ChemPubSoc Europe

DOI: 10.1002/cmdc.201500578

CHEMMEDCHEM Communications

Inhibition of *para*-Hydroxyphenylpyruvate Dioxygenase by Analogues of the Herbicide Nitisinone As a Strategy to Decrease Homogentisic Acid Levels, the Causative Agent of Alkaptonuria

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Alkaptonuria (AKU) is a rare multisystem metabolic disease caused by deficient activity of homogentisate 1,2-dioxygenase (HGD), which leads to the accumulation of homogentisic acid (HGA). Currently, there is no treatment for AKU. The sole drug with some beneficial effects is the herbicide nitisinone (**1**), an inhibitor of *p*-hydroxyphenylpyruvate dioxygenase (4-HPPD). **1** has been used as a life-saving drug in infants with type I tyrosinemia despite severe side effects due to the buildup of tyrosine. Four clinical trials of nitisinone to treat AKU have shown that **1** consistently decreases HGA levels, but also caused the accumulation of tyrosine in blood serum. Moreover, the human preclinical toxicological data for **1** are incomplete. In this work, we performed pharmacodynamics and toxicological evaluations of **1**, providing the first report of LD_{50} values in human cells. Intracellular tyrosinemia was also evaluated. Three additional 4-HPPD inhibitors with a more favorable profile than that of **1** in terms of IC_{50}, LD_{50}, and tyrosine accumulation were also identified among commercially available compounds. These may be promising starting points for the development of new therapeutic strategies for the treatment of AKU.

Alkaptonuria (AKU, OMIM no. 203500) is a serious, debilitating, and rare multisystem metabolic disorder caused by the accumulation of homogentisic acid (HGA) due to a deficiency in homogentisate 1,2-dioxygenase (HGD, EC: 1.13.11.5) activity in the tyrosine catabolic pathway. In a process known as ochronosis, HGA is oxidized into a melanin-like polymeric pigment deposited in connective tissues—particularly cartilage—leading to severe early-onset arthritis, heart disease, and significant disability.[1,2] Experimental evidence has demonstrated that the expression of HGD also occurs in cells of the osteoarticular compartment, where the disease is mainly set, and thus contributes to induction of local ochronosis in AKU arthropathy.[3]

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ORCID(s) from the author(s) for this article is/are available on the WWW under http://dx.doi.org/10.1002/cmdc.201500578.

As yet, there is no licensed treatment for AKU. Several therapeutic approaches have been tried, but they were palliative and therefore did not tackle the intrinsic cause of AKU.[4,5] The sole drug with some beneficial effects is the herbicide 2-(2-nitro-4-trifluoromethylbenzoyl)cyclohexane-1,3-dione (nitisinone, **1**).[6] **1** is a potent time-dependent (tight-binding) inhibitor of rat and human liver 4-hydroxyphenylpyruvate dioxygenase (4-HPPD), which is a key iron(II)-dependent, non-heme oxygenase involved in the catabolism of tyrosine. In particular, 4-HPPD catalyzes the transformation of 4-hydroxyphenylpyruvate (HPP) to HGA.[7] On this basis, **1** should prevent or slow the damage from AKU by inhibiting 4-HPPD and therefore decreasing HGA accumulation.

1 has been approved by the US Food and Drug Administration (FDA) for use "under exceptional circumstances" as a life-saving agent in infant patients of hereditary type I tyrosinemia (HT-1), but it may cause severe side effects, such as visual disorders, liver failure, convulsions, and cognitive difficulties, which are related to the chronic high plasma levels of tyrosine.[8–11] Because pre-clinical toxicological and pharmacokinetic data for **1** are mainly derived from comprehensive records based on different animal models,[12–14] human safety margins cannot yet be fully determined.[9,10] However, the theoretical basis of therapy with **1** in AKU cases is convincing in terms of decreasing the plasma levels of HGA.

