digitised using CED Micro 1401 (Cambridge, UK) analogue-to-digital converter with the sampling rate of 10 kHz. A standard depolarising sequence contained 10 voltage ramps applied every 20 s depolarising the cell membrane from -100 mV up to +40 mV; ramp duration was 340 ms and holding potential -90 mV. The linear (ohmic) component of the current was subtracted off-line from the final record. All experiments were carried out at room temperature (22-24°C).

**RESULTS AND DISCUSSION**

Examples of whole-cell potassium currents recorded in TL in the presence of tested compounds are depicted in Figs. 5-7. The currents were recorded using a standard depolarising sequence with voltage ramps (see Materials and Methods) under control conditions, upon application of the tested compound and after
wash-out. In each case application of a voltage ramp evoked a current, which contained two components: a linear non-specific leak current subtracted during the off-line analysis followed by a non-linear outward current activated upon membrane depolarisation. Our previous experiments demonstrated that the non-linear component was predominantly due to activation of Kv1.3 channels (29).

Fig. 5. Examples of whole-cell potassium currents as a function of time recorded in T lymphocytes using voltage ramps from -100 mV to +40 mV in the presence of tested stilbenes: A) compound 1, B) compound 2, C) compound 3. Records "a" - control conditions, records "b" - application of the tested compound, records "c" - wash-out. The voltage ramp is drawn schematically in panel A above the records.

Fig. 6. Examples of whole-cell potassium currents as a function of time recorded in T lymphocytes using voltage ramps from -100 mV to +40 mV in the presence of tested flavonoids: A) compound 4, B) compound 5, C) compound 6, D) compound 7. Records "a" - control conditions, records "b" - application of the tested compound, records "c" - wash-out. The voltage ramp is drawn schematically in panel A above the records.

Fig. 5. depicts examples the currents recorded in the presence of tested stilbenes (compounds 1-3). Apparently, the Kv1.3 component of the currents was significantly reduced in the presence of compound 2 at a concentration of 30 µM (Fig. 5B, record "b"). Wash-out of the drug caused a recovery of Kv1.3 component (Fig. 5B, record "c"). On the other hand, application of compound 1 at a concentration of 40
µM and compound 3 at a concentration of 30 µM did not affect significantly Kv1.3 currents (Fig. 5A,C).

Fig. 6. depicts examples of the currents recorded in the presence of tested flavonoids (compounds 4-7). Apparently, the Kv1.3 currents were significantly reduced in the presence of compounds 5 and 6 applied at a concentration of 30 µM (Fig. 6B,C records "b"). Wash-out of the drugs caused a recovery of the currents (Fig. 6B,C records "c"). However, application of compounds 4 and 7 at a concentration of 30 µM did not affect significantly Kv1.3 currents (Fig. 6A,D).

Fig. 7A. depicts examples of the currents recorded in the presence of compound 9 applied at a concentration of 30 µM. Apparently, application of this compound did not affect significantly the currents. Similar results were obtained in case of compound 8 applied at the same concentration (not shown). Fig. 7B depicts the currents recorded in the presence of 30 µM of compound 12. Application of this compound did not affect significantly the currents. Lack of current reduction was also observed in case of compounds 10 and 11 applied at the same concentration (not shown).

The results are summarised in Table 1. This table depicts in [%] average peak current ratio calculated by dividing peak current values recorded at ±40 mV (the highest voltage of the voltage ramp) upon application of the tested compounds by the peak currents recorded at the same membrane potential under control conditions. Data are presented as an average value ± standard deviation.

Obtained results show that a reversible reduction of the current amplitude occurred upon application of compounds 2, 5 and 6. The inhibitory effect was strongest in case of compound 5. The effects exerted by compounds 2 and 6 were comparable to each other. Application of compound 1 at a concentration of 40 µM and compounds 3, 4, 7-12 at a concentration of 30 µM did not significantly affect the recorded currents (Table 1).

Apparently, all the channel inhibitors are methoxy-derivatives of piceatannol (1) and naringenin (4). However, not all the methoxy-
derivatives tested were effective inhibitors of Kv1.3 channels. Application of scopoletin (9), which is a methoxy-derivative of esculetin (8, Fig. 3), did not inhibit significantly the currents (Table 1).

The results provide evidence that not all tested compounds applied at a concentration of 30 µM appeared to be effective inhibitors of Kv1.3 channels. This confirms our previous suggestions that the ability to inhibit Kv1.3 channels is not a property of all inhibitors of the membrane multidrug transporter MRP1, but only of some of them. At the present it is not possible to explain, why some tested compounds inhibit the channels at this concentration and others do not. It could only be mentioned that difference in a chemical structure between the inhibitors of Kv1.3 channels and non-inhibitors are very subtle. For example, piceatannol (1) does not inhibit Kv1.3 channels, whereas resveratrol, which has only one hydroxyl group less in the molecule structure, was shown to be an effective inhibitor of the channels (20). On the other hand, 3,5,3',4'-tetramethoxy-piceatannol (2), is a more effective inhibitor of the channels than resveratrol: application of 30 µM of compound 2 inhibits the currents to ca. 31% of their control value, whereas resveratrol inhibited the currents to a half of the control value only at 40 µM concentration (20). The two methylated derivatives of naringenin (4) differ from its structure by the presence of one or two methoxyl, instead of hydroxyl groups (Fig. 2). Thus, it can be suggested that the channel inhibition is due to specific interactions of the inhibitor molecules with the channel proteins. Mechanism of such an inhibition is probably different for each of the inhibitors. More detailed studies are needed to elucidate this problem.

