Keywords

Thienopyrimidines, autophagy, mitochondrial respiration, complex I, inhibitor

Abstract

Autophagy ensures cellular homeostasis by the degradation of long-lived proteins, damaged organelles and pathogens. This catabolic process provides essential cellular building blocks upon nutrient deprivation. Cellular metabolism, especially mitochondrial respiration, has a significant influence on autophagic flux, and complex I function is required for maximal autophagy. In Parkinson's disease mitochondrial function is frequently impaired and autophagic flux is altered. Thus, dysfunctional organelles and protein aggregates accumulate and cause cellular damage. In order to investigate the interdependency between mitochondrial function and autophagy, novel tool compounds are required. Herein, we report the discovery of a structurally novel autophagy inhibitor (Authipyrin) using a high content screening approach. Target identification and validation led to the discovery that Authipyrin targets mitochondrial complex I directly, leading to the potent inhibition of mitochondrial respiration as well as autophagy.

Main Text

Macroautophagy, hereafter referred to as autophagy, is a highly regulated and conserved biological process. Induced during periods of nutrient deprivation, autophagy leads to the bulk degradation of cytoplasmic constituents, whose building blocks are used as an alternative energy supply.[1] Furthermore, this catabolic process regulates the clearance of damaged organelles and long-lived proteins to ensure cellular homeostasis.[2] The initiation phase of autophagy is characterized by formation of an isolation membrane, which engulfs selected cellular components. Upon expansion and membrane closure, the autophagosome fuses with the lysosome. Lysosomal hydrolases degrade the autophagosomal cargo and release the respective catabolites into the cytoplasm.[3,4] Dysregulation of autophagy is involved in various pathological conditions, such as neurodegenerative disorders and cancer.[2] However, by clearing malfunctioning organelles, as well as misfolded and aggregated proteins, autophagy significantly contributes to disease prevention.[5,6] Damaged mitochondria for example can release reactive oxygen species, which have harmful effects on DNA and cellular macromolecules. Altered autophagy and dysfunctional mitochondria are frequently observed in patients that suffer from Parkinson's disease. This prevents the selective degradation of mitochondria by autophagy, which is referred to as mitophagy.[7] On

the contrary, autophagy is regulated by ATP levels, which represent the available energy of a cell. Published findings postulate a connection between the inhibition of mitochondrial respiration and modulation of autophagy.[8] Furthermore, altered mitochondrial respiration, especially complex I function, impairs autophagic flux.[9] However, the exact mechanism that underlies this interplay is still unknown. Thus, the development of novel tool compounds, to investigate the connection between oxidative phosphorylation and autophagic flux is of utmost importance. Herein, we report the discovery of a novel, highly potent autophagy inhibitor termed Authipyrin, which targets mitochondrial complex I. Its potency and selectivity make it a useful tool to study the interplay between mitochondrial respiration and autophagy further.

To identify structurally novel autophagy inhibitors, a medium throughput screen of our in-house library of approximately 160,000 compounds was performed.[10] In the screening assay, MCF7 cells stably transfected with eGFP-tagged light chain 3 (LC3), were employed, which can be detected by automated fluorescence microscopy. Upon initiation of autophagy, cytosolic LC3-I is conjugated to phosphatidylethanolamine to produce LC3-II which localises to the autophagosomal membrane. The autophagosomes are represented as green punctae, while LC3-I fluorescence is visible as a diffuse signal throughout the cytosol. Autophagy inhibitors should reverse the phenotype of autophagosome formation, while autophagy enhancement results in an increase.[11] Based on this screen, thienopyrimidines were identified as potential autophagy inhibitors (see the Supporting Information, Table S1). The most potent of these compounds was Authipyrin (Figure 1 A-C, Table S1 entry 1). Authipyrin inhibits starvation-induced autophagy with an IC_{50} of 0.02 ± 0.01 µM and rapamycin-induced autophagy with an IC_{50} of 0.18 ± 0.07 µM. Furthermore, the influence of Authipyrin on the autophagy marker LC3 was determined in different cell lines (Figure 1D).[12] An autophagy inhibitor should prevent LC3-II formation. Authipyrin significantly reduced LC3-II lipidation under starvation in the presence of chloroquine. This confirms the inhibitory effect of Authipyrin on autophagy. Furthermore, selective toxicity of Authipyrin under nutrient deprivation was investigated. While Authipyrin had no influence on cell viability under normal conditions, it had a pronounced dose-dependent toxicity upon glucose starvation (see the Supporting Information; Figure S1).

