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Genistein and Resveratrol: Inhibitors of Kv1.3 Channels in Cancer Cells

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Abstract

Background: genistein and resveratrol are bioactive compounds isolated from plants, recognised for their diverse biological activities including anti-cancer properties. Both compounds are also known as modulators of potassium channels, including the Kv1.3 ones. These channels are expressed in both normal and cancerous tissues. Their activity is crucial in regulating cell proliferation and apoptosis in cells that express Kv1.3 channels. The potential clinical application of channel inhibitors may extend to treating cancers characterized by an over expression of Kv1.3 channels. Methods: this study investigates the inhibitory effects of genistein and resveratrol on Kv1.3 channels in cancer cells – human leukemic Jurkat T cells, applying the whole-cell patch-clamp technique. Results: applying both compounds at concentrations ranging from 3 μM to 90 μM leads to a dose-dependent inhibition of the channel activity, reducing it to approximately 50% of control level. This inhibitory effect was reversible and associated with a significant reduction of the activation rate. When combined with simvastatin, the inhibitory effect exhibited synergy; however, it was additive when co-applied with mevastatin. Conclusion: the inhibition of Kv1.3 channels is likely linked to the anti-cancer activities of these compounds on Kv1.3 channel- expressing cancer cells, especially when co-applied with the statins.

Keywords: Kv1.3 channel; Jurkat T cell; genistein; resveratrol; cancer cell proliferation; cancer cell apoptosis

1. Introduction

Genistein (Figure 1a) is a plant-derived isoflavone found in several vegetables, recognised primarily as a potent inhibitor of protein tyrosine kinase (PTK). It has also been identified as a naturally occurring anti-cancer agent [1]. Additionally, genistein serves as an effective inhibitor of various ion channels, including several potassium channels types [2]. Importantly, its inhibitory action on these channels occurs via a PTK-independent mechanism likely involving direct interactions with channel proteins [2].

Resveratrol (3,4',5 – trihydroxystilbene – Figure 1b) is another biologically-active polyphenol derived from plants – most notably present in red grapes and red wine - exerting various biological effects, including an anti-cancer activity [3]. Like genistein, resveratrol modulates different potassium channel activities [2].

The voltage-gated potassium channel Kv1.3, encoded by the KCNA3 gene, is widely distributed across diverse tissues [4] and can be found not only within plasma membranes but also embedded within inner mitochondrial membranes (mito Kv1.3) [5]. The functionality of these channels plays an essential role in regulating cellular proliferation and apoptosis among cells that express them [4–14]. Alterations in their expression may occur significantly during certain cancer conditions [6–14].

Inhibitors targeting these channels hold promise for clinical applications aimed at treating various disorders – including specific cancers like melanoma, pancreatic ductal adenocarcinoma

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(PDAC), multiple myeloma and B-type chronic lymphocytic leukaemia (B-CLL) – that exhibit overexpression of Kv1.3 channels [6–18].

Research suggests that some lipophilic small-molecule organic inhibitors targeting Kv1.3 channels may possess anti-proliferative and pro-apoptotic properties against cancers expressing these channels while sparing normal cells [6–18].

Among these inhibitors are certain flavonoids, chalcones and statins [12,15,17], whose efficacy can be enhanced when combined with statins, such as simvastatin or mevastatin [15,17]. The augmented inhibitory effect on the channels may be co-related to an improved pro-apoptotic activity of these compounds, applied in a combination, on Kv1.3 channel-expressing cancer cells [15,17].

Previous studies have established that both genistein and resveratrol inhibit Kv1.3 channels in normal human T lymphocytes via concentration-dependent mechanisms accompanied by significant reductions in activation rates without altering the kinetics of inactivation [19,20]. The inhibitory effects of genistein and resveratrol were additive when both compounds were co-applied at the concentration of 50 μM [20].

It is well-known that function of membrane proteins, including ion channels, may be changed in cancer cells when compared to normal ones. Our previous studies have shown that sensitivity of Kv1.3 channels expressed in cancer cells Jurkat T to inhibition by naturally occurring compounds, including resveratrol, was lower than in case of the same channels in normal human T lymphocytes [Gąsiorowska – unpublished observations]. However, the influence of resveratrol on the activity of Kv1.3 channels in cancer cells was not studied in detail. Moreover, it still remains unknown whether putative inhibitory effects of genistein and resveratrol on Kv1.3 channels in cancer cells are augmented upon a co-application with the statins: simvastatin and mevastatin.

The objective of this research was to analyze how genistein and resveratrol influence the activity of Kv1.3 channels in cancer cells - specifically upon co-administration with simvastatin and mevastatin (Figure 1c and 1d). Given that Kv1.3 channels are abundantly expressed in human leukemic cell line Jurkat T – a relevant model system - this study aims to elucidate how the selected compounds affect channel activity in these cells [21,22].

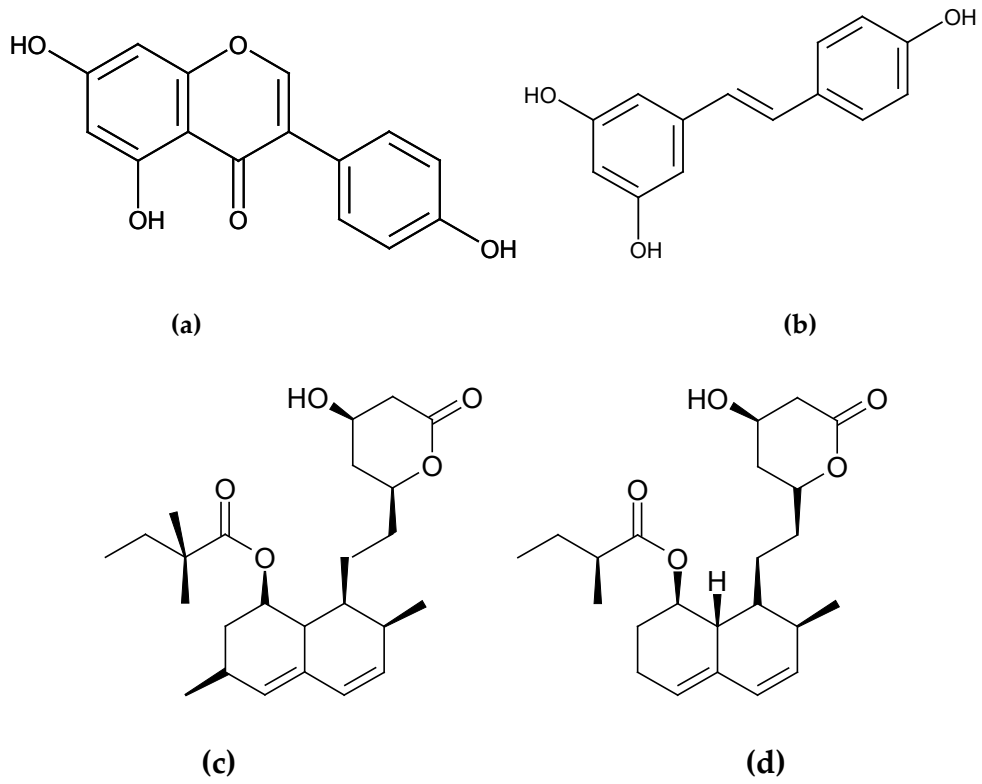

Figure 1. Structural formulas of genistein (**a**), resveratrol (**b**), simvastatin (**c**) and mevastatin (**d**).

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Our findings reveal that both genistein and resveratrol effectively inhibit Kv1.3 channels in Jurkat T cells; however, their effects were less pronounced than those observed previously in normal human T lymphocytes [19,20]. Notably, a combined application of each compound with the statins resulted in significantly augmented inhibitory effects compared to an individual application of each compound alone.