A total of four clinical trials on the use of **1** to treat AKU have been undertaken (NCT00107783; NCT01390077; NCT01828463, SONIA1; and NCT01916382, SONIA2).[4,15–18] Each study showed that **1** consistently decreases HGA, but also causes the accumulation of serum tyrosine levels. In the SONIA1 clinical trial,[4,18] undertaken by the DevelopAKUre consortium aiming to obtain marketing authorization from the European Medicines Agency for the use of nitisinone to treat AKU, it was found that **1** decreases urinary excretion of HGA in a concentration-dependent manner and that the observed increase in tyrosine is not clearly correlated with concentrations of **1**. Moreover, pharmacokinetic data for **1**, such as area under the curve (AUC), maximum concentrations (c_{max}), and median oral clearance were obtained.[4,18] Nevertheless, the pathophysiological and clinical significance of tyrosinemia consequent to treatment with **1** is not yet known, and long-term monitoring of tyrosine is required to ensure the safe use of unlicensed **1** to treat AKU.[19,20] This is particularly the case, because de-

spite the lower dosage of **1** needed to treat AKU than for HT-1, nitisinone-induced tyrosinemia levels surpassed the recommended threshold of 400–500 μM.[4,18,20] In this context, the importance of obtaining complete preclinical data for **1** is evident, especially at the human cellular level, as is the need to evaluate alternative 4-HPPD inhibitors to significantly decrease HGA with minimal tyrosinemia, which is surely a pioneering goal for the treatment of AKU.

In this work, **1** and twelve commercial analogues of **1** (Figure 1) were submitted to a pharmacodynamic and toxicological study to evaluate their IC_{50}, LD_{50}, and tyrosine buildup values. **3** (tembotrione) and **4** (mesotrione) have been reported as inhibitors of 4-HPPD.[21,22] The other compounds were selected on the basis of their structural similarity to **1** and on their commercial availability. Inhibition of 4-HPPD activity was determined in rat liver cytosol as the source of 4-HPPD by using an oxygen consumption enzyme assay (SDR SensorDish Reader), and the data are expressed as IC_{50} values. LD_{50} values were determined in vitro by MTT cell viability assay, using human cells (human primary fibroblasts [HFb]) for the first time in this context; HFb were proven to express HGD by western blot analysis (data not shown). Intracellular tyrosinemia in HFb was determined by LC–MS/MS analysis.

Enhanced potency (lower IC_{50} values toward 4-HPPD inhibition) and decreased cytotoxicity (higher LD_{50} values) relative to **1** (Table 1) prompted us to select three compounds (**3**, **4**, and **6**) for subsequent tyrosine dosage assays. Data obtained for the other compounds were not encouraging: four of them, namely **7**, **8**, **9**, and **12**, did not show inhibitory activity toward 4-HPPD, and the remaining compounds presented a bad profile. In fact, they showed higher IC_{50} and lower LD_{50} values than those of **1**, and thus an enhanced propensity to give rise to tyrosine accumulation.

1 showed sub-micromolar inhibitory activity (173 nM) toward 4-HPPD (Figure 2 A). Regarding cytotoxicity (Table 1), a short-term LD_{50} value could not be calculated, as >50% of