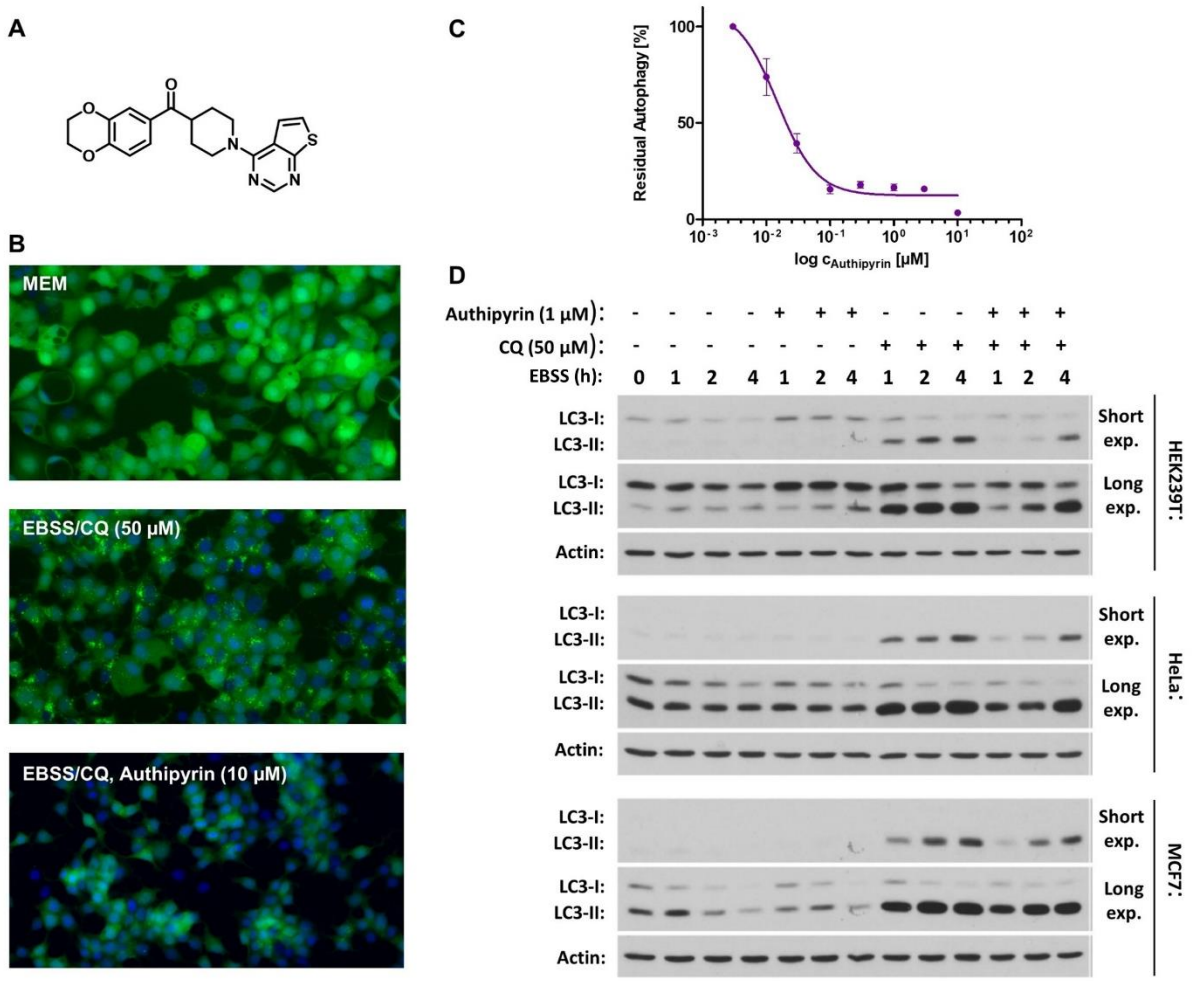

Figure 1: Effect of Authipyrin on autophagic flux. **A**: Structure of Authipyrin. **B**: Phenotypic autophagy assay based on MCF7 cells stably transfected with eGFP-LC3 (green). Non-starved cells were treated with MEM, starved cells with EBSS and 50 µM CQ. Simultaneously, decreasing concentrations of Authipyrin were added. After 3 h incubation, the cells were fixed with paraformaldehyde and the nuclear DNA was stained with Hoechst (blue). **C**: Dose dependent effect of Authipyrin on autophagosome formation in phenotypic autophagy assay. **D:** Effect of Authipyrin (1 µM) on the autophagy marker LC3 in starved cells with and without CQ (50 µM). Different cell lines were starved with EBSS for the annotated time points with/without CQ or Authipyrin. Cells were then lysed and immunoblots performed. All experiments are n = 3, representative images and blots shown. CQ: Chloroquine; Exp: Exposure.