2. Materials and Methods

Cell Culture and Solutions

Jurkat (clone E6-1) human leukemic T cells were procured from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS, 10 mM HEPES along with glutamate (2 mM). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. During the experiments cells were placed in the external solution, which contained: 150 mM NaCl, 4.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, pH =7.35, adjusted with NaOH. The internal (pipette) solution contained: 150 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, pH =7.2, adjusted with KOH. The calculated concentration of free calcium ions in the internal solution was below 100 nM [23]. Such a low calcium concentration was necessary to prevent the activation of calcium-activated K^+ channels $K_{Ca}2.2$, which are abundantly expressed in Jurkat T cells, in addition to Kv1.3 channels [23,24]. Activation of these channels would produce an additional potassium current, irrelevant for this study [24]. The chemicals were purchased from the Polish Chemical Company (POCH, Gliwice, Poland), except of HEPES and EGTA that were purchased from SIGMA. Genistein and resveratrol were purchased from Alexis Biochemicals (Lausen, Switzerland).

Patch-Clamp Recordings

Dishes with cells were placed under an inverted Olympus IMT-2 microscope. Solutions containing tested compounds were applied using eight-channel gravitation perfusion system (ALA Scientific Instruments, Farmingdale, NY, USA). Pipettes were pulled from a borosilicate glass (Hilgenberg, Germany). The pipette resistance was higher than 3 MΩ.

Whole-cell potassium currents in TL were recorded applying the patch-clamp technique [25]. The currents were recorded using an EPC-7 Amplifier (HEKA, Germany), low-pass filtered at 3 kHz, digitised using a CED Micro 1401 analogue-to-digital converter (Cambridge, UK) with a sampling rate of 10 kHz. The influence of selected compounds on the activity of the channels was studied by applying the voltage ramp protocol. Voltage ramps gradually depolarised cell membranes from –100 mV up to +40 mV. Time duration of a single "ramp" was 340 ms, time interval between successive "ramps" was 30 s. At the time between "ramps" examined cells were kept at a holding potential of –90 mV. Upon application of the voltage ramp protocol, potassium currents in Jurkat T cells could be stably recorded for at least 20 minutes after "break-in" to the whole-cell configuration. During the off-line analysis the value of Kv1.3 current at the end of a voltage ramp (+40 mV) was calculated. For this purpose, the leak current estimated at +40 mV was subtracted from the total ramp current recorded at this voltage.

In order to study the influence of selected compounds on the channel activation and inactivation kinetics in more detail another protocol of depolarising voltage stimuli was applied. This protocol contained depolarising voltage steps from the holding potential of – 90 mV to +60 mV (500 ms step duration) applied every 30 s.

All experiments were carried out at room temperature (22-24°C).

Unless otherwise stated the data are presented as mean ± standard deviation.

Data Analysis

The inhibition of the channel is presented in terms of a relative current recorded upon application of the studied compounds, defined as I/I_{contr}; where: I – Kv1.3 current upon an application

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of an examined compound at + 40 mV, I_{contr} – Kv1.3 current recorded on the same cell at +40 mV under control conditions. Activation kinetics were measured in terms of a the time-to-peak (T_{peak}). The higher was the value of T_{peak} the slower was the activation kinetics. Since the T_{peak} values varied remarkably among examined cells, slowing of the activation kinetics upon an application of the compounds was measured in terms of a relative kinetics slowing (RKS), defined as:

$$RKS = ((T_{peakcompound} - T_{peakcontrol})/ T_{peakcontrol})*100\% \quad (1)$$

Where:

$T_{peakcompound}$ – mean time-to-peak value upon an application of the compound [ms],

$T_{peakcontrol}$ – mean time-to-peak value under control conditions [ms].

Inactivation kinetics were fitted by a single exponential function, defined as:

$$I(t) = A_1*\exp(-t/\tau) + A_2 \quad (2)$$

Where: A_1 and A_2 – fit constants, t – time, τ – inactivation time constant [ms]

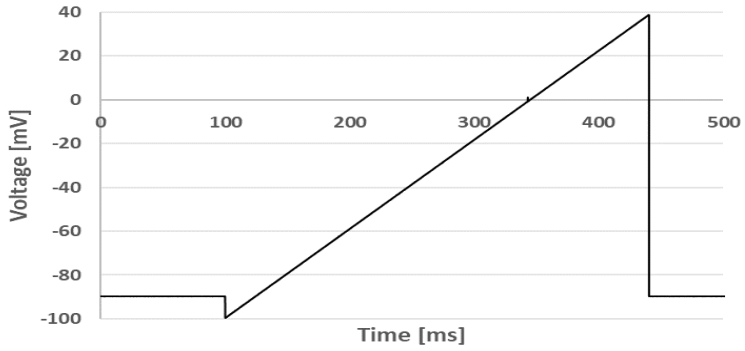
The τ value is the measure of the inactivation rate. The slower the inactivation is, the higher is the value of the τ.

Statistical analysis was performed applying the Student's unpaired t test or one-way ANOVA test. The results were considered statistically significant when $p<0.05$.

3. Results

Figure 2 depicts examples of the whole-cell ramp currents recorded in a Jurkat T cell applying the voltage ramp (defined in Materials and Methods, Figure 2a) under control conditions, upon an application of genistein and resveratrol at the concentration of 30 μM, and after wash-out of the compound (Figure 2b and 2c, respectively).

The figure depicts raw ramp currents (without leak subtraction), which contained two components: small linear and much bigger non-linear ones. The linear component was an unspecific leak current. This current was irrelevant for this study and it was subtracted from the total current during the off-line analysis. On the other hand, the non-linear component was due to an activation of Kv1.3 channels [26]. Visibly, an application of both genistein and resveratrol significantly diminished the amplitude of the Kv1.3 currents. The current recovered after the wash-out of genistein and resveratrol. This indicates that both compounds exerted an inhibitory effect on the current and this effect was fully reversible.



(a)

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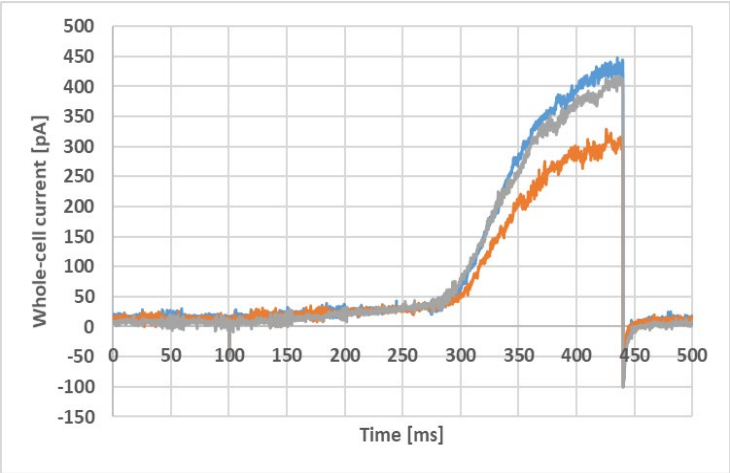
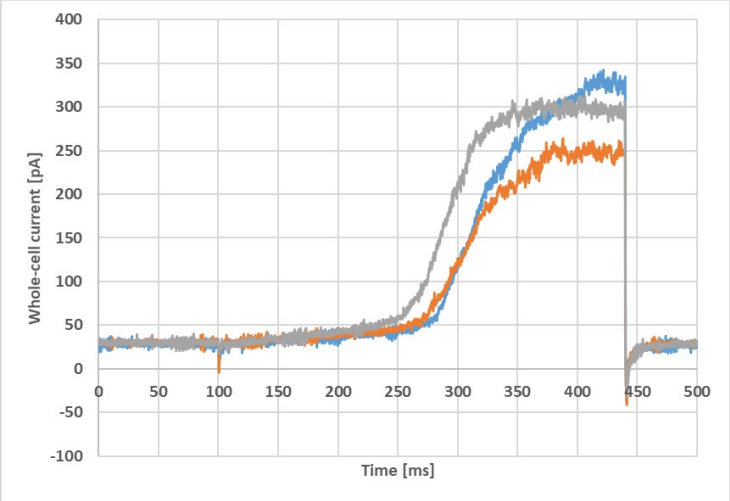


(b)



(c)

Figure 2. Whole-cell currents recorded in Jurkat T cells (**b** and **c)** applying the "voltage ramp" (**a**), under control conditions (blue traces), upon an application of 30 μM genistein (**b** -orange trace), 30 μM resveratrol (**c** -orange trace) and during wash-out of the drugs (grey traces).

