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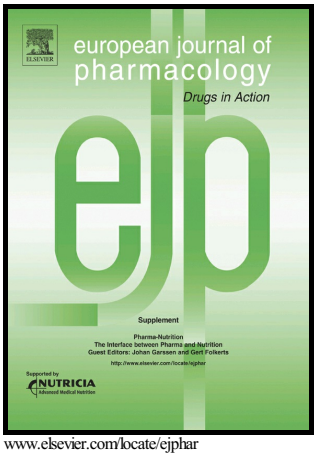

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"Effect of polygodial and its direct derivatives on the mammalian Na^+/K^+-ATPase activity"

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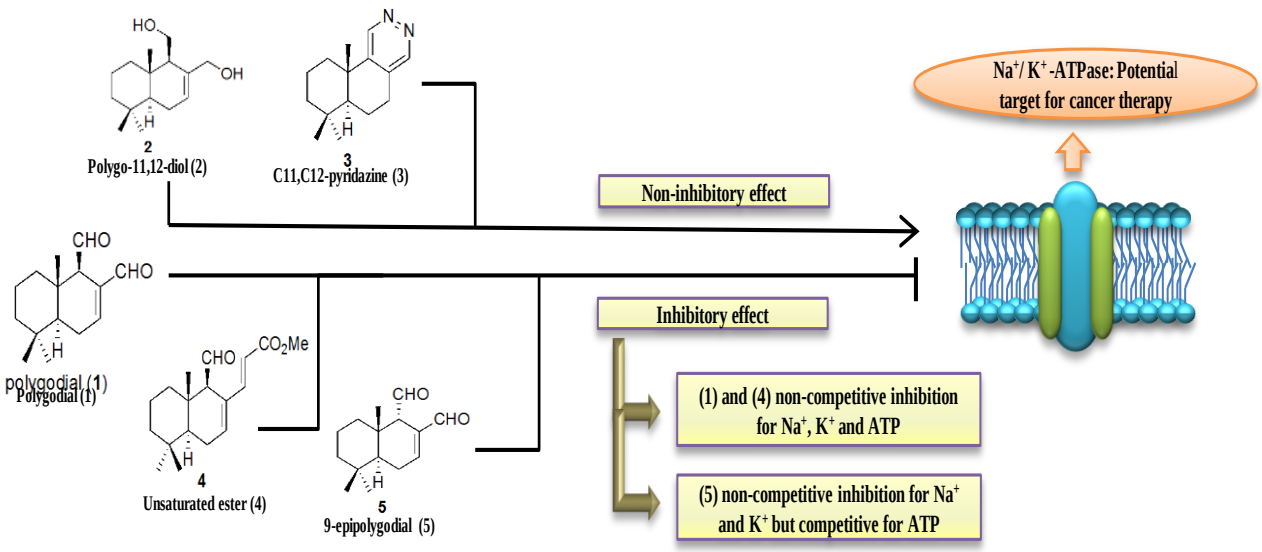
ABSTRACT

The sesquiterpene polygodial is an agonist of the transient receptor potential vanilloid 1 (TRPV1). Our group recently reported the synthesis and anticancer effects of polygodial and its derivatives, and showed that these compounds retain activity against apoptosis- and multidrug-resistant cancer cells. Herein, we tested the inhibitory effect of these compounds on the activity of the enzyme Na^+/K^+-ATPase (NKA) from kidney (α_1 isoform) and brain (α_2 and α_3 isoforms) guinea pig extracts. Polygodial (**1**) displayed a dose-dependent

inhibition of both kidney and brain purified NKA preparations, with higher sensitivity for the cerebral isoforms. Polygo-11,12-diol (**2**) and C11,C12-pyridazine derivative (**3**) proved to be poor inhibitors. Unsaturated ester (**4**) and 9-epipolygodial (**5**) inhibited NKA preparations from brain and kidney, with the same inhibitory potency. Nevertheless, they did not achieve maximum inhibition even at higher concentration. Comparing the inhibitory potency in crude homogenates and purified preparations of NKA, compounds **4** and **5** revealed a degree of selectivity toward the renal enzyme. Kinetic studies showed a non-competitive inhibition for Na^+ and K^+ by compounds **1**, **4** and **5** and for ATP by **1** and **4**. However, compound **5** presented a competitive inhibition type. Furthermore, K^+-activated *p*-nitrophenylphosphatase activity of these purified preparations was not inhibited by **1**, **4** and **5**, suggesting that these compounds acted in the initial phase of the enzyme's catalytic cycle. These findings suggest that the antitumor action of polygodial and its analogues may be linked to their NKA inhibitory properties and reinforce that NKA may be an important target for cancer therapy.

Graphical abstract



KEYWORDS: Na^+/K^+-ATPase; Polygodial; Sesquiterpene; Anticancer agents

1. INTRODUCTION

The Na^+/K^+-ATPase (NKA) is a plasma membrane protein complex transferring the stored energy of ATP to the active transport of Na^+ and K^+ ions across the cell membrane. The ionic transport performed by this enzyme creates an electrochemical gradient through the cell plasma membrane, which is essential for the maintenance of muscle and nerve cell excitability (Skou, 1965, 1957). The NKA also participates in cell signaling events, which are mediated via the NKA caveolar pool, affecting cell–cell interactions, as well as cell proliferation, differentiation, and death (Gonçalves-de-Albuquerque et al., 2017; Jaitovich and Bertorello, 2006; Liu et al., 2003; Skou, 2004).

Alterations in the expression and activity of the NKA are implicated in the pathophysiology of several diseases such as leptospirosis (Burth et al., 1997; Gonçalves-de-Albuquerque et al., 2014), cardio-metabolic diseases (Liu and Songu-Mize, 1997; Obradovic et al., 2017; Tsimaratos et al., 2001), Alzheimer's disease (Dickey et al., 2005; Ohnishi et al., 2015), and cancer (Chen et al., 2006; Lefranc et al., 2008; Yu, 2003). Moreover, suppression or over-expression of some NKA isoforms were detected at early stages of tumorigenesis (Mijatovic et al., 2008; Sakai et al., 2004). The involvement of the NKA in the pathogenesis of many other diseases and its signal transduction properties make this enzyme an attractive target for drug development (Aperia, 2007; Gonçalves-de-Albuquerque et al., 2017).