Several thienopyrimidine derived kinase inhibitors were reported.[13-15] A subset of these kinase inhibitors are also active in autophagy.[16,17] Therefore, a single point full kinase panel with Authipyrin was performed at a concentration of 1 µM (see the Supporting Information, Table S2). This resulted in the identification of four out of 485 kinases that featured a

reduction in activity by more than 50% (Table 1). The affected kinases were PEAK1 (83%), the inactive version of the cyclin-dependent kinase CDK11 (70%), as well as haspin (51%) and the mutant DDR2 (N456S) protein (58%). Subsequently, the respective IC_{50} values for these kinases were determined. PEAK1 possessed the lowest IC_{50} of 0.65 µM. The cell-based IC_{50} of Authipyrin for autophagy inhibition upon starvation was 0.02 ± 0.01 µM. The IC_{50} for the purified target protein, which causes the inhibitory effect on autophagic flux should be comparable or even lower. Thus, it can be assumed that autophagy modulation by Authipyrin is not dependent on the inhibition of a kinase.

Table 1: Results from SelectScreen™ Kinase profiling with Authipyrin. Single point inhibition was determined at 1 µM. Four kinases with an inhibition > 50 % were chosen for IC_{50} measurements. Kinases were screened against decreasing concentrations of Authipyrin with the highest concentration of 1 µM.

Kinase	Single point inhibition [%]	IC_{50} Inhibition [%]	IC50 [µM]
PEAK1	83	68	0.62
CDK11 (inactive)	70	47	>1.0
DDR2 (N456S)	58	38	>1.0
Haspin	51	33	>1.0

Autophagy is an essential mechanism to maintain cellular homeostasis, by clearance of damaged organelles, including mitochondria.[18] Furthermore, alteration of metabolic processes, such as oxidative phosphorylation, results in modulation of autophagic processes.[19-21] Therefore, the influence of Authipyrin on mitochondrial respiration was investigated by means of a Mito Stress Test assay employing the Seahorse XF analyzer. The readout is based on two different fluorophores. One fluorophore is sensitive to changes in the pH, which represents the extracellular acidification rate (ECAR). The ECAR is influenced by lactate excretion due to anaerobic glycolysis. The second fluorophore detects the cellular oxygen consumption rate (OCR). Initially, the basal respiration under resting conditions was determined (Figure 2A and B). Subsequently, the inhibitor of interest was added to the cells, to determine the influence on mitochondrial respiration (point a). Injection of the known complex V inhibitor oligomycin reflects the amount of oxygen required for ATP production (point B). Subsequent addition of carbonyl cyanide-4-(trifluoro-methoxy)phenylhydrazone (FCCP) disrupts the proton gradient and causes a rise of mitochondrial respiration to maximal capacity (point c).[22] This represents the cellular respiration under stress conditions. The range between basal and maximal OCR is the

cellular spare capacity available to respond to increased energy requirement. The known complex I and III inhibitors rotenone and antimycin A are finally employed for complete inhibition of mitochondrial respiration (point c). The Mito Stress Test showed that Authipyrin inhibits oxidative phosphorylation in a dose-dependent manner. At a concentration of 0.1 µM and 1 µM cell treatment with Authipyrin resulted in a decrease of the OCR by 25% and 75% respectively (Figure 2A). Simultaneously the ECAR showed a strong increase. (Figure 2B). However, downregulation of mitochondrial respiration could be a general characteristic of this scaffold. Therefore, two structurally similar thienopyrimidines (Table S1, entry 15 and 27) were analyzed by means of MitoStress Test assay. These compounds were inactive in the initial autophagy screen. Both compounds did not display any effect on the OCR. (see supporting information Figure S2) Therefore, downregulation of mitochondrial respiration by Authipyrin is connected to the inhibition of autophagy.