The inhibitory effects of genistein and resveratrol on Kv1.3 whole-cell currents in Jurkat T cells was studied at different concentrations of the drugs. Figure 3 depicts relative peak currents upon an application of genistein and resveratrol at various concentrations ranging from 3 μM to 90 μM. Upon an application of genistein at the concentrations of 3 μM, 6 μM, 12 μM, 15 μM, 30 μM, 60 μM and 90 μM the relative peak currents were reduced to: 0.78±0.17 (n=17), 0.84±0.12 (n=18), 0.76±0.15 (n=21), 0.69±0.18 (n=18), 0.59±0.13 (n=13), 0.51±0.16 (n=21) and 0.50±0.10 (n=20) of the control value, respectively (Figure 3a, Supplementary Table 1). The inhibitory effect of genistein on Kv1.3 currents in Jurkat T cells was statistically significant ($p<0.05$) at all the concentrations applied and weakly dose-dependent (Figure 3a). Upon an application of resveratrol at the concentrations of 3 μM, 4.5 μM, 7.5 μM, 15 μM, 30 μM, 60 μM and 90 μM the relative peak currents were reduced to: 0.89±0.07 (n=19), 0.87±0.15 (n=19), 0.79±0.19 (n=21), 0.74±0.09 (n=24), 0.63±0.12 (n=11), 0.52±0.11 (n=11) and 0.47±0.07 (n=20) of the control value, respectively (Figure 3b, Supplementary Table 2). The inhibitory effect of resveratrol on Kv1.3 currents in Jurkat T cells was statistically significant ($p<0.05$) at all the concentrations and weakly dose-dependent (Figure 3b).

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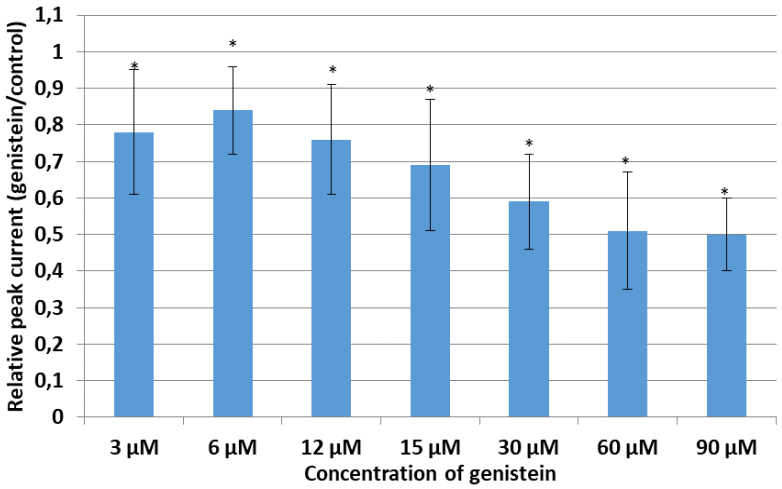
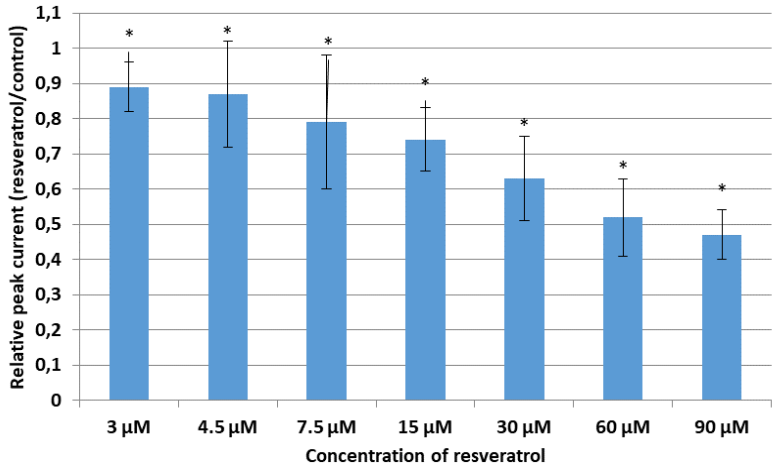


(a)



(b)

Figure 3. Relative peak current upon an application of genistein (**a**) and resveratrol (**b**) at different concentrations. Error bars are shown. * - statistical significance.

Our previous studies performed on Kv1.3 channels expressed in normal human T lymphocytes have demonstrated that the channel inhibition upon an application of genistein and resveratrol was accompanied, in both cases, by a significant slowing of the channel activation rate without any significant change of the inactivation rate [19,20]. Therefore, it was of interest to study whether such a slowing also occurred in case of the channels expressed in cancer cells. Figure 4 depicts whole-cell Kv1.3 channel currents recorded applying the voltage step protocol (see Materials and Methods, Figure 4a) under control conditions and upon an application of genistein at the concentration of 30 µM (b) and resveratrol at the concentration of 90 µM (c).

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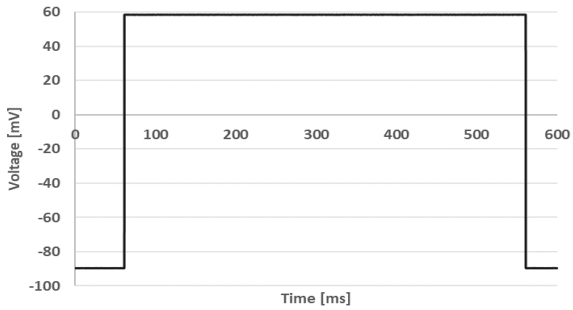
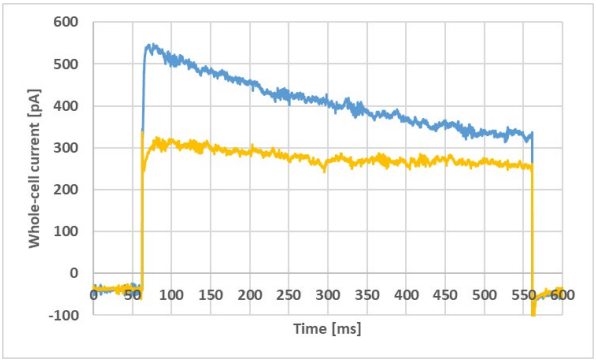


(a)



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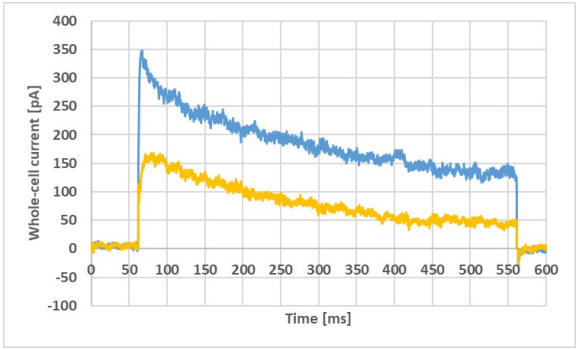

(c)

Figure 4. Whole-cell currents recorded in Jurkat T cells (**b** and **c**) applying the voltage step (**a**), under control conditions (blue traces), upon an application of 30 μM genistein (**b**-yellow trace), or 90 μM resveratrol (**c**-yellow trace). Traces for wash-out of the compounds were omitted for clarity.