Cardiac glycosides, which are classical NKA selective inhibitors, have been widely used in patients with chronic cardiovascular diseases. However, due to their high toxicity, the search for inotropic drugs having a better therapeutic index is of great importance (Pôças et al., 2003). Incidentally, the first indication of their anticancer properties emerged from the fact that a low breast cancer mortality rate was detected in cardiac women treated with such glycosides due to their cardiovascular problems (Stenkvist, 1999). This fact generated new interest in cardiac glycosides as anticancer drugs (Kometiani et al., 2005; Lefranc and Kiss, 2008; Mijatovic et al., 2007b; Winnicka et al., 2010). These observations have been confirmed in several *in vitro* and *in vivo* studies, and substances based on the structures of cardiac glycosides have already been tested in clinical trials for cancer treatment (Gonçalves-de-Albuquerque et al., 2017; Mijatovic et al., 2007b, 2007a; Prassas and Diamandis, 2008).

On the other hand, the importance of NKA in cancer therapy has also been suggested using compounds unrelated to the cardiac glycoside structure (Garcia et al., 2015; Lefranc et al., 2013). In this sense, the current investigation describes the inhibitory effect on the NKA activity by polygodial and its synthetic derivatives (Fig. 1). Polygodial is a terpenenoid dialdehyde isolated from *Persicaria hydropiper* (L.) (Polygonaceae), a plant once used as a pepper substitute in Europe and still a popular condiment for sashimi in Japan (Ohsuka, 1963). It is a known agonist of the transient receptor potential vanilloid 1 (TRPV1) showing cytotoxic effects on cancer cells. We found, however, that several polygodial derivatives exert their antiproliferative action mainly through cytostatic effects. The anticancer evaluation of these compounds revealed their promising activity against human cancer cells displaying various forms of resistance, including resistance to proapoptotic agents and multidrug resistance (Dasari et al., 2015a, 2015b).

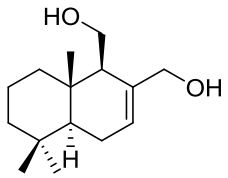
Present work investigated the inhibitory properties of polygodial and its derivatives on the NKA from guinea pig kidney and brain tissues.

2. MATERIALS AND METHODS

2.1 Compound Synthesis

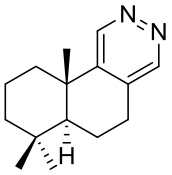
General: All reagents, solvents, and catalysts were purchased from commercial sources (Acros Organics and Sigma–Aldrich) and used without purification. All reactions were performed in oven-dried flasks open to the atmosphere or under nitrogen or argon and monitored by thin-layer

chromatography (TLC) on TLC pre-coated (250 mm) silica gel 60 F254 glass-backed plates (EMD Chemicals Inc.). Visualization was accomplished with UV light, iodine, and *p*-anisaldehyde stains. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 & 500 spectrometer. Chemical shifts (d) are reported in ppm relative to the residual solvent signal internal standard. Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), HRMS spectra were recorded on a Waters Synapt G2 UPLC Mass Spectrometer. Polygodial (**1**) was purchased from VWR.

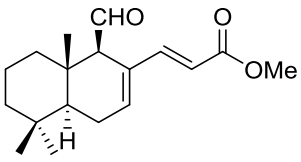


Compound 2: To a solution of **1** (3 mg, 0.0128 mmol) in MeOH (2 ml) was added sodium borohydride (1.0 mg, 0.027 mmol). The mixture was stirred at room temperature for 2 h. After completion of the reaction, as monitored by TLC, added water to the reaction mixture and evaporated MeOH. The reaction mixture was diluted with ethyl acetate and organic phase was washed with 1N HCl and water, dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by preparative TLC (10/90 EtOAc/Hexane) to obtain 2.4 mg of **2** (80% yield); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.83 – 5.78 (m, 1H), 4.38 – 4.32 (m, 1H), 3.99 (d, *J* = 12.1 Hz, 1H), 3.91 (dd, *J* = 10.9, 2.2 Hz, 1H), 3.69 (dd, *J* = 10.9, 8.2 Hz, 1H), 2.86 (brs, 1H), 2.14 (brs, 1H), 2.13 – 2.04 (m, 1H), 2.01 – 1.83 (m, 2H), 1.59 – 1.39 (m, 3H), 1.28 – 1.11 (m, 4H), 0.88 (s, 3H), 0.87 (s, 3H), 0.76 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.9, 127.6, 67.5, 61.5, 54.5, 49.4, 42.0, 39.3, 35.6, 33.2, 33.0, 23.6, 21.9,

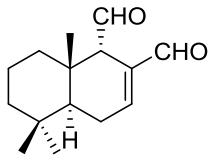
18.8, 14.5; HRMS (ESI) calcd for $C_{15}H_{26}NaO_2$ (M+Na) 261.1830, found 261.1829.



Compound 3: To a solution of **1** (3 mg, 0.0128 mmol) and hydrazine hydrate (0.7 µl, 0.014 mmol) in MeOH (1 ml) were added 4A molecular sieves. The mixture was stirred at room temperature for 20 h. After completion of the reaction, as monitored by TLC, the reaction mixture was filtered and filtrate was concentrated under reduced pressure. The crude product was purified by preparative TLC (4/96 MeOH/CHCl3) to obtain 2.8 mg of **2** (95% yield); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.02 (s, 1H), 8.84 (s, 1H), 2.99 – 2.90 (m, 1H), 2.82 (ddd, *J* = 18.7, 11.1, 7.7 Hz, 1H), 2.37 – 2.31 (m, 1H), 2.06 – 1.98 (m, 1H), 1.86 – 1.65 (m, 3H), 1.58 – 1.51 (m, 1H), 1.42 (td, *J* = 12.7, 3.9 Hz, 1H), 1.31 – 1.23 (m, 2H), 1.23 (d, *J* = 0.7 Hz, 3H), 0.98 (s, 3H), 0.95 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.2, 148.3, 145.8, 135.8, 49.5, 41.2, 36.95, 36.4, 33.4, 33.0, 26.9, 24.2, 21.5, 18.6, 17.7; HRMS (ESI) calcd for $C_{15}H_{23}N_2$ (M+H) 231.1861, found 231.1861.