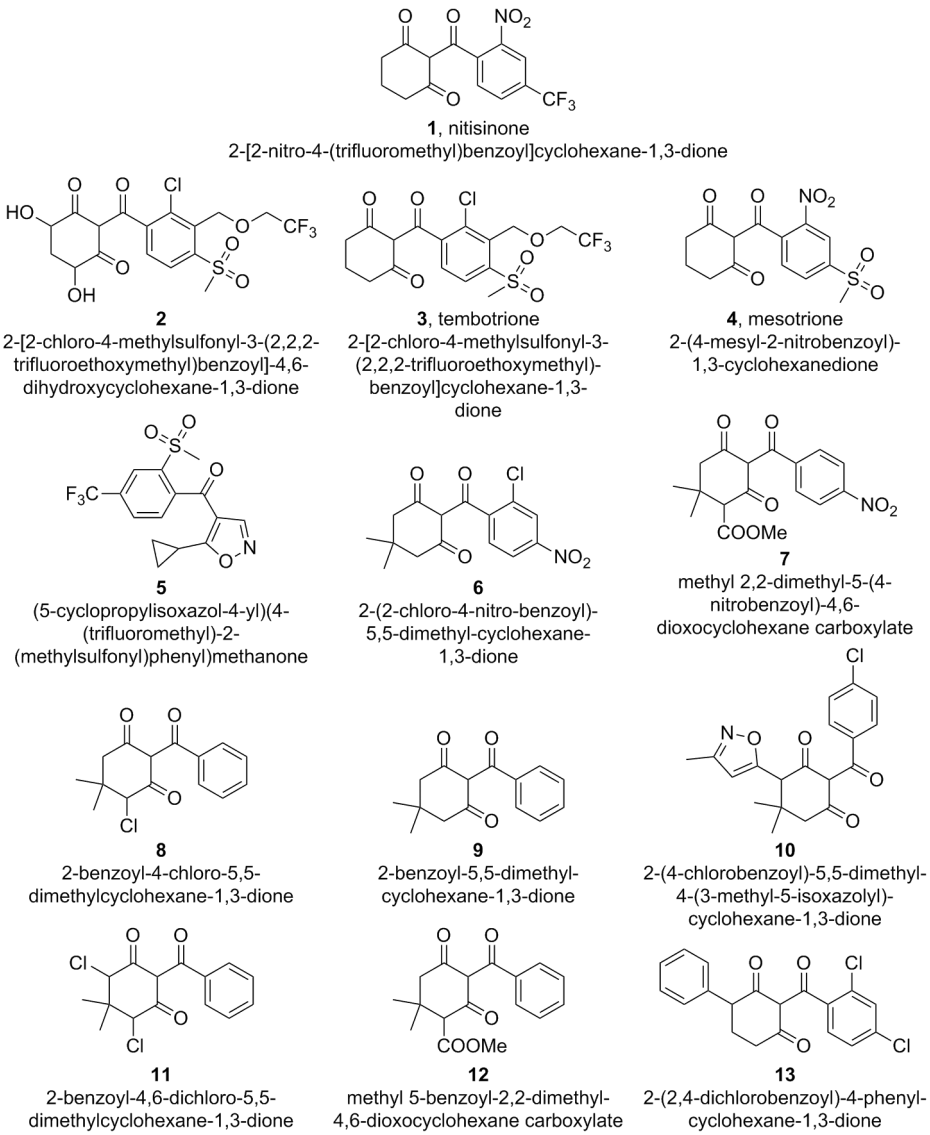

Figure 1. Test compounds used in this study.

Table 1. LD_{50} and IC_{50} values obtained for compound **1** and other commercial 4-HPPD inhibitors.

Compd	LD_{50} [mM][a]		IC_{50} [nM][a]
	24 h	144 h	
1	>1[c]	~1	173±1
2	0.48±0.04	0.25±0.03	227±9
3[b]	>1[c]	>1[c]	152±6
4[b]	1.1±0.1	>1[c]	36.7±9.7
5	>1[c]	0.11±0.07	399±19
6[b]	>1[c]	>1[c]	36.4±9.3
7	0.63±0.12	>1[c]	n.i.[d]
8	>1[c]	>1[c]	n.i.[d]
9	>1[c]	>1[c]	n.i.[d]
10	>1[c]	0.82±0.20	59.9±1.8 μM
11	>1[c]	0.29±0.08	192±2
12	>1[c]	>1[c]	n.i.[d]
13	>1[c]	0.36±0.15	279±6

[a] Values are the mean±SD of $n=3$ determinations performed in triplicate. [b] Compounds with more favorable IC_{50} and LD_{50} values with respect to **1**. [c] An LD_{50} value >1 mM indicates that at a compound concentration of 1 mM, more than 50% of cells were still viable after the indicated treatment period, and thus it was confidently considered to be >1 mM. [d] No inhibition.