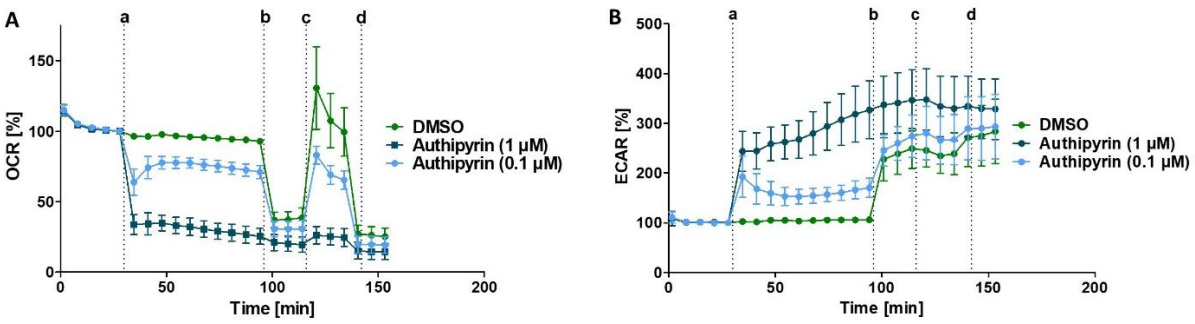

Figure 2: Influence of Authipyrin on mitochondrial respiration determined by means of Seahorse XF Mito Stress Test. **A**: Measurement of the OCR. **B**: Measurement of the ECAR. (Injection of **a**: DMSO or Authipyrin (1 µM and 0.1 µM) **b**: Oligomycin (1 µM); **c**: FCCP (0.25 µM); **d**: Mixture of rotenone and antimycin A (0.5 µM). Data is mean ± SD, n = 3.

In order to determine which complex of the electron transport chain is targeted by Authipyrin, a semi-intact assay was performed.[23] The substrates of each complex were added separately in combination with Authipyrin or the respective control inhibitor. Seahorse XF plasma membrane permeabilizer (PMP) was used to ensure substrate availability. Authipyrin selectively inhibited complex I (Figure 3A), but had no effect on the activity of complex II to IV (Figure 3B-D). These results are in line with previously published data on the autophagy inhibitor Aumitin. Aumitin also targets mitochondrial complex I and has an inhibitory effect on autophagy. Furthermore, the complex I inhibitor Rotenone was identified as a modulator of autophagy.[8]

Authipyrin could cause complex I inhibition by two different mechanisms. On the one hand, the compound could directly target complex I to interfere with mitochondrial respiration. On the other hand Authipyrin could target a protein involved in NADH generation and thus interfere with the NADH supply chain. This would result in complex I substrate depletion and consequently decrease of oxidative phosphorylation. To investigate, whether the inhibitory effect of Authipyrin on complex I is direct or indirect, an assay with isolated bovine heart mitochondria was performed. This assay showed that Authipyrin dose-dependently impaired NADH-Coenyme Q reductase activity (Figure 3E). This assay requires higher concentrations of Authipyrin in comparison to the semi-intact assay in order to achieve complex I inhibition. This is presumably due to the fact that isolated bovine heart mitochondria were employed, which results in a higher concentration of complex I than under cellular conditions. Similar results were also observed for the complex I inhibitor Aumitin.[8] Conclusively, this assay shows that Authipyrin is a direct inhibitor of complex I (Figure 3E).