Remarkably, an application of both compounds caused a significant slowing of the activation rate of the currents (Supplementary Tables 3 and 4). The slowing of the activation rate was measured in terms of the relative kinetics slowing (RKS), defined by the formula (1) in the section Materials and Methods. Figure 5 depicts the RKS values calculated for the currents recorded upon an application of genistein and resveratrol at different concentrations (Figure 5a, b). The RKS values upon an application of genistein at the concentrations of 3 μM, 6 μM, 12 μM, 15 μM, 30 μM, 60 μM and 90 μM were equal to 51,7%, 52,0%, 106,5%, 145,4%, 129,5%, 191,8% and 279,5%, respectively (Figure 5a). Activation kinetics slowing was statistically significant ($p<0.05$) at all applied concentrations. The RKS values upon an application of resveratrol at the concentrations of 3 μM, 4.5 μM, 7.5 μM, 15 μM, 30 μM, 60 μM and 90 μM were equal to -0.96%, 0,0%, 7,6,%, 17,4%, 24,0%, 100,0% and 116,0%, respectively (Figure 5b). Activation kinetics slowing was statistically significant ($p<0.05$) only at

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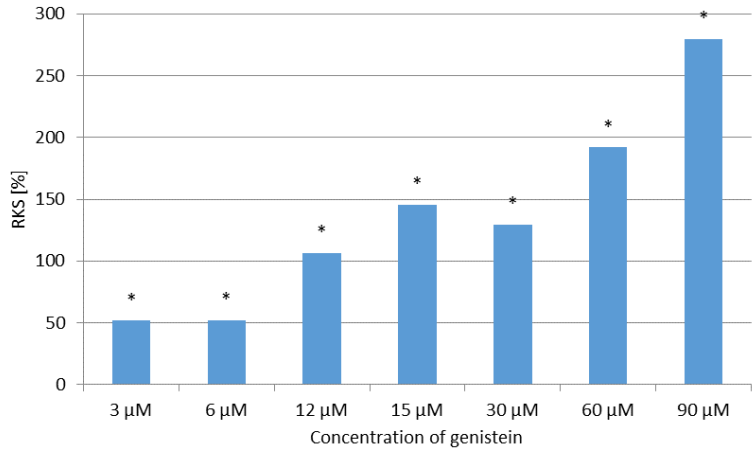
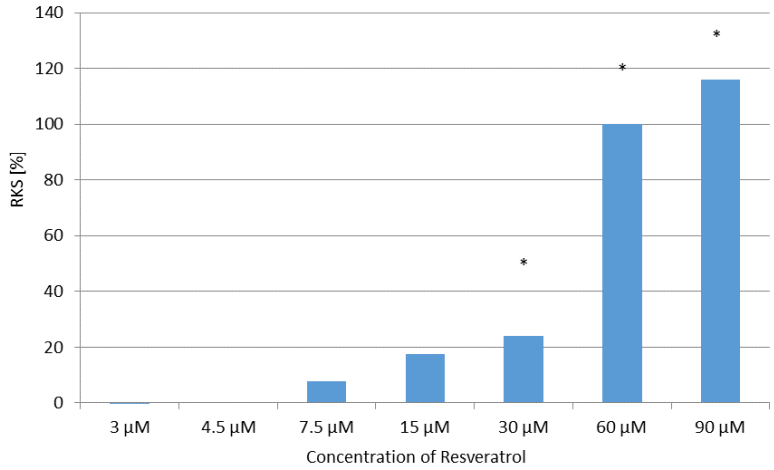
concentrations of 30 μM, 60 μM and 90 μM. Apparently, the RKS values were higher upon an application of genistein than when resveratrol was applied.



(a)



(b)

Figure 5. Relative kinetics slowing (RKS- defined in Materials and Methods) upon an application of genistein (**a**) and resveratrol (**b**) at different concentrations. * - statistical significance.

It was also proven whether an application of genistein and resveratrol changed the inactivation rate, measured in terms of the inactivation time constant τ calculated by applying the equation 2 (see Materials and Methods, Supplementary Table 5). Upon an application of genistein at the concentration of 30 μM, the τ value was equal to 205.8±40.62 ms (n=16). This value was not significantly different ($p>0.05$) from the control value of 201.6±28.07 ms (n=16). Lack of influence of genistein application on the inactivation kinetics was also observed at other concentrations of this compound (not shown). Upon an application of resveratrol at the concentration of 90 μM, the τ value was equal to 175.4±25.97 ms (n=16). This value was not significantly different ($p>0.05$) from the control value of 178.89±30.69 ms (n=14). Lack of influence of resveratrol application on the inactivation kinetics was also observed at lower concentrations of this compound (not shown).

Results of our previous study has shown that the inhibitory effects of genistein and resveratrol on Kv1.3 channels in normal human T lymphocytes were additive [20]. Therefore, it was of interest to prove, whether such an additive inhibition of the channels also occurred in case of a co-application of genistein and resveratrol in cancer Jurkat T cells.

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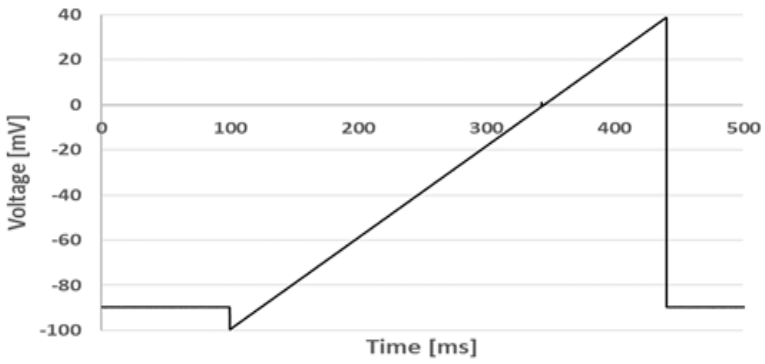
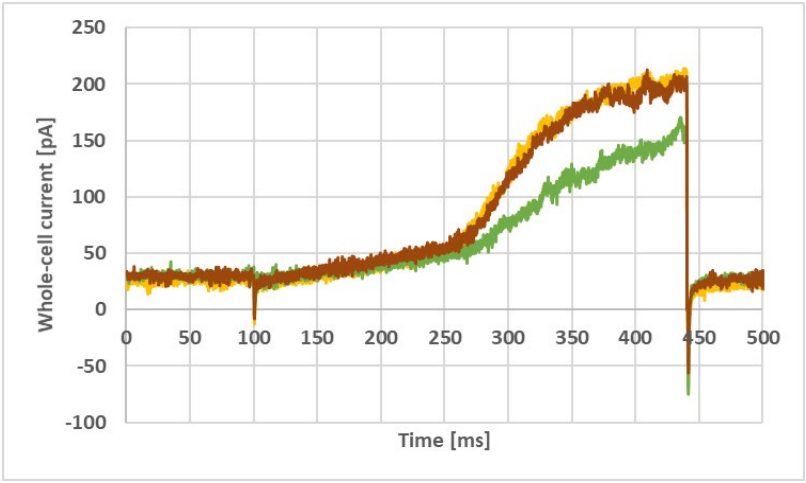
Figure 6b depicts an example of whole-cell current recorded under control conditions, upon a co-application of genistein and resveratrol at 30 μM and after wash-out of the mixture.



(a)



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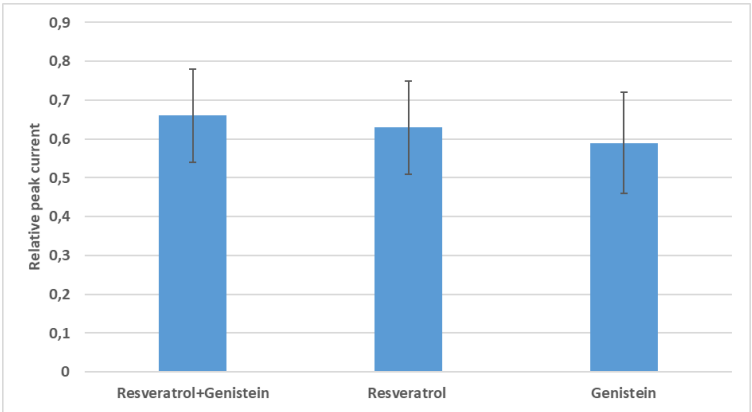

(c)

Figure 6. Whole-cell currents recorded in Jurkat T cells (**b**) applying the "voltage ramp" (**a**), under control conditions (**b** - yellow trace), upon co-application of 30 μM genistein with the same concentration of resveratrol (**b** - green trace) and during wash-out of the mixture (b - red trace). Relative peak currents upon a co-application of genistein and resveratrol at 30 μM concentration and genistein and resveratrol at the same concentration alone (**c**). Error bars are shown. * - statistical significance.

