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Modified (2′-deoxy)adenosines activate autophagy primarily through AMPK/ULK1-dependent pathway

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ABSTRACT

Autophagy is a conserved self-digestion process, which governs regulated degradation of cellular components. Autophagy is upregulated upon energy shortage sensed by AMP-dependent protein kinase (AMPK). Autophagy activators might be contemplated as therapies for metabolic neurodegenerative diseases and obesity, as well as cancer, considering tumor-suppressive functions of autophagy. Among them, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAr), a nucleoside precursor of the active phosphorylated AMP analog, is the most commonly used pharmacological modulator of AMPK activity, despite its multiple reported "off-target" effects. Here, we assessed the autophagy/mitophagy activation ability of a small set of (2′-deoxy)adenosine derivatives and analogs using a fluorescent reporter assay and immunoblotting analysis. The first two leader compounds, 7,8-dihydro-8-oxo-2′-deoxyadenosine and -adenosine, are nucleoside forms of major oxidative DNA and RNA lesions. The third, a derivative of inactive N^6-methyladenosine with a metabolizable phosphate-masking group, exhibited the highest activity in the series. These compounds primarily contributed to the activation of AMPK and outperformed AICAr; however, retaining the activity in knockout cell lines for *AMPK (ΔAMPK)* and its upstream regulator *SIRT1 (ΔSIRT1)* suggests that AMPK is not a main cellular target. Overall, we confirmed the prospects of searching for autophagy activators among (2′-deoxy)adenosine derivatives and demonstrated the applicability of the phosphate-masking strategy for increasing their efficacy.

Autophagy is a fundamental molecular process that involves the elimination of damaged or malfunctioning cellular components through lysosome-mediated degradation. Disruptions in autophagy can lead to various pathologies, such as neurodegenerative diseases[1] and obesity.[2]

The main stimuli for activating autophagy are energy and amino acid starvation in the cell. Serine/threonine protein kinase mTOR (mammalian target of rapamycin) is the central regulator of autophagy. In cells, mTOR serves as a core component of two complexes — mTORC1 and mTORC2, which have distinct functions. mTORC2 mainly regulates growth factor signaling pathways by phosphorylating the AKT and SGK kinases, affecting cell survival and suppressing apoptosis.[3] Meanwhile, mTORC1 inhibits autophagy by suppressing the activity of ULK1 and initiates protein biosynthesis via phosphorylation of the main mRNA translation regulators, such as proteins of the 4E-BP group that inhibit the protein synthesis initiation factor eIF4E.[3] mTORC1 is located on the surface of lysosomes, where it can be activated by amino acids.[4] Inhibition of mTOR due to starvation or the action of chemical molecules leads to ULK1 autophosphorylation and conformational changes. This

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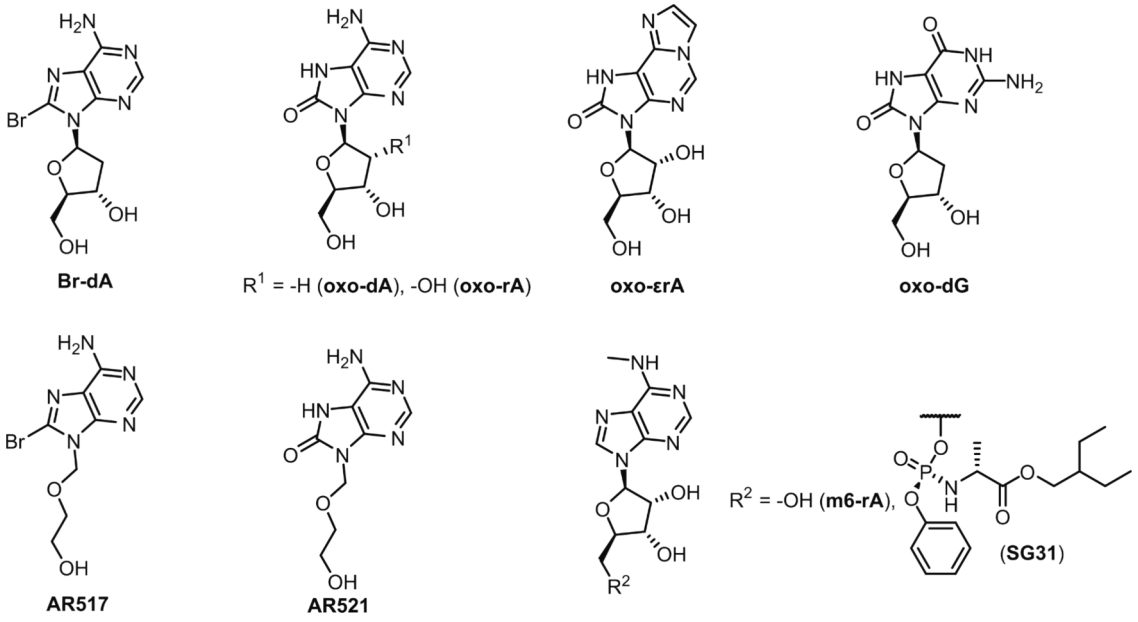