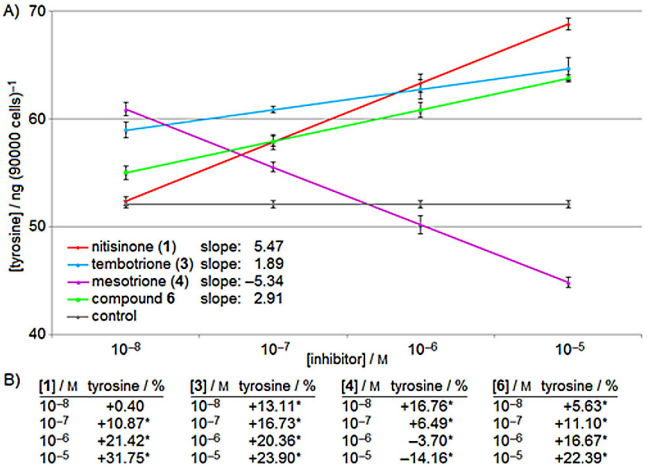

Figure 3. A) Tyrosine levels in HFb treated with **1**, **3**, **4**, and **6** (as well as vehicle control) for 72 h. Data are the mean±SD of $n=3$ determinations performed in triplicate. B) Accumulation of tyrosine in HFb treated with **1**, **3**, **4**, and **6** at various concentrations relative to control (untreated HFb); positive values indicate an increase relative to control, while negative values indicate a decrease; *$p<0.05$ compared with untreated HFb.

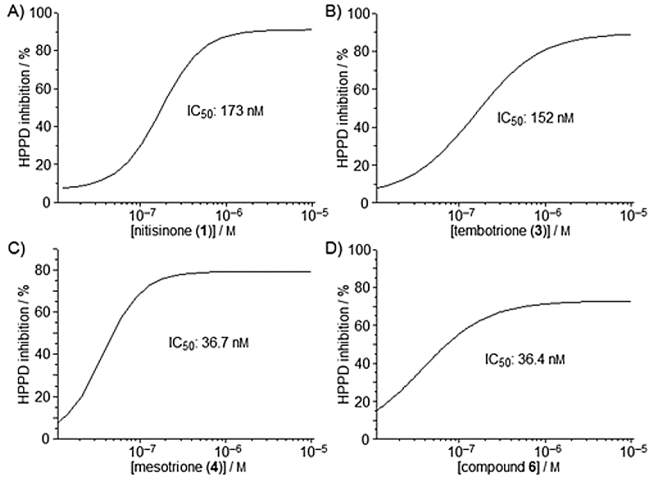

Figure 2. Inhibition of 4-HPPD activity by compounds A) **1**, B) **3**, C) **4**, and D) **6**.

cells were still viable after 24 h. On the basis of this result, short-term LD_{50} was considered to be >1 mM. On the other hand, long-term LD_{50} was about 1 mM. A long-term LD_{50} value lower than the short-term LD_{50} supports the hypothesis that the toxicity of **1** is linked to its mechanism of action (i.e., the irreversible inhibition of 4-HPPD leading to in vivo hypertyrosinemia after repeated drug administration).[9,14,17,18] These data were further confirmed by results obtained from the tyrosine dosage assay, which provided the first evidence of massive intracellular accumulation of tyrosine in human cell cultures after treatment with **1**. Indeed, as shown in Figure 3B, in HFb treated with **1**, intracellular tyrosine increased by about 21 and 32% with respect to control at drug dosages of 10^{-6} and 10^{-5} M, respectively. These two concentrations reflected the plasma levels of free **1** observed in AKU patients.[17,18] Although a 10- to 14-fold increase in tyrosine levels in AKU patients following long-term treatment with **1** was observed relative to untreated AKU patients,[4,17,18] the tyrosine accumulation observed in our in vitro cellular model (1.1- to 1.3-fold change relative to control) is still notable, considering that these data reflect a very short period of treatment with **1** (only 72 h). Moreover, Figure 3A showed that tyrosine accumulation during administration of **1** appears to be clearly dose-dependent.

3 possesses a very interesting activity profile, with an IC_{50} value of ~152 nM (Figure 2B) and an LD_{50} (>1 mM) higher than that of **1** (Table 1). This means that such a compound is able to inhibit 4-HPPD in a manner similar to that of **1**, but with a better cytotoxicity profile. These results are in agreement with and are corroborated by tyrosine dosage. In fact, the increase in intracellular tyrosine levels was lower with respect to that caused by treatment with **1**, as shown by comparing the slopes of the respective plots (Figure 3A). Therefore, from the perspective of cytotoxicity related to the mechanism of action, it is reasonable to consider **3** safer than **1**, while still being effective on 4-HPPD.