Compound 4: To a solution of **1** (3 mg, 0.0128 mmol) in toluene (3 ml) was added Wittig reagent (12.8 mg, 0.0384 mmol). The resultant mixture was stirred at room temperature for 20 h. After completion of the reaction, as monitored by TLC, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by preparative TLC (9/91 EtOAc/Hexane) to obtain **4** (94% yield); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.47 (d, *J* = 4.8 Hz, 1H), 7.33 (d, *J* = 16.3 Hz, 1H), 6.53 – 6.49 (m, 1H), 5.50 (d, *J* = 16.3 Hz, 1H), 3.71 (s, 3H), 2.83 (s, 1H), 2.37 – 2.16 (m, 2H), 1.87 – 1.80 (m, 1H), 1.53 – 1.44 (m, 3H), 1.38 – 1.30 (m, 1H), 1.23 – 1.16 (m, 2H), 1.00 (s, 3H), 0.94 (s, 3H), 0.90 (s, 3H); ^{13}C NMR (100 MHz, C_6D_6): *δ* 203.8, 167.2, 146.9, 141.1, 130.7, 116.8, 62.9, 51.2, 48.3, 41.9, 40.2, 37.2, 33.2, 32.9, 24.7, 22.2, 18.3, 15.3; HRMS (ESI) calcd for $C_{18}H_{26}NaO_3$ (M+Na) 313.1780, found 313.1779.



Compound 5: To a solution of **1** (3 mg, 0.0128 mmol) in dry toluene (1.5 ml) were added 4A molecular sieves and PTSA (cat.). The mixture was stirred at room temperature for 40 h. Solid $NaHCO_3$ was added to the reaction mixture and stirred for 10 minutes, filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by preparative TLC (8/92 EtOAc/Hexane) to obtain 1.2 mg of **5** (40% yield); ^{1}H NMR (400 MHz, C_6D_6) δ 9.69 (d, *J* = 2.4 Hz, 1H), 9.19 (s, 1H), 6.20 (dd, *J* = 4.9, 2.4 Hz, 1H), 1.96 – 1.88 (m, 1H), 1.62 – 1.51 (m, 1H), 1.45 – 1.39 (m, 2H), 1.34 – 1.25 (m, 2H), 1.20 – 1.12 (m, 2H), 0.96 – 0.87 (m, 2H), 0.65 (s, 3H), 0.63 (s, 3H), 0.57 (d, *J* = 0.7 Hz,

3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.2, 192.8, 153.4, 137.4, 58.5, 44.2, 42.0, 37.7, 37.1, 32.9, 32.7, 25.5, 21.9, 21.5, 18.4; HRMS (ESI) calcd for $C_{15}H_{22}NaO_2$ (M+Na) 257.1517, found 257.1519.

2.2 NKA preparation

Guinea pig brain and kidney were used in the preparation of homogenates and NKA-enriched fractions according to Jørgensen's procedure (Jørgensen, 1977). Animals were kept in compliance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services, NIH) and according to the in-house regulations of our institution.

The final enzyme suspensions (adjusted to 0.35 mg of protein/ml) were prepared in a solution containing 30 mM Tris-HCl pH 7.5, 1 mM EDTA and 1% sucrose. Aliquots of these suspensions were poured into dark flasks, lyophilized and stored at −20 °C. The lyophilized preparation could be stored for more than 6 months without losing its activity. Freeze-dried aliquots suspended in water and kept at 0 °C could be used within 24 h after the suspension. Enzyme specific activities were 112 and 120 μmol of Pi formed/h/mg of protein for kidney and brain, respectively. Ouabain insensitive ATPase activity was not detected in these purified preparations. NKA specific activities in whole kidney and brain homogenates were 1.4 and 1.8 μmol of Pi formed/h/mg of protein, respectively.

2.3 NKA assays

Enzyme activity was determined in microtiter plates. The final incubation volume contained 110 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.6, compounds (absent in controls) dissolved in 100% dimethyl sulfoxide (DMSO), enzyme preparation and 5 mM ATP. Assays were performed as detailed previously (Burth et al., 1997). In all experiments (including controls and blanks), the final DMSO concentration in the incubation mixture was kept below 1% (v/v), a condition that did not affect the enzyme activity. The amount of the enzyme preparation used in all experiments (including tissue homogenates) corresponded to an activity of 0.12–0.13 µmol Pi formed/h in control assays (100% enzyme activity). Preparations were preincubated for 10 min before starting the enzyme reaction with ATP and this reaction was stopped 40 min later. In the blanks, the enzyme was added only after stopping the reaction. NKA activity in whole tissue homogenates was calculated from the difference between the total ATPase activity (in the absence of ouabain) and Mg^{++}-ATPase activity (in the presence of 3.6 mM ouabain). For kinetic studies varying Na^+, K^+ or ATP concentrations, the concentration of the compounds were fixed about 50% the enzyme activity.

2.4 K^+ activated-*p*-nitrophenylphosphatase (K^+-pnppase) Assay

K^+-pnppase assay was performed according to Rodriguez de Lores Arnaiz *et al*. (2003). For basal measurements, KCl was omitted and substituted by 1 mM ouabain. K^+-pnppase activity was the difference between total and basal activities.

2.5 Statistics

Prism 5.0 software (GraphPad Inc., CA, USA) was used for graphical presentation and statistical analysis. The statistical analyses included Student's *t*-test and Two-Way ANOVA followed by Bonferroni test. The data are expressed as the means ± standard deviation (S.D.) of at least three independent experiments. Significance was determined at $P < 0.05$.

3. RESULTS

3.1 NKA inhibition

3.1.1 Purified preparations

We evaluated the synthesized compounds' inhibitory effect on the activity of purified NKA from guinea pig kidney and brain. Fig. 2-A shows a polygodial (**1**) dose-dependent inhibition of both purified NKA preparations. The IC_{50} values were 9.9 μM (± 0.1) for the brain enzyme and 53 μM (± 5.1) for the kidney enzyme, indicating a higher sensitivity for the NKA cerebral isoforms ($P < 0.001$, Two-Way ANOVA Bonferroni post-test). Compounds **2** and **3** presented a weak effect (Fig. 2-B and 2-C), reaching 50% inhibition of both enzymes only at high concentrations: the IC_{50} for **2** was above 100 μM, while **3** did not achieve 100% enzymatic inhibition even at 1000 μM. Compounds **4** and **5** inhibited the activity of purified kidney and brain NKA preparations (Fig. 2-D

and 2-E) with similar potencies (IC_{50} ≈ 50 μM). Neither of these two compounds achieved a NKA total inhibition at the concentrations tested.