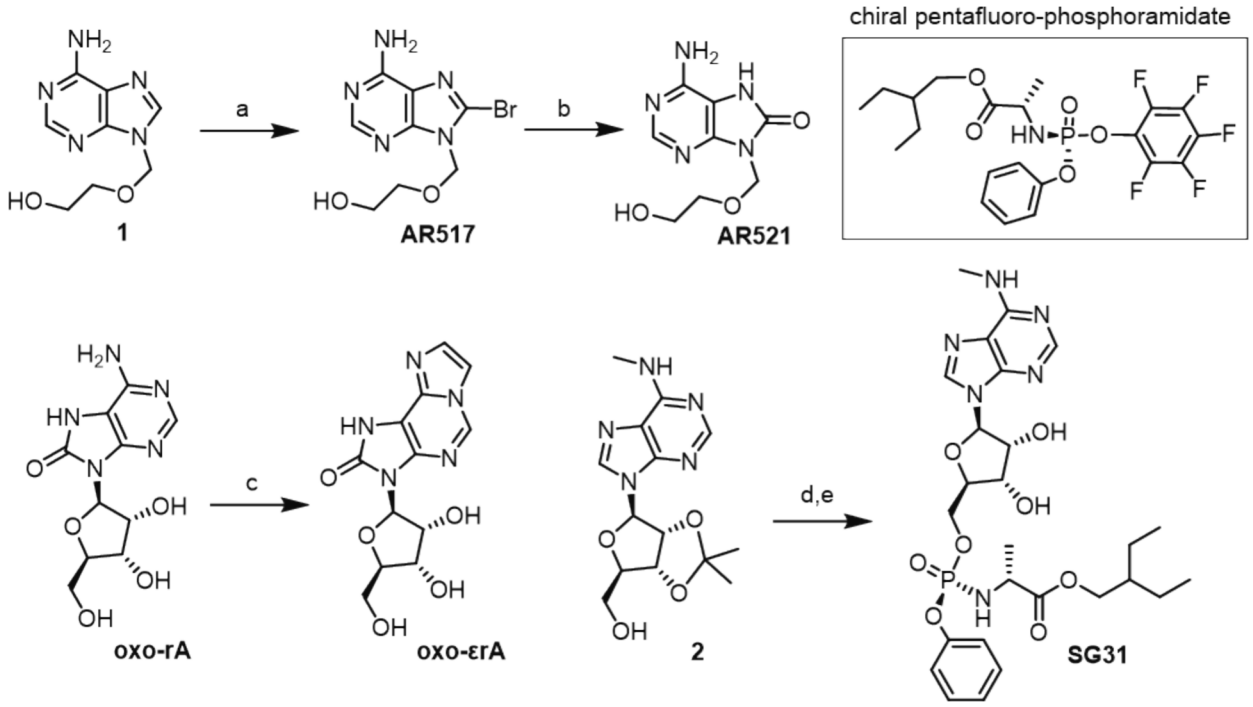
Fig. 1. Chemical structure of (2′-deoxy)adenosine derivatives and analogs.

process initiates the assembly of a platform, which then leads to the formation of an autophagosome.[5] The main component of this platform is Beclin 1 (BECN1), which activates the assembly of the autophagophore.