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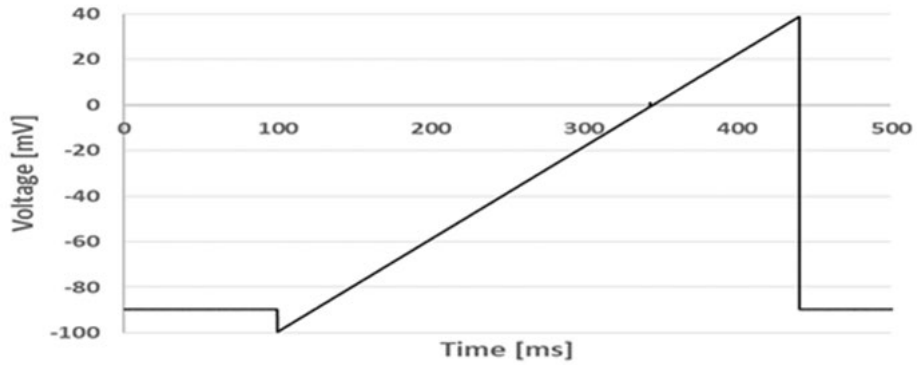
The inhibitory effects upon an application of a mixture of two examined compounds could be additive, synergistic or non-additive [15]. In case of an additive inhibitory effect (1+1=2) the relative peak current upon an application of a mixture is equal to the product of multiplication of relative peak currents upon an application of each compound alone. In case of a synergistic inhibitory effect (1+1>2) the relative peak current upon an application of a mixture is lower than the product of multiplication of relative peak currents upon an application of each compound alone [15]. In case of a non-additive inhibitory effect (1+1<2) the relative peak current upon an application of a mixture is higher than the product of multiplication of relative peak currents upon an application of each compound alone [15].

The relative peak currents recorded upon an application of genistein and resveratrol alone at 30 μM concentration were equal to 0.59 and 0.63, respectively (see above). The theoretical value of the relative peak current upon a co-application of genistein and resveratrol at this concentration would be equal to 0.59*0.63= 0.37. However, the whole-cell current recorded upon a co-application of genistein and resveratrol was not significantly lower than the currents recorded upon an application of each compound alone, at the same concentration (compare Figures 2 and 6). In accordance to this observation, the calculated relative peak current upon a co-application of genistein and resveratrol at the concentration of 30 μM was equal to 0.66±0.11 (n=21). This value was not significantly different ($p>0.05$) from the values obtained upon an application of each compound alone, at the same concentration (Figure 6c). On the other hand, this value was significantly ($p<0.05$) higher than the theoretical value calculated for an additive inhibitory effect (Table 1). This indicates that the inhibitory effects of genistein and resveratrol were not additive (Table 1) and they were not significantly different from an effect exerted by each compound applied alone.

The next stage of our study was to investigate whether a co-application of genistein and resveratrol with simvastatin and mevastatin led to an additive or synergistic inhibitory effect on the channel, such as it occurred, in most cases, upon a co-application of the flavonoids and chalcones with the statins [15,17].

Figure 7b depicts an example of whole-cell current recorded under control conditions, upon a co-application of genistein at 30 μM concentration and simvastatin at 6 μM concentration and after wash-out of the mixture. Figure 7c depicts the same current recorded under control conditions, upon a co-application of resveratrol at 30 μM concentration and simvastatin at 6 μM concentration and after wash-out of the mixture.



(a)

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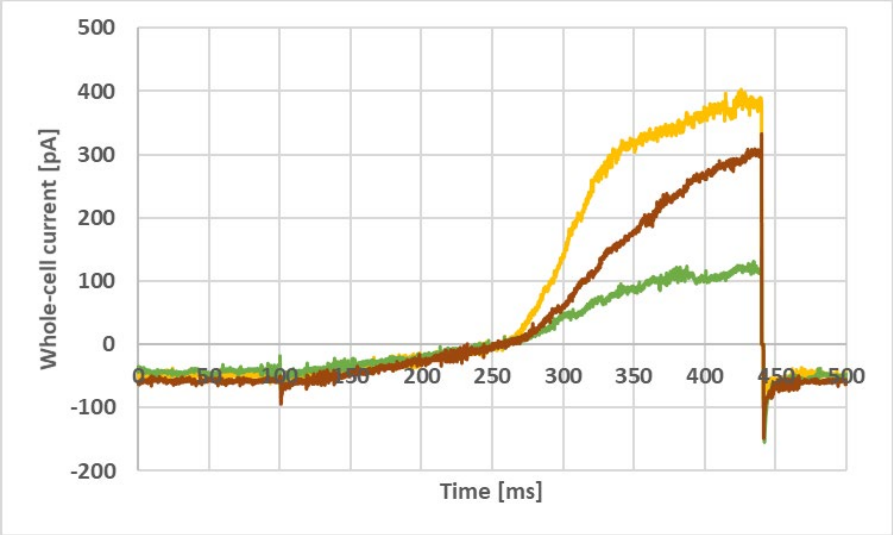

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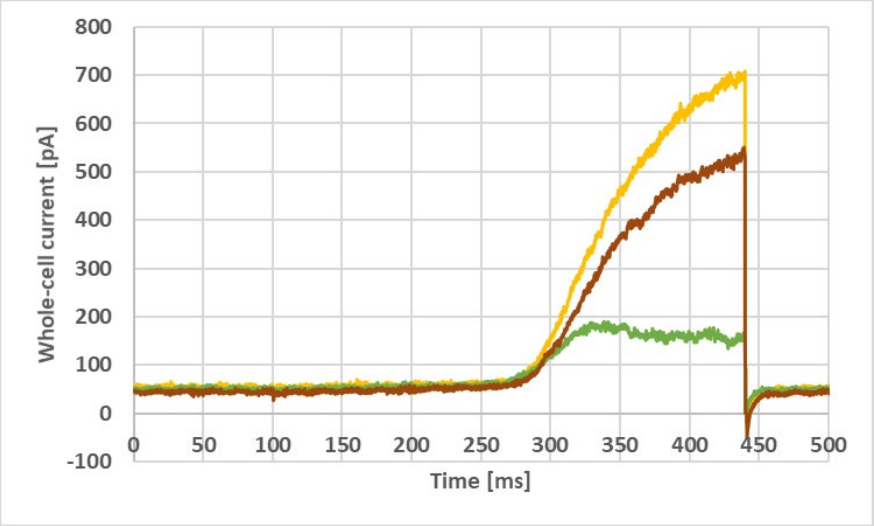

(c)

Figure 7. Whole-cell currents recorded in Jurkat T cells (**b** and **c**) applying the "voltage ramp" (**a**), under control conditions (yellow traces), upon co-application of 30 μM genistein with 6 μM of simvastatin (**b** – green trace), 30 μM of resveratrol with 6 μM of simvastatin (**c** -green trace) and after wash-out of the mixture (red traces).

Apparently, the whole-cell currents recorded upon a co-application of genistein and resveratrol with simvastatin were significantly lower than the currents recorded upon an application of both genistein and resveratrol alone (compare Figures 2 and 7). Moreover, the currents did not recover completely upon a wash-out of the mixture. This is probably due to the fact that an inhibitory effect of simvastatin on Kv1.3 channels was partially irreversible [27].