3.1.2 Crude preparations

Inhibitory potencies in crude homogenates compared to purified preparations of NKA are shown in Fig. 3. This approach can serve as an indication of selectivity. In the crude homogenates, a nonspecific binding to different targets may occur and it would decrease the inhibitory effects toward the NKA. Our results suggest good selectivity level for compounds **4** and **5**, because the inhibition in the kidney homogenate was similar to the purified enzyme (Fig. 3-B and 3-C). Compound **1** did not display the same pattern (Fig. 3-A). The results were different however when the brain tissue was used. The compounds did not show selectivity for cerebral NKA isoforms (Fig. 3-D to 3-F).

3.2 Kinetic studies

Kinetic studies evaluating the influence of the compounds at fixed concentrations on the activation of purified kidney NKA preparations by Na^+, K^+ and ATP can be observed from double reciprocal plots shown in Figs. 4, 5 and 6. A non-competitive inhibition for Na^+ (Fig. 4-A to 4-C) and K^+ (Fig. 5-A to 5-C) was found for compounds **1**, **4** and **5**. Altering the ATP concentration, compounds **1** and **4** also displayed a non-competitive inhibition (Fig. 6-A and 6-B) but **5** exhibited a competitive inhibition (Fig. 6-C).

3.3 Influence on K^+-activated *p*-nitrophenylphosphatase activity

Considering the two phases (I and II) of the NKA catalytic cycle, the K^+-activated *p*-nitrophenylphosphatase (K^+-pnppase) measures phase II activity (K+-activated enzyme dephosphorylation). As shown in Fig. 7, compound **1**, **4** and **5** did not affect this phosphatase in a purified kidney NKA preparation.

4. DISCUSSION

Polygodial is an agonist of the transient receptor potential vanilloid 1 (TRPV1) (Andre et al., 2004; André et al., 2006; D'Acunto et al., 2010; Della Monica et al., 2007; Iwasaki et al., 2009; Mendes et al., 1998; Sterner and Szallasi, 1999), a temperature-sensitive ion channel with the preference for Ca^{2+} ions (Caterina et al., 1997; Caterina and Julius, 2001). In addition to its expression in sensory neurons, TRPV1 is upregulated in several human cancer cells (Gkika and Prevarskaya, 2009; Hartel et al., 2006; Premkumar and Bishnoi, 2011). As an example, in human glioma cells the activation of TRPV1 leads to cell death by endoplasmic reticulum stress (Stock et al., 2012). In our previous investigations, polygodial and a series its analogues were investigated for TRPV1-agonistic and anticancer activities, showing cytotoxic and cytostatic effects depending on a specific derivative (Dasari et al., 2015a, 2015b). Furthermore, our previous results suggested that in contrast to polygodial (**1**), the antiproliferative activity of compounds **4** and **5** were non-TRPV1-mediated.

Inhibition of various types of ion channels is a strategy used to combat several types of cancers, including those associated with dismal prognoses. We

have been studying the NKA, especially its α_1 subunit, which is overexpressed in gliomas, melanomas, and non-small cell lung cancer (Garcia et al., 2015; Lefranc et al., 2008, 2013; Mathieu et al., 2009; Mijatovic et al., 2007a). When the activity of the NKA α_1 subunit is impaired, cancer cells, including those with multidrug resistance phenotype, undergo cell death through apoptosis or autophagy (Gonçalves-de-Albuquerque et al., 2017; Lefranc et al., 2007; Lefranc and Kiss, 2008; Mijatovic et al., 2009, 2012). These effects on cancer cells have been observed using either natural or synthetic NKA inhibitors with steroidal (e.g., cardiac glycosides, classical NKA inhibitors) or non-steroidal structure (Garcia et al., 2015; Lefranc et al., 2013, 2008).

Herein, the brain enzyme, rich in NKA α_2 and α_3 isoforms, showed a higher sensitivity to compound **1**. The inhibition of this enzyme was ca. 500% more potent than that of the kidney enzyme, expressing α_1 isoform. On the other hand, compounds **2** and **3** were found to be poor inhibitors of the NKA enzyme activity. Furthermore, compounds **4** and **5** acted with the same potency on the kidney and brain NKA, indicating that all isoforms are equally inhibited.

The inhibitory capacity of ouabain, a well-known NKA specific inhibitor, was ca. 3.5 times higher for brain than for kidney preparations from guinea-pig (Garcia et al., 2009), while this ratio is around 1000 for rat enzymes (Pôças et al., 2003). In contrast to the rat, the affinity of all human NKA α-subunit isoforms for ouabain is similar (Crambert et al., 2000; Wang et al., 2001). These results indicate large differences of isoform sensitivity to cardiac glycosides among animal species. Therefore, we cannot predict, at this time, the inhibition behavior of human isoforms to these compounds.

Aiming to determine the magnitude of selectivity of these compounds towards NKA, we performed another experiment using organ crude homogenates instead of purified preparations. Using kidney homogenate, polygodial (**1**) did not show selectivity. Compounds **4** and **5**, however, seem to selective for the NKA α_1 isoform. Using brain homogenates, no level of selectivity was found. The IC_{50} ratio between the kidney purified and crude enzyme preparations for compounds **4** and **5** was 1.5 and 1.0, respectively. Drawing a comparison with our previous work (Garcia et al., 2009), the selectivity was similar to ouabain (1.2) and significantly lower for a nonselective inhibitor, oleic acid (3.3) (Table 1). These data point to a high degree of selectivity of compound **4** and, mainly, compound **5** in binding NKA. These finding are consistent with the fact that polygodial (**1**) has other targets in the cell, such as TRPV1, while additional interactions for compounds **4** and **5** have not been revealed. Also consistent is the fact that compound **5** has antiproliferative potency significantly exceeding that of **1** and was found to maintain potency against apoptosis-resistant cancer cells as well as those displaying the multidrug-resistant (MDR) phenotype (Dasari et al., 2015b). Given the small number of compounds tested herein and the lack of information on the structural requirements for binding to NKA, it is premature to speculate on the structure-activity relationship in this series of compounds. This will be the subject of future studies.