4 also showed a very interesting profile, both in terms of in vitro enzyme inhibition and cytotoxicity. In fact, the IC_{50} (36.7 nM) toward 4-HPPD was fourfold better than that found for **1** (Figure 2C), indicating improved potency in 4-HPPD inhibition. On the other hand, the inhibition curve of **4** did not reach the same plateau of **1** (79 versus 90%, respectively), suggesting a minor blockage of 4-HPPD with possible consequent minor tyrosine buildup. The LD_{50} value after treatment for 144 h was higher than that of **1** (Table 1), indicating lower cytotoxicity of the compound, especially in long-term treatment. Surprisingly, the tyrosine dosage assay revealed that at the lowest compound concentration, intracellular levels of this

amino acid is about 17% higher than control levels ($p < 0.05$). In contrast, when the inhibitor dose was increased, intracellular tyrosine levels underwent a significant decrease ($p < 0.05$). In particular, at the highest dose of inhibitor, the tyrosine concentration decreased by about 14% relative to control ($p < 0.01$; Figure 3A,B). This could suggest a massive blockage of 4-HPPD, as also indicated by low IC_{50}, which in turn could activate alternative (usually inactive) pathways of tyrosine catabolism.[21] Moreover, this hypothesis was also validated by results of the cytotoxicity assay. In fact, the lower short-term LD_{50} value with respect to long-term LD_{50} (Table 1) could be explained by the initial potentially damaging accumulation of tyrosine. The trend of tyrosine levels shown upon administration of **4** surely represents a beneficial effect for a potential drug for the treatment of AKU, as the accumulation of HGA could be decreased without an increase in tyrosine concentration. On the other hand, decreasing intracellular tyrosine levels below the control threshold could create drawbacks in terms of maintaining cellular homeostasis.

The overall biological results suggested **6** as the most promising compound in terms of inhibitory activity toward 4-HPPD, cytotoxicity, and tyrosine accumulation profile. Its IC_{50} value of ~36.4 nM (Figure 2D) makes this compound effective in vitro at very low concentrations, as well as safe, with the residual activity of the enzyme being nearly 30% and the viability of HFb after 144 h nearly 75% at 1 mM. This concentration is 500-fold higher than plasma levels of **1** observed in AKU patients in a clinical trial,[4,17,18] thereby underscoring the safety of **6**. Moreover, at the highest concentration tested, we observed an intracellular tyrosine accumulation about 7% lower than **1** ($p < 0.01$) after treatment for three days. Therefore, even if comparison between in vitro and in vivo models should be done very cautiously, data obtained for **6** suggest that the modulation of 4-HPPD activity in vivo could be achieved easily and safely. Indeed, in a therapeutic context such as AKU, the ideal characteristic of a drug is represented by its capacity to modulate enzymes involved in HGA production, without the severe and undesired accumulation of tyrosine.

Notably, for both **4** and **6**, IC_{50} and LD_{50} values differed significantly. This is surely an advantage for a drug that could be able to produce the desired pharmacological effect without generating unwanted side effects linked to the mechanism of action.

In conclusion, we have determined toxicological data for **1**, providing the first determination of LD_{50} values in human cells following both acute (24 h) and chronic (144 h) treatment. Moreover, because delineating the most appropriate dose of **1** or developing new 4-HPPD inhibitors that significantly decrease HGA with minimal tyrosinemia is an important goal for the treatment of AKU, especially for a drug that should be administered over a lifetime, we suggest the use of alternative 4-HPPD inhibitors: three 4-HPPD commercial inhibitors with a more favorable profile than **1** in terms of IC_{50}, LD_{50}, and tyrosine accumulation. These represent a promising scaffold to be used as starting points in the development of new therapeutic strategies for the treatment of AKU.

Experimental Section

Compounds: All commercial reagents were obtained from Sigma–Aldrich, as were **1** and other 4-HPPD inhibitors. Compounds were determined by HPLC to be ≥95% pure, and were solubilized in aqueous saline or in DMSO.