Figure 8 depicts relative peak currents calculated upon a co-application of 30 μM of genistein (a) and the same concentration of resveratrol (b) with 6 μM of simvastatin. For comparison, relative peak currents upon an application of each compound alone at the same concentrations are also presented. Upon a co-application of genistein with the statin, the relative peak current was equal to 0.16±0.07 (n=32) of the control value. This value was significantly ($p<0.05$) lower than the values upon an

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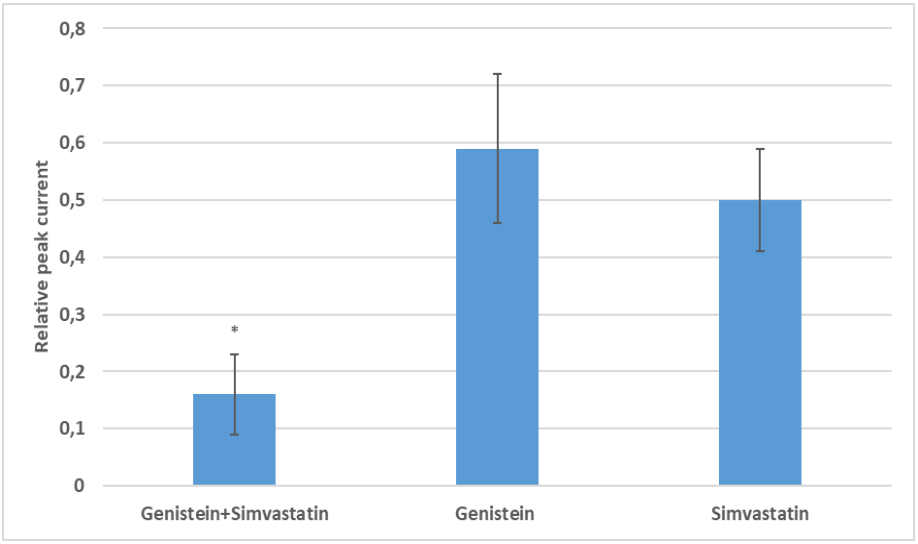
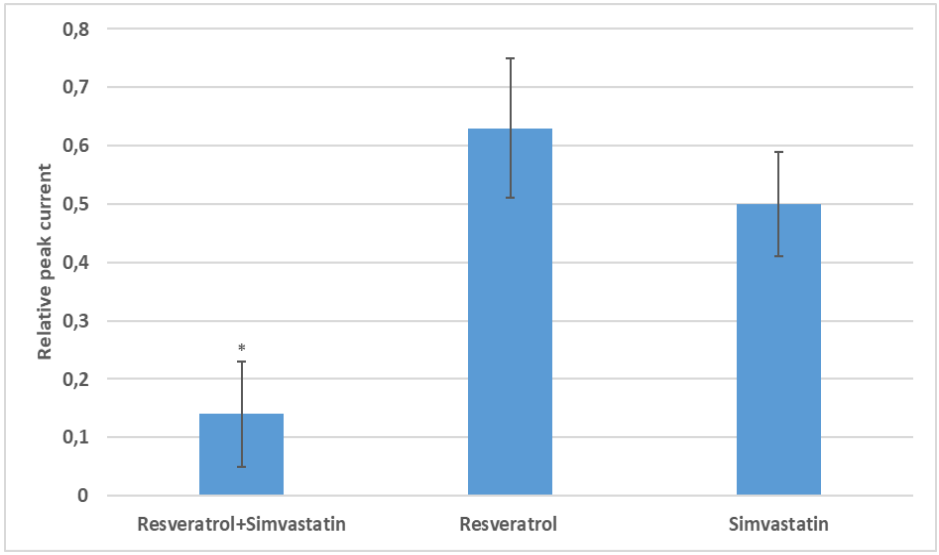
application of genistein alone (this study) and simvastatin alone (0.50±0.10, n=16) at the same concentration [15]. The relative peak current upon the co-application (0.16) was significantly ($p<0.05$) lower than the product of multiplication of the currents recorded upon the application of each compound alone (0.295) (Table 1). This indicates that the inhibitory effects of genistein and simvastatin was synergistic (Table 1).

Upon a co-application of resveratrol and simvastatin, the relative peak current was equal to 0.14±0.09 (n=17) of the control value. This value was significantly ($p<0.05$) lower than the values upon an application of resveratrol alone (this study) and simvastatin alone at the same concentration [15]. The relative peak current upon the co-application (0.14) was significantly ($p<0.05$) lower than the product of multiplication of the currents recorded upon the application of each compound alone (0.315) (Table1). This indicates that the inhibitory effects of resveratrol and simvastatin was synergistic (Table 1).



(a)



(b)

Figure 8. Relative peak current upon a co-application of genistein at 30 μM concentration with simvastatin at 6 μM concentration, genistein at the same concentration applied alone and simvastatin at the same concentration applied alone (**a**).. Relative peak current upon a co-application of resveratrol at 30 μM concentration with

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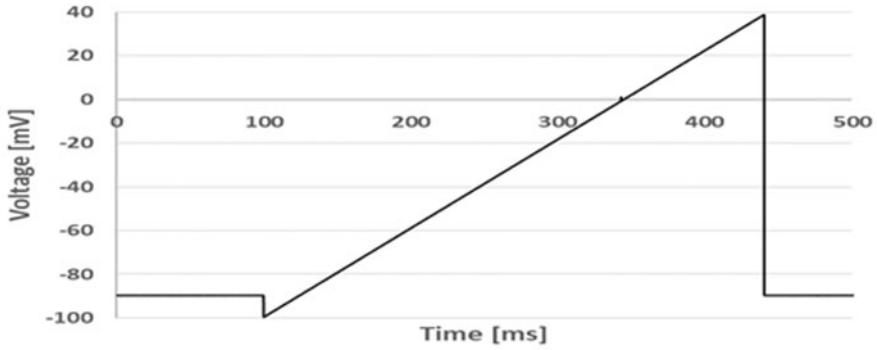
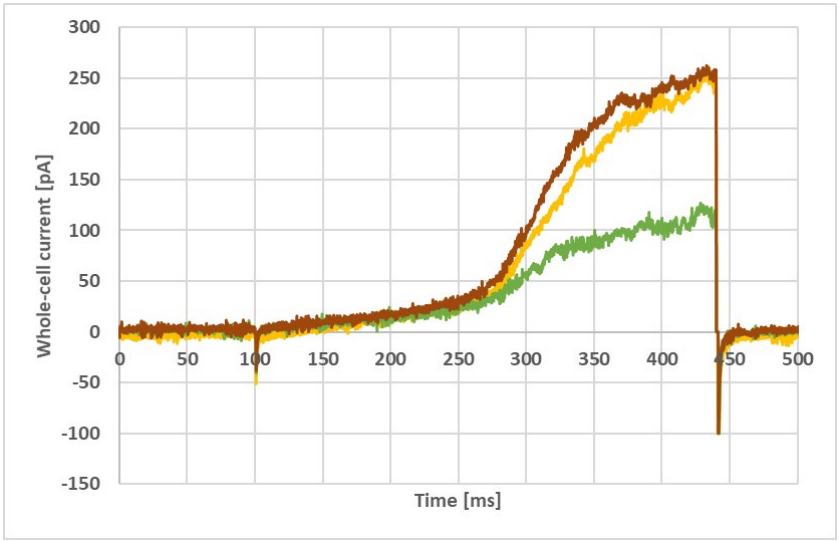
simvastatin at 6 μM concentration, resveratrol at the same concentration applied alone and simvastatin at the same concentration applied alone (**b**). Error bars are shown. * - statistical significance.

Figure 9b depicts an example of whole-cell current recorded under control conditions, upon a co-application of genistein at 30 μM concentration and mevastatin at 6 μM concentration and after wash-out of the mixture. Figure 9c depicts the same current recorded under control conditions, upon a co-application of resveratrol at 30 μM concentration and mevastatin at 6 μM concentration and after wash-out of the mixture.