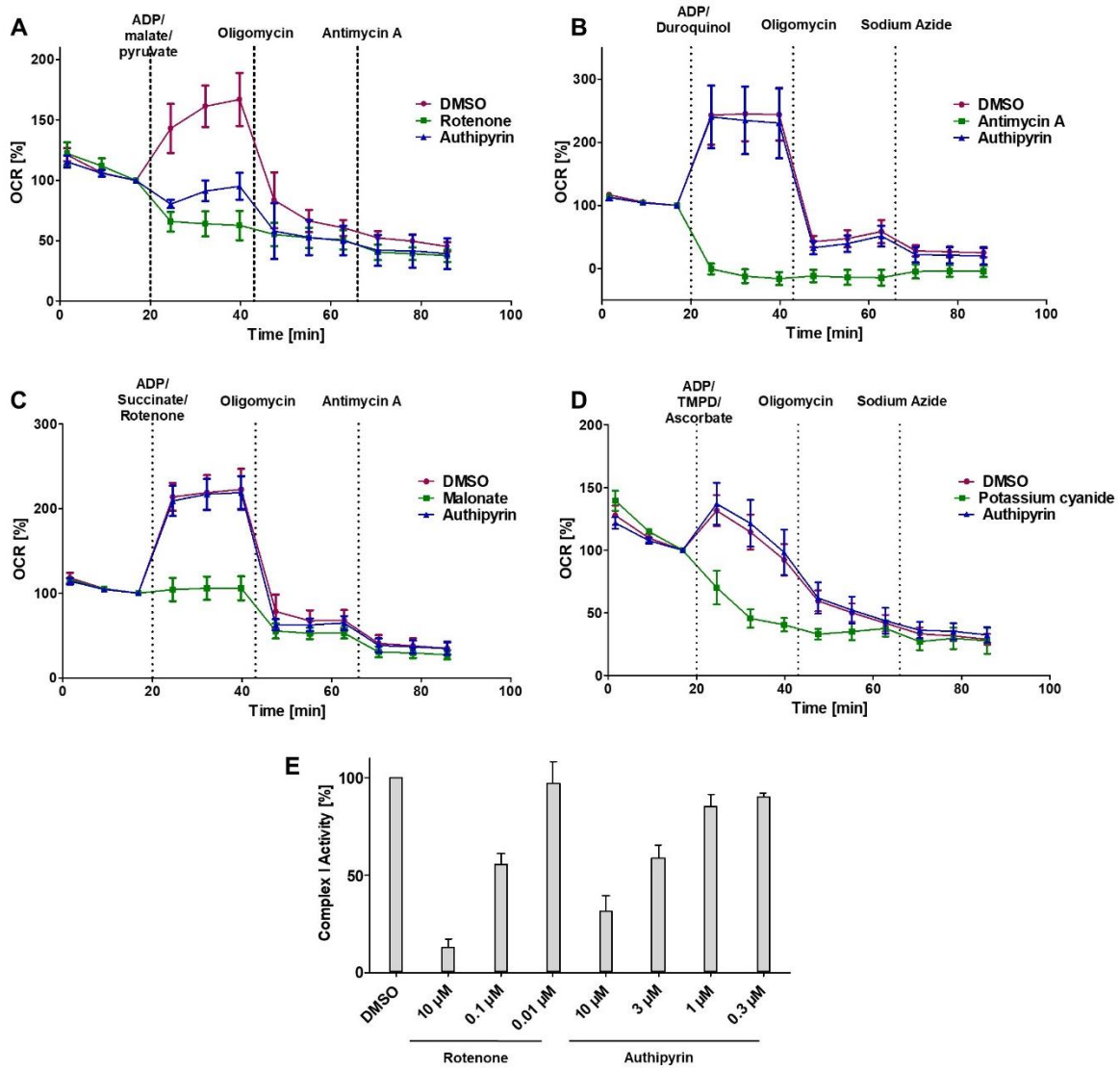

Figure 3: Semi-intact assay with Authipyrin (1 µM). MCF7/LC3 cells were permeablized with Seahorse XF plasma membrane permeabilizer. DMSO, Authipyrin or a control inhibitor were added in combination with the respective substrates, followed by oligomycin and antimycin A. Influence of Authipyrin on **A**: Complex I, **B**: complex II, **C**: complex III and **D**: complex IV. Data is mean ± SD, n = 3. **E**: Determination of NADH-Coenzyme Q reductase activity in isolated bovine heart mitochondria. Rotenone was employed as a control inhibitor. Data is mean ± SD, n = 2.

In conclusion, we demonstrated that the thienopyrimidine-containing molecule Authipyrin is a highly potent inhibitor of starvation, as well as rapamycin induced autophagy. Thus, it is likely that Authipyrin acts downstream of mTOR. Although thienopyrimidine is a known kinase inhibitory scaffold, autophagy modulation of Authipyrin is not connected to a kinase target. We demonstrated that Authipyrin downregulates mitochondrial respiration by affecting

mitochondrial complex I. An assay with isolated bovine heart mitochondria showed that Authipyrin has a direct effect on NADH-CoQ reductase activity. Alterations of mitochondrial metabolism and complex I activity are known to influence autophagic flux. Furthermore, dysfunctional mitochondria and impaired autophagy are characteristic for Parkinson's disease. However, the exact mechanism underlying the interplay between autophagic flux and mitochondrial respiration remains to be elucidated. Thus, structurally novel tool compounds, such as Authipyrin are highly valuable for investigation of this interdependency and can facilitate the development of more efficient therapeutics.

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