At the present we can not reject the hypothesis that the compounds tested in this study and ineffective at a concentration of 30 µM might inhibit the channels if applied at higher concentrations. To verify this hypothesis more studies will have to be done. However it should be taken into account that at higher concentations at least some of tested compounds could be toxic for the cells. More experiments are also needed to study in detail the concentration dependence of the inhibitory effect observed for compounds 2, 5 and 6.

It should be pointed out that the compounds 2 and 6, which are effective inhibitors of Kv1.3 channels are also effective inhibitors of the membrane multidrug transporter MRP1 (22). However, naringenin-4',7-dimethylether (5), which is the most potent inhibitor of Kv1.3 channels among all of the tested compounds, was a weak inhibitor of the MRP1 transporter (22). On the other hand, the compounds such as piceatannol (1), naringenin (4) or aromadendrin (7), which are effective inhibitors of MRP1 transporter (22), do not inhibit Kv1.3 channels when applied at a concentration of 30 µM. There is also no correlation between ability to inhibit MRP1 transporter by the derivatives of piceatannol and naringenin and their ability to inhibit Kv1.3 channels. When comparing the results shown in Table 1 with the earlier published data on the inhibition of MRP1 transporter (22) one can conclude that piceatannol (1) and naringenin (4), which are not inhibitors of Kv1.3 channels, are much more potent inhibitors of MRP1 transporter than the methoxy-derivatives of these compounds, which inhibit the channels. Thus, the processes of inhibition of Kv1.3 channels and MRP1 transporters can be considered as two different processes, which occur independently on each other.

It is also interesting that the inhibition of Kv1.3 channels by compounds 2 and 6 occurs at the concentrations comparable to those, which are necessary to inhibit MRP1 transporter (22). It may have significance in case of cancers, such as colon cancer, for example, in which there is an overexpression of both Kv1.3 channels and MRP1 transporters (10). It may be possible that, in such a case, both the Kv1.3 channels and MRP1 transporters could be inhibited simultaneously. Since the inhibition of Kv1.3 channels may inhibit the cancer cell proliferation, the inhibition of MRP1 transporters by the compounds may be accompanied by an inhibition of the cell proliferation. This may have significance for a potential application of these compounds in the case of cancers, in which there is an overexpression of both Kv1.3 channels and MRP1 transporters.

It is also worthy to note that Kv1.3 channels are not the only voltage-gated potassium channels, which can be inhibited by the tested compounds simultaneously with MRP1 transporters. Recently published results showed that naringenin (4) inhibited HERG channels expressed in Xenopus oocytes and HEK cells with a half-blocking concentration (IC_{50}) equal to 102.6 µM nad 36.5 µM, respectively (30-31). Although these experiments were done in relation to the channels expressed in cardiac cells, it is known that the HERG channels human ether-a-go-go channels, classified as Kv11.1 channels in the IUPHAR nomenclature (4) are also present in various types of cancer cells including colon cancer, where their overexpression is highly correlated with invasive phenotype (1). It was shown that inhibition of the HERG channels decreases cell proliferation in the G2 phase (1). Since naringenin also inhibits MRP1 transporters with a similar potency (75 µM of the compounds inhibits the MRP1 activity by 44.24%) (22), it may be possible...
that both the inhibition of HERG channels and MRP1 transporters in cancer cells occurred simultaneously.

It is well-known that the most potent inhibitors of Kv1.3 channels, such as oligopeptides (e.g. Noxistoxin, Margatoxin, Kaliotoxin, Agitoxin 1 and 2) or nonpeptide small-molecule organic compounds (e.g. correolide, Psora-4) inhibit the channels when applied at nanomolar or picomolar concentrations (16, 32). When compared with these high-affinity inhibitors, the compounds 2,5 and 6 tested in this study, which inhibit Kv1.3 channels at the concentration of 30 µM, are much less potent. On the other hand, these compounds are much more potent than the standard potassium channel inhibitor, tetraethylammonium (TEA), which inhibit Kv1.3 channels at low millimolar concentrations (14, 32). Therefore, these compounds could be considered as inhibitors with an "intermediate" potency. Other well-known small-molecule organic compounds that inhibit Kv1.3 channels with an "intermediate" potency are: nifedipine, verapamil, quinine, diltiazem, progesterone, propranolol and veratridine (14, 32).

Moreover, the compounds inhibit the channels at the concentration comparable to those reported previously for other multidrug resistance modulators such as trifluoperazine, thiouracil, tamoxifen, genistein and resveratrol (17, 18, 20). At the present it is known that trifluoperazine, genistein and resveratrol are not specific inhibitors of Kv1.3 channels, but they inhibit various types of ion channels, including some types of voltage-gated potassium channels different than Kv1.3 (17, 18, 20). It remains unknown whether the compounds 2,5 and 6 are specific inhibitors of Kv1.3 channels. More experiments on other types of voltage-gated potassium channels are needed to study in detail this problem.

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Author’s address: Prof. Dr Andrzej Teisseyre; Phone: +48 71 7841414; Fax: +48 71 7840088; e-mail: ateiss@biofiz.am.wroc.pl