(a)



(b)

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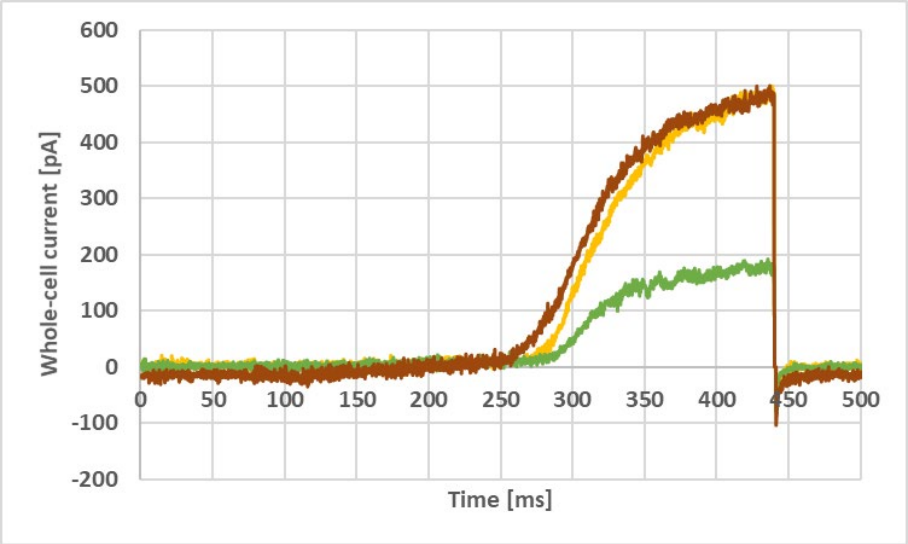

(c)

Figure 9. Whole-cell currents recorded in Jurkat T cells (**b** and **c**) applying the "voltage ramp" (**a**), under control conditions (yellow traces), upon co-application of 30 μM genistein with 6 μM of mevastatin (**b** – green trace), 30 μM of resveratrol with 6 μM of mevastatin (**c** -green trace) and after wash-out of the mixture (red traces).

Apparently, the whole-cell currents recorded upon a co-application of genistein and resveratrol with mevastatin were significantly lower than the currents recorded upon an application of both genistein and resveratrol alone (compare Figures 2 and 9). The currents recovered completely upon a wash-out of the mixture. This is in agreement with the observation that an inhibitory effect of mevastatin on Kv1.3 channels was reversible [27].

Figure 10 depicts relative peak currents calculated upon a co-application of 30 μM of genistein (a) and the same concentration of resveratrol (b) with 6 μM of mevastatin. For comparison, relative peak currents upon an application of each compound alone at the same concentrations are also presented. Upon a co-application of genistein with the statin, the relative peak current was equal to 0.26±0.07 (n=37) of the control value. This value was significantly ($p<0.05$) lower than the values upon an application of genistein alone (this study) and mevastatin alone (0.41±0.10, n=21) at the same concentration [15]. The relative peak current upon the co-application (0.26) was not significantly ($p>0.05$) higher than the product of multiplication of the currents recorded upon the application of each compound alone (0.24) (Table 1). This indicates that the inhibitory effects of genistein and mevastatin were additive (Table 1). Upon a co-application of resveratrol and mevastatin, the relative peak current was equal to 0.31±0.12 (n=31) of the control value. This value was significantly ($p<0.05$) lower than the values upon an application of resveratrol alone (this study) and mevastatin alone at the same concentration [15]. The relative peak current upon the co-application (0.31) was not significantly ($p>0.05$) higher than the product of multiplication of the currents recorded upon the application of each compound alone (0.26) (Table 1). This indicates that the inhibitory effects of resveratrol and mevastatin were additive (Table 1).

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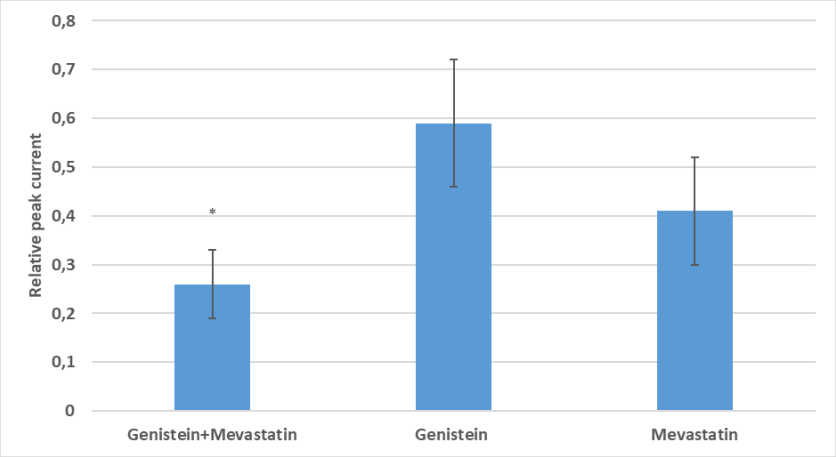
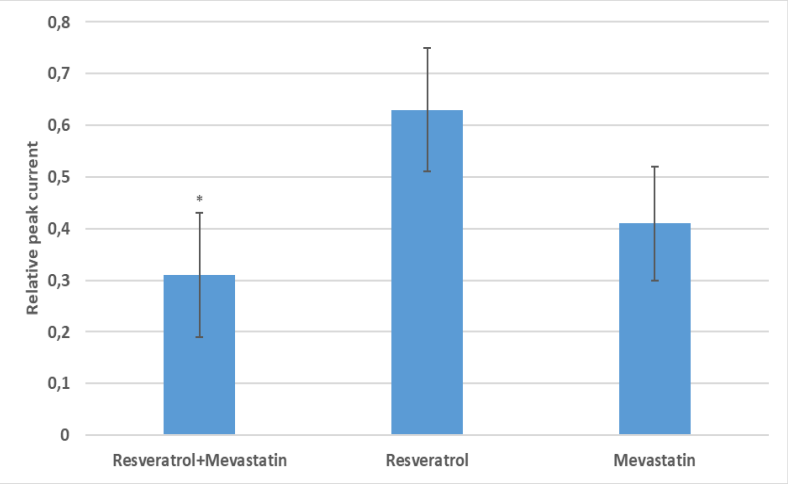


(a)



(b)

Figure 10. Relative peak current upon a co-application of genistein at 30 μM concentration with mevastatin at 6 μM concentration, genistein at the same concentration applied alone and mevastatin at the same concentration applied alone (**a**). Relative peak current upon a co-application of resveratrol at 30 μM concentration with mevastatin at 6 μM concentration, resveratrol at the same concentration applied alone and mevastatin at the same concentration applied alone (**b**).. Error bars are shown. * - statistical significance.

Table 1. Comparisons of theoretical and experimental relative peak currents upon a co-application of genistein with resveratrol and co-application of each compound with simvastatin and mevastatin. * -experimental values significantly higher, **-experimental values significantly lower, than theoretical ones, no asterisk next to experimental values – no significant difference from theoretical ones.

Combination of the compounds/ concentration	Relative peak currents- theoretical values	Relative peak currents- experimental values	Channel inhibition upon a co-application
Genistein [30 μM] + Resveratrol [30 μM]	0.37	0.66*	Not additive
Genistein [30 μM] + Simvastatin [6 μM]	0.295	0.16**	Synergistic
Resveratrol [30 μM] + Simvastatin [6 μM]	0.315	0.14**	Synergistic
Genistein [30 μM]+	0.24	0.26	Additive

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Mevastatin [6 μM]			
Resveratrol [30 μM] + Mevastatin [6 μM]	0.26	0.31	Additive

4. Discussion

The results of this study indicate that both genistein – a known isoflavone - and resveratrol – a polyphenolic compound - inhibit Kv1.3 channel activity in Jurkat T cells dose-dependently but not completely even at concentrations like 90 μM where relative peak currents drop to a half of the control value. The channel inhibition was accompanied by a significant slowing of the current activation rate without significantly affecting the inactivation rate. The inhibitory effects of genistein and resveratrol were not additive. On the other hand, the inhibitory effects were additive when genistein and resveratrol were co-applied with mevastatin and synergistic upon the co-application with simvastatin.