Measurement of HPPD activity: 4-HPPD activity was measured in male Wistar rat liver cytosol by monitoring oxygen consumption using an SDR SensorDish Reader. Briefly, a tube containing an oxygen sensor spot was filled with 0.2 M sodium phosphate buffer (pH 7.4), 1.7 mM ascorbic acid as a cofactor, and 10.92 mg rat enzyme preparation (4-HPPD source). The mixture was equilibrated at 37 °C for 5 min, and the reaction was initiated by the addition of substrate (0.2 M HPP). Incubation was carried out in the presence of **1** or other test compounds at various concentrations (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M). For each compound tested, non-interference with the assay components was confirmed by a negative control experiment using an enzyme-free solution. Percent inhibition of enzyme activity was calculated as the ratio of the slopes (decrease in O_2 concentration [μM] per minute) of samples versus untreated controls corrected with blank values. IC_{50} was calculated by regression analysis in the concentration range using Origin Data Analysis and Graphing Software with sigmoidal curve fitting, by plotting compound concentration versus oxygen consumption values expressed as percentage of 4-HPPD inhibition, reflected in oxygen decrease correlated with enzyme inhibition.

Evaluation of cytotoxicity: HFb (12×10^4 well^{-1}) were exposed to compounds at various concentrations (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M) for 24 and 144 h. Cell viability was quantified by MTT assay. Negative controls were carried out with vehicle at a concentration corresponding to the highest dose of the test compound. The final DMSO concentration in any case did not exceed 0.2% (*v*/*v*) and did not affect the parameters analyzed. LD_{50} calculations were done with Origin Data Analysis and Graphing Software using sigmoidal curve fitting. HFb were isolated (after written informed consent was obtained) and cultured as described.[23]

Tyrosine dosage: Intracellular tyrosine was dosed in HFb (9×10^4 cells well^{-1}) treated for 72 h with compounds at various concentrations (10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M); controls were done with the vehicle at a concentration corresponding to the highest dose of the test compound. At the end of treatment, cells were collected by scraping in presence of norvaline (10^{-3} M, as internal standard) and ice-cold methanol (40 μL), and then lysed by freeze–thaw cycles in dry-ice/nitrogen/ice-bath. The supernatant was finally collected, evaporated, and re-suspended with 100 μL 0.05% formic acid. Each sample was analyzed by LC–MS/MS. In particular, analysis was carried out with Varian chromatographic equipment managed by Varian MS Workstation System Control 6.9 software and composed of a dual pump (212-LC) connected to a triple quadrupole Varian detector (320-LC) equipped with a Varian ESI ionization source. Subsequently, the amount of intracellular tyrosine per 9×10^4 cells was plotted versus each compound concentration, and the final dose–response curve was obtained using Origin Data Analysis and Graphing Software with linear curve fitting.

Statistical analysis: All experiments were carried out with $n = 3$ determinations performed in triplicate; data are presented as average values with standard deviation. Unpaired Student's *t*-test was used when necessary. Differences with a *p*-value of at least ≤0.05 were considered significant.

Acknowledgements

This work was supported by Telethon Italy grant GGP10058. The authors thank the Associazione Italiana Malati di Alcaptonuria (AimAKU) (grant ORPHA263402), Siena Biotech S.P.A., Toscana Life Sciences Orphan_1 project, and FMPS 2008–2010.

Keywords: alkaptonuria · enzymes · inhibitors · nitisinone · *p*-hydroxyphenylpyruvate dioxygenase

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Received: November 11, 2015
Published online on ■■ ▮▮, 0000

COMMUNICATIONS

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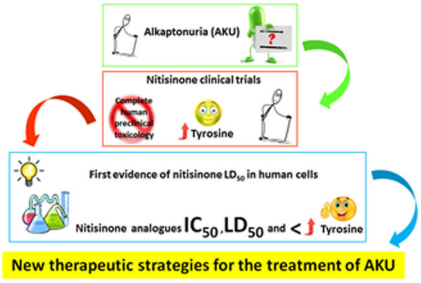
Inhibition of *para*-Hydroxyphenylpyruvate Dioxygenase by Analogues of the Herbicide Nitisinone As a Strategy to Decrease Homogentisic Acid Levels, the Causative Agent of Alkaptonuria



A better stop 4-HPPD: Alkaptonuria (AKU) is a rare and serious multisystem debilitating disease with no licensed treatment. Nitisinone is used to treat AKU despite severe hypertyrosinemia and incomplete preclinical human toxicological data. This study provides the first determinations of LD_{50} values in human cells and suggests the use of alternative compounds as promising scaffolds for developing new therapeutic strategies for treatment of AKU.