The possibility thus remains that the antiproliferative effect of polygodial, which is not a cardiotonic steroid, and its derivatives could be mediated via the NKA caveolar pool at lower concentrations, because it is independent of the changes in intracellular Na^+ and K^+ concentrations. Importantly, cardiotonic

steroids display *in vitro* growth inhibitory effects usually in nM ranges and, yet, they have not been developed clinically to combat various types of cancer. The fact remains that various types of cancer display significantly higher amounts of "NKA receptors" (mainly the NKA alpha-1 subunit) when compared to their normal tissues (Lefranc et al., 2008; Mathieu et al., 2009; Mijatovic et al., 2008, 2007a). Thus, on the one hand, the concentration expected to inhibit the NKA activity in the type of assay used may be higher than the one required for inhibition of cell proliferation. On the other hand, it is known that the high potency of cardiotonic steroids in inhibition of the NKA activity is related to their ability to link / bridge / block the fifth loop of the alpha-1 subunit in the plasma membrane, as demonstrated with the alpha-1 NKA subunit in rodents, in which it is doubly mutated and is therefore 1000 times less sensitive to the toxic effects of cardiotonic steroids (Mijatovic et al., 2007b).

Kinetic studies show a non-competitive inhibition for Na^+ and K^+ by compounds **1**, **4** and **5**. Moreover, the same type of inhibition was found for **1** and **4** when ATP concentration was changed. Remarkably, a competitive inhibition was presented by compound **5**. In competitive inhibition, the substrate (ATP) and inhibitor compete for access to the active site of the enzyme. Thus, the maximum velocity of the reaction was unchanged, while the apparent affinity of the substrate to the binding site decreased. Although a direct interaction of **5** with a specific enzyme site may be involved, we cannot rule out the possibility that this compound binds to the lipid microenvironment involving the enzyme. Furthermore, compounds **1**, **4** and **5** have no effect on the second phase of the NKA catalytic cycle, the K^+-activated *p*-nitrophenylphosphatase (K^+-pnppase). Consistent with that, unlike cardiac glycosides, polygodial and its derivatives

acted on the first phase of the NKA cycle, which starts with ATP binding to the enzyme and is followed by Na^+-activated enzyme phosphorylation by ATP (Rodríguez de Lores Arnaiz et al., 2003).

In general, diverse mechanisms of cell death are triggered via NKA but the induction of apoptosis has been reported more frequently (Garcia et al., 2015; Kulikov et al., 2007; Yang et al., 2014; Yu et al., 2008). Nonetheless, the cytostatic effects in different cancer cell models can be exerted by impairing the NKA as well (Bloise et al., 2009; Schneider et al., 2017). Our manuscript describes a new way of blocking the alpha-1-related NKA activity, which could be successful in combating cancers with dismal prognoses, because all our data suggest that the polygodial derivatives under study do not display their antiproliferative activity through the sole inhibition of the alpha-1 NKA subunit, and definitively not through the fifth loop of the alpha-1 NKA subunit.

5. CONCLUSION

The present communication provides for the first time the evidence that polygodial and its derivatives are NKA inhibitors. Interestingly, the minor structural differences found among these compounds, especially between **1** and **5**, which are epimers, strikingly altered their inhibitory and selective properties on NKA. Further experiments are necessary to define the effects of these compounds on the activation of NKA-dependent signaling cascades leading to cell death. It is important to note that we are not in search of NKA inhibitors more potent than cardiotonic steroids, but of NKA inhibitors that could be clinically manageable. Therefore, the effects of these compounds on NKA may

encourage the development of polygodial analogues showing superior inhibitory properties in a search of new anticancer agents.

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CONFLICTS OF INTEREST: None.

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FIGURE LEGENDS

Fig. 1. Synthesis of compounds **2-5**.

Fig. 2: Inhibition of purified preparations of NKA from kidney (light gray) or brain (dark gray) by compounds 1 (A), 2 (B), 3 (C), 4 (D) and 5 (E). Each point is the mean ± S.D. of three different experiments conducted in triplicate.

Fig. 3: Inhibition of crude (homogenate) preparations of NKA from kidney (light gray) or brain (dark gray) by compounds 1 (A and D), 4 (B and E) and 5 (C and F). Inhibition data for purified preparations shown in Fig. 2 were also included here for comparative purposes (hatched). Each point is the mean ± S.D. of three different experiments conducted in triplicate.

Fig. 4: Influence of the compounds 1 (A), 4 (B) and 5 (C) on the NKA activation by Na+. Double reciprocal plots (inverse of velocity versus inverse of Na+ concentration) in absence of the compounds (filled circle) and in presence of the compounds (filled diamond). Each point is the mean ± S.D. of three different experiments conducted in triplicate.

Fig. 5: Influence of the compounds 1 (A), 4 (B) and 5 (C) on the NKA activation by K+. Double reciprocal plots (inverse of velocity versus inverse of K+ concentration) in absence of the compounds (filled circle) and in presence of the compounds (filled triangle). Each point is the mean ± S.D. of three different experiments conducted in triplicate.

Fig. 6: Influence of the compounds 1 (A), 4 (B) and 5 (C) on the NKA activation by ATP. Double reciprocal plots (inverse of velocity versus inverse of ATP concentration) in absence of the compounds (filled circle) and in presence of the compounds (filled square). Each point is the mean ± S.D. of three different experiments conducted in triplicate.

Fig. 7: Influence of compounds 1, 4 and 5 on K+-pnppase activity of the purified kidney NKA. Control K+-pnppase activity (100%) was 0.1 µmol p-nitrophenol formed per min. Each point is the mean ± S.D. of three different experiments conducted in triplicate.

TABLE

Table 1. Compound concentrations inhibiting 50% (IC_{50}) the activities of Na+/K+ ATPase from several sources

Compound	Enzyme source	IC_{50} (µM)	Ratio IC_{50} Homog/IC_{50} purif
Ouabain	Kidney Purified	1.6[a]	1.2
	Kidney homogenate	1.9[a]	
Unsaturated ester (4)	Kidney Purified	50[b]	1.5
	Kidney homogenate	73.5[b]	
9-epipolygodial (5)	Kidney Purified	50[b]	1.0
	Kidney homogenate	50.1[b]	
Oleic acid	Kidney Purified	115[a]	3.3
	Kidney homogenate	375_a	

[a] IC_{50} calculated from data published in previous wok (Garcia et al., 2009).
[b] IC_{50} calculated from data shown in graphs.

FIGURES

Fig.1

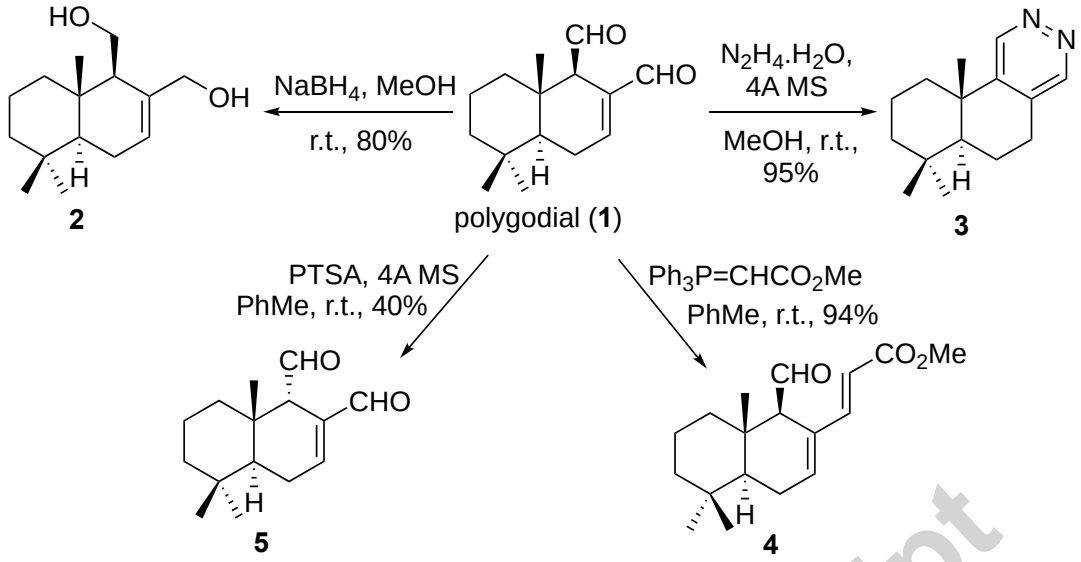

Fig. 2

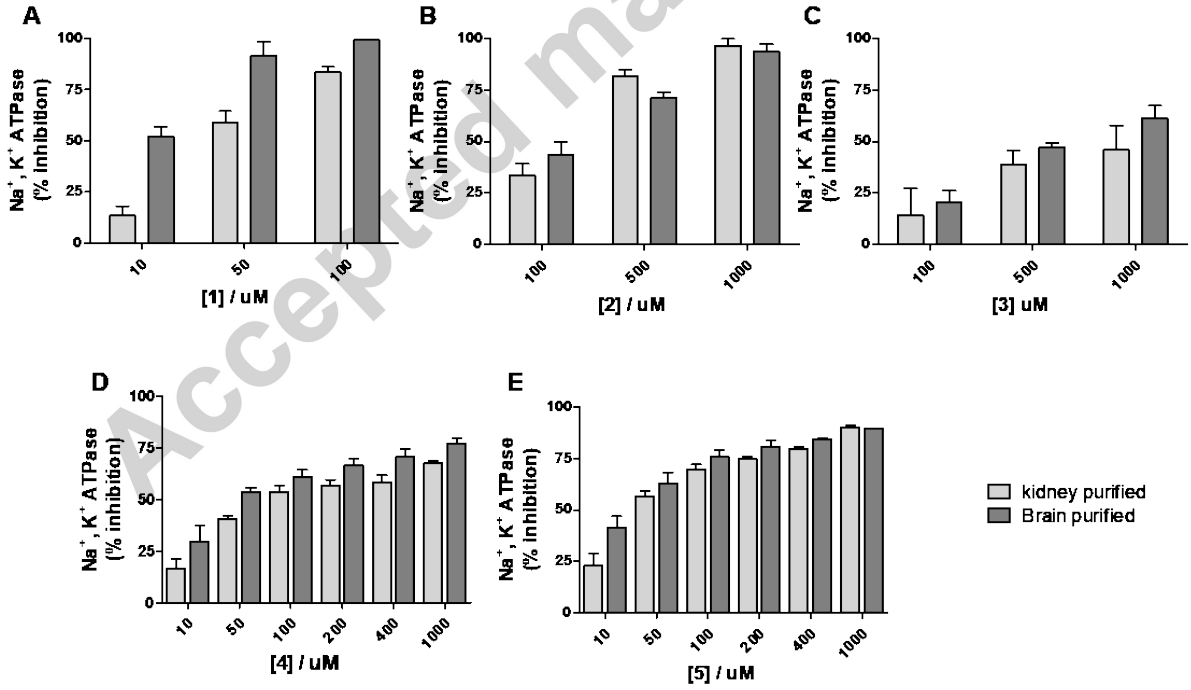

Fig. 3

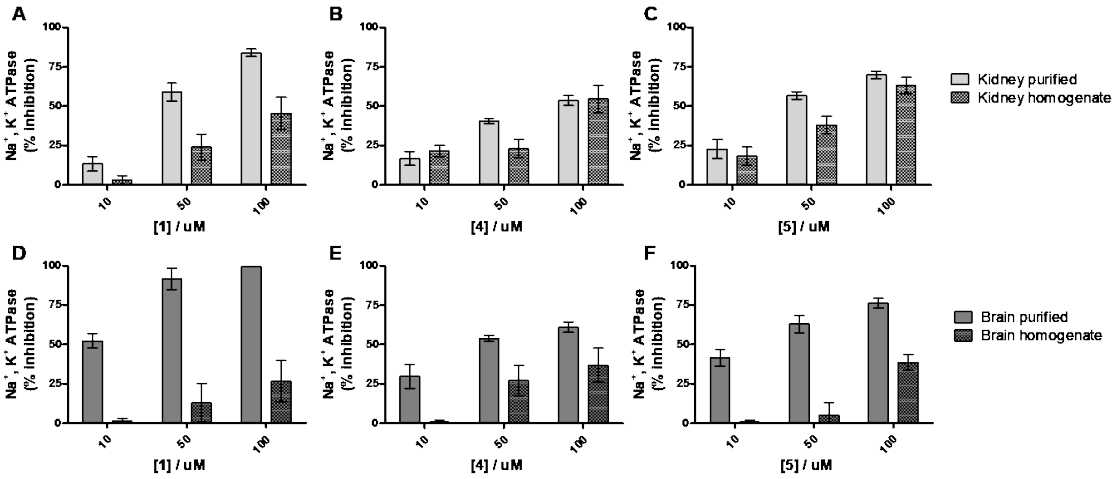

Fig. 4

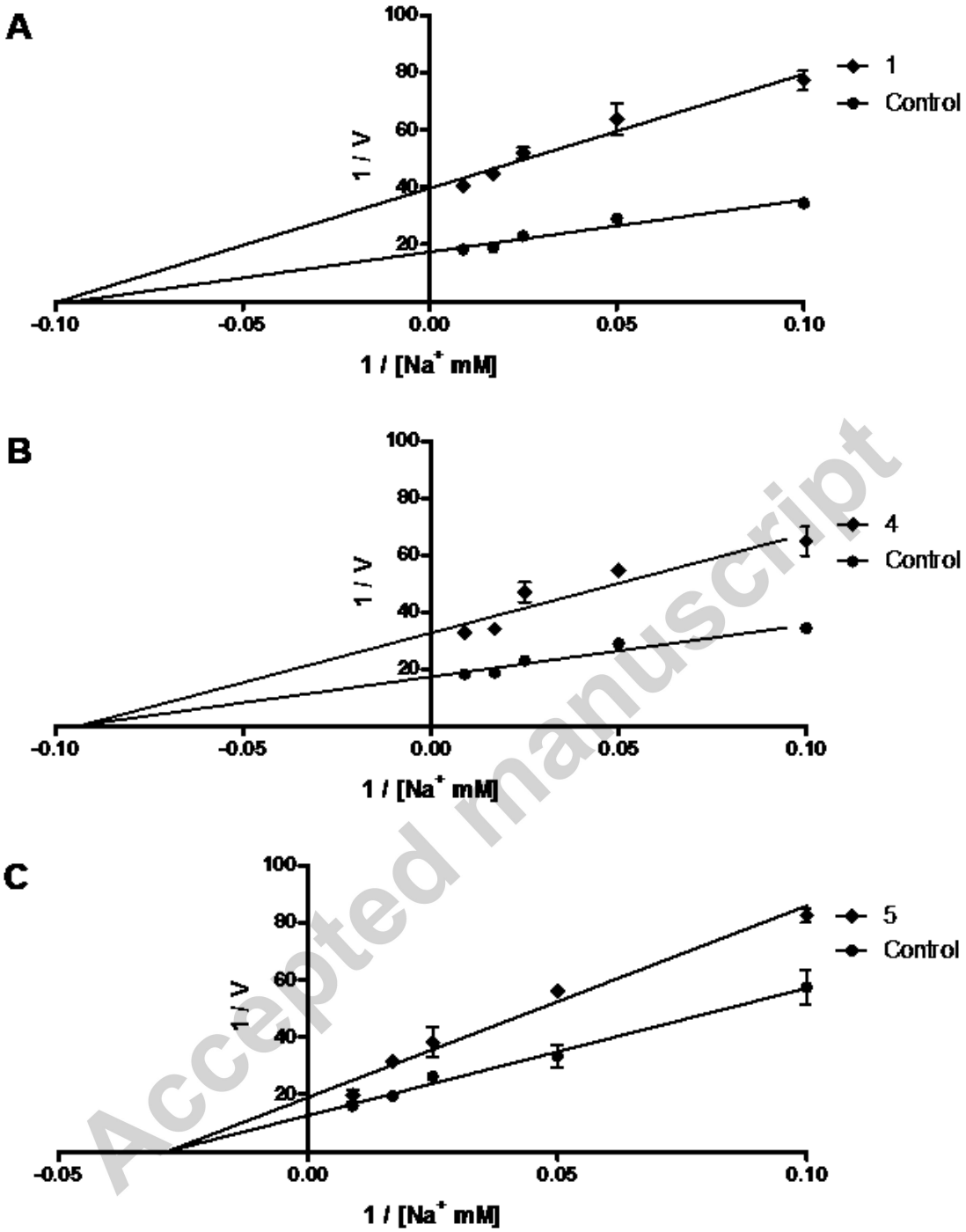

Fig. 5

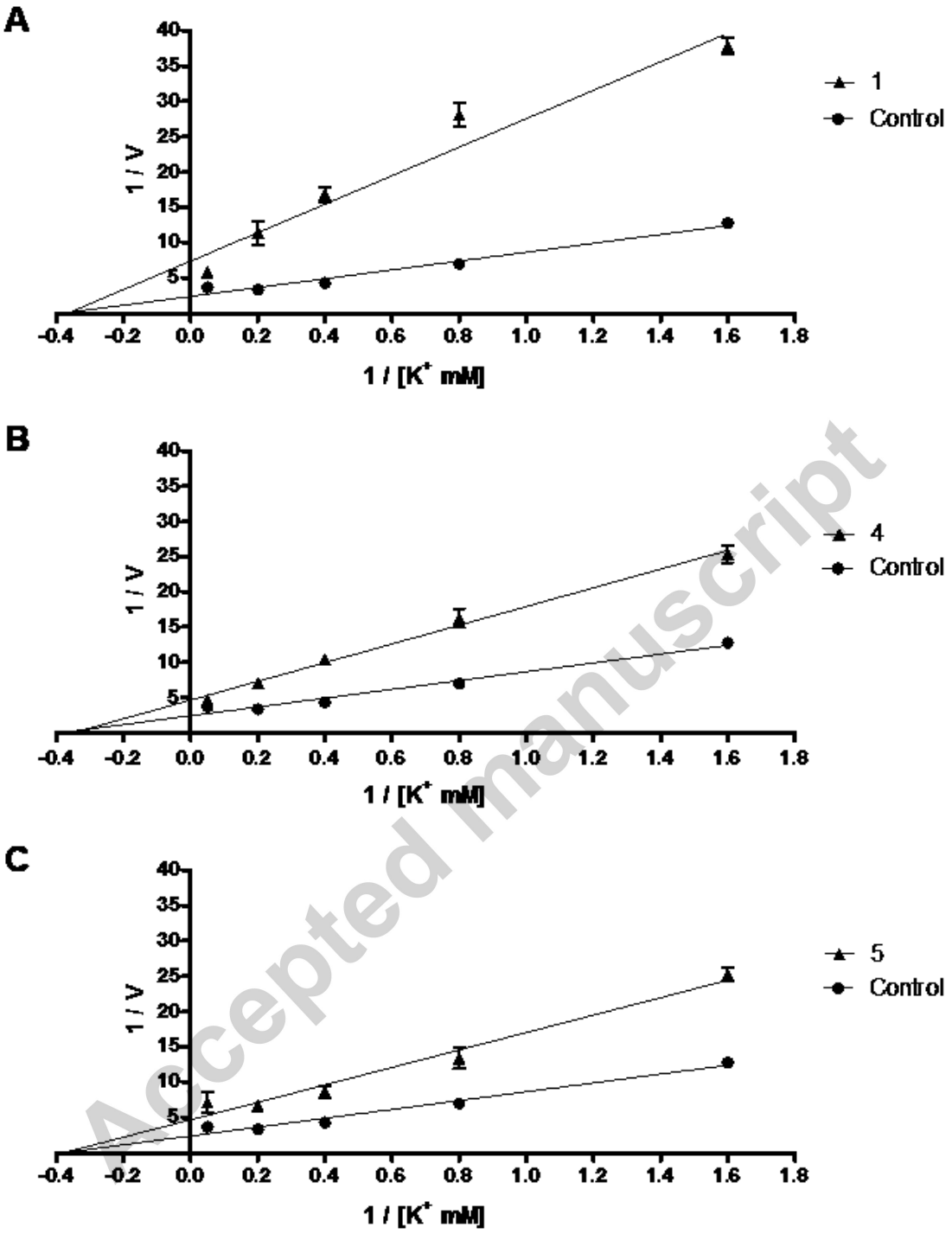

Fig. 6

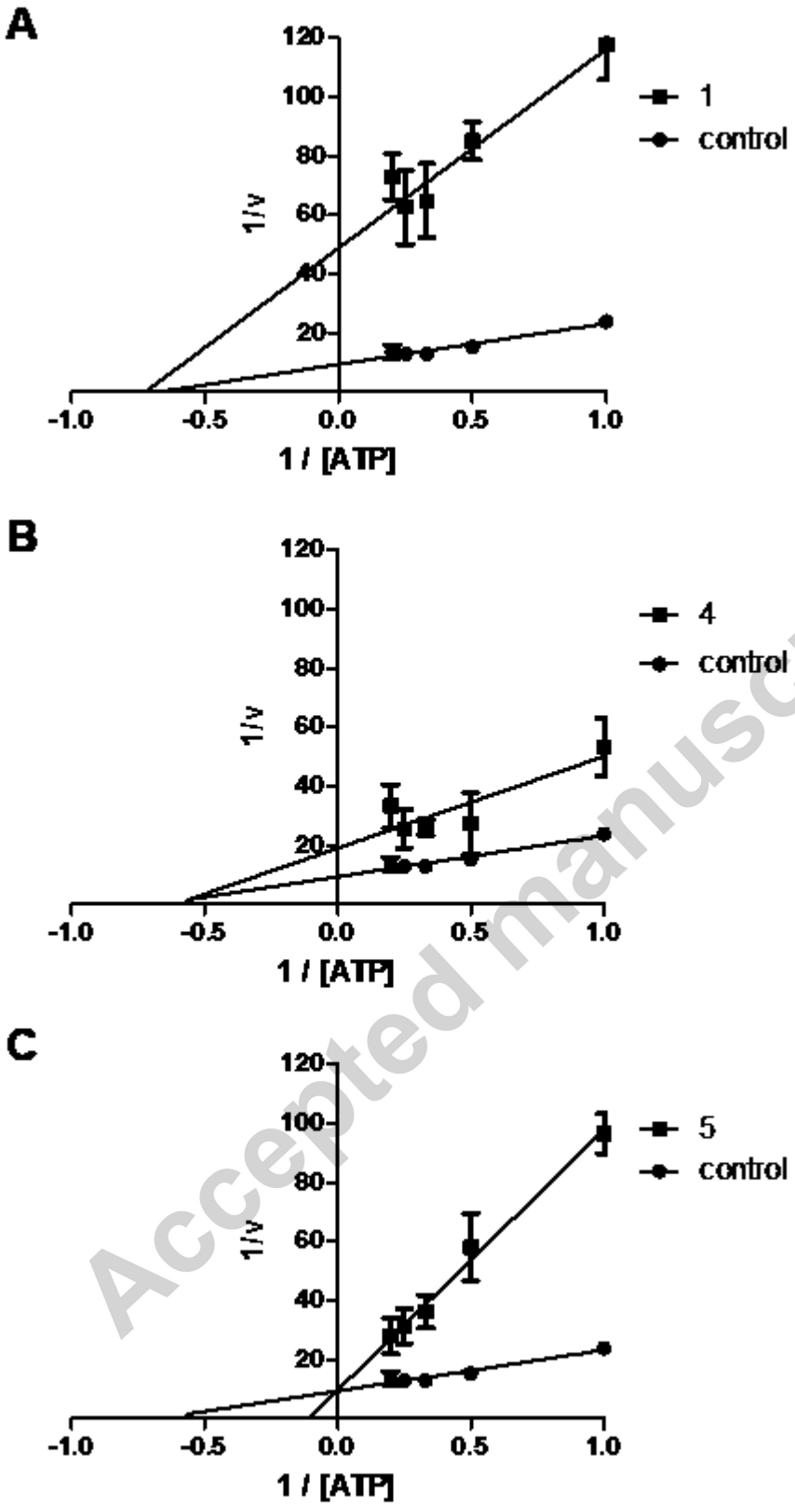

Fig. 7