Our previous studies have shown that both genistein and resveratrol dose-dependently inhibited the activity of Kv1.3 channels in normal T lymphocytes [19,20]. The half-blocking concentration was about 40 μM for both compounds [19,20]. The channel activity was inhibited to about 0.23 of the control value in case of application of genistein at the concentration of 80 μM and to about 0.25 upon an application of resveratrol at the concentration of 100 μM [19,20]. These values were considerably lower than calculated in this study for Kv1.3 channels in cancer cells.

This indicates than channel inhibition in normal cells was stronger than observed in this study for cancer cells. Moreover, in contrast to what was observed in case of Jurkat T cells, the inhibitory effects of genistein and resveratrol on Kv1.3 channels in normal human T lymphocytes were additive [20].

On the other hand, similarly to what was observed in case of Kv1.3 channels in cancer Jurkat T cells, the inhibition of the channels in normal cells was accompanied by a significant slowing of the activation rate without changing of the inactivation rate [19,20]. This may indicate that the putative mechanism of the channel inhibition remains the same in normal and cancer cells. It could be proposed that an application of genistein and resveratrol stabilized the channel proteins in the closed state leading to a delay of their opening. Once the channel is open, the inhibitor's molecules probably dissociated from the channel's protein and re-bond to the channel after its closure. Nevertheless, the extent of the channel inhibition is different. This may be due to the fact that the channels are expressed in different model systems. Different sensitivity of Kv1.3 channels expressed in different model systems for inhibition by the same compounds has widely been observed ([28], Gąsiorowska – unpublished observations).

According to our previous observations upon a co-application of flavonoids with the statins: simvastatin and simvastatin, additive or synergistic inhibitory effect were exerted on the channels [15,17]. More effective was the co-application with mevastatin [15,17]. In contrast to those observations, results of this study have shown that the channel inhibition was more pronounced upon the co-application with simvastatin. It was shown that the relative peak currents upon the co-application of genistein and resveratrol with simvastatin were significantly lower than theoretical values calculated assuming that the inhibitory effects were additive. This indicates that these effects were synergistic. On the other hand, additive inhibitory effects were observed upon the co-application of genistein and resveratrol with mevastatin.

Additivity or synergism are important from the point of view of a putative application of genistein and resveratrol to support cancer therapy. The co-application with the statins improves a modest inhibitory effect of genistein and resveratrol applied alone. This may enable reduction of a putative required therapeutic dose and minimize risk of unwanted side effects [15,17].

Interestingly, the inhibitory effect upon a co-application of genistein and resveratrol with simvastatin is partially irreversible. Our previous study has shown that an application of simvastatin at the concentration of 15 μM and 30 μM exerted a partially irreversible inhibitory effect on Kv1.3 channels in Jurkat T cells [27]. This may be due to irreversible perturbations in structure of membrane

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lipid bilayer. It was shown that an application of simvastatin at 10 μM concentration caused an irreversible decrease of the membrane capacitance [29]. This was probably a consequence of an irreversible increase of membrane thickness, probably due to accumulation of the drug in the plasma membrane [29]. Accumulated drug molecules may directly or indirectly interact with channel protein irreversibly reducing the potassium current [29]. Results of this study have shown that a partial irreversibility of the inhibitory effect occurred upon a co-application of simvastatin at 6 μM concentration with genistein or resveratrol. On the other hand, our previous study has shown that an application of simvastatin alone at the concentrations of 6 μM and 7.5 μM caused a significant, but reversible inhibitory effect on the channels [27]. A partial irreversibility observed in this study upon the co-application could be a consequence of synergistic interactions of simvastatin combined with genistein or resveratrol with membrane lipid bilayers, leading to irreversible perturbations in its structure.

The inhibition of Kv1.3 channels may be related to anti-cancer activities of genistein and resveratrol [1,2]. It is known that lipophilic inhibitors of Kv1.3 channels may simultaneously inhibit the proliferation of Kv1.3 channel-expressing cancer cells and induce of apoptosis of these cells via an activation of the mitochondrial pathway of this process [5–12]. The inhibition of proliferation is related to an inhibition of Kv1.3 channels in the plasma membrane, whereas an induction of the mitochondrial pathway of apoptosis is a result of an inhibition of mitoKv1.3 channels [5–12]. Importantly, the apoptosis is selectively induced in cancer cells, whereas normal cells remain alive [6–18]. It is known that genistein inhibits proliferation in Kv1.3 channel-expressing breast cancer cell lines MCF-7 and MDA-MB- 231 [30], colon cancer cell line HT-29 [31] and induces apoptosis in colon cancer cell line HT-29 [31]. Genistein is considered as a putatively potent anti-breast cancer agent [32,33]. Resveratrol inhibits proliferation and induces apoptosis of Kv1.3 channel-expressing colon cancer cell line Caco-2 [34]. It also induces apoptosis of Kv1.3 channel-expressing U937 and MOLT-4 leukaemia cells, MCF-7 breast cancer cells and A549 lung cancer cells [35]. It may be possible that these anti-proliferative and pro-apoptotic effects of genistein and resveratrol are, at least partially due to an inhibition of mito Kv1.3 channels. More studies are necessary to further elucidate this problem.

Altogether, results of this study may indicate that both genistein and resveratrol may putatively be applied to support chemotherapy of cancer disorders, with an over-expression of Kv1.3 channels [6–18], especially when co-applied with the statins: simvastatin and mevastatin. For this purpose, more studies are necessary to provide evidence that an application of genistein and resveratrol with and without statins leads to a selective elimination of cancer cells without killing normal ones, such as it occurs upon an application of “mitochondriotropic” inhibitors of mito Kv1.3 channels [7–18].

5. Conclusions

Results of this study have shown that both genistein and resveratrol are inhibitors of Kv1.3 channels in cancer cells. The mechanism of the channel inhibition is probably the same as in the case of the channels expressed in normal cells. Inhibition of the channels in cancer cells by genistein and resveratrol is weaker than in normal cells. On the other hand, the channel inhibition is significantly ameliorated upon a co-application of the compounds with the statins: simvastatin and mevastatin. The channel inhibition may putatively be involved in anti-cancer activity of the compounds, however, more research studies are necessary to elucidate this question.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Table 1, Supplementary Table 2, Supplementary Table 3, Supplementary Table 4, Supplementary Table 5, Supplementary Table 6.

Author Contributions: Conceptualization, A.T., AP.-Ł. and K.Ś.-P..; methodology, A.T and A.U..; software, A.T.; validation, A.T.; formal analysis, A.T.; investigation, A.T; resources, AP.-Ł., K.Ś.-P and A.U.; data curation, , A.T., AP.-Ł. and K.Ś.-P; writing—original draft preparation, A.T..; writing—review and editing, , A.T., AP.-Ł. and K.Ś.-P.; visualization, , A.T., AP.-Ł. and K.Ś.-P; supervision, , A.T., AP.-Ł. and K.Ś.-P; project administration, ,

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Conflicts of Interest: The Authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Kv1.3 - voltage-gated potassium channel studied
I – Kv1.3 current upon an application of an examined compound at + 40 mV
I_{contr} – Kv1.3 current recorded on the same cell at +40 mV under control conditions
T_{peak} - time-to-peak
RKS – relative kinetics slowing
$T_{peakcompound}$ – mean time-to-peak value upon an application of the compound
$T_{peakcontrol}$ – mean time-to-peak value under control conditions
τ – inactivation time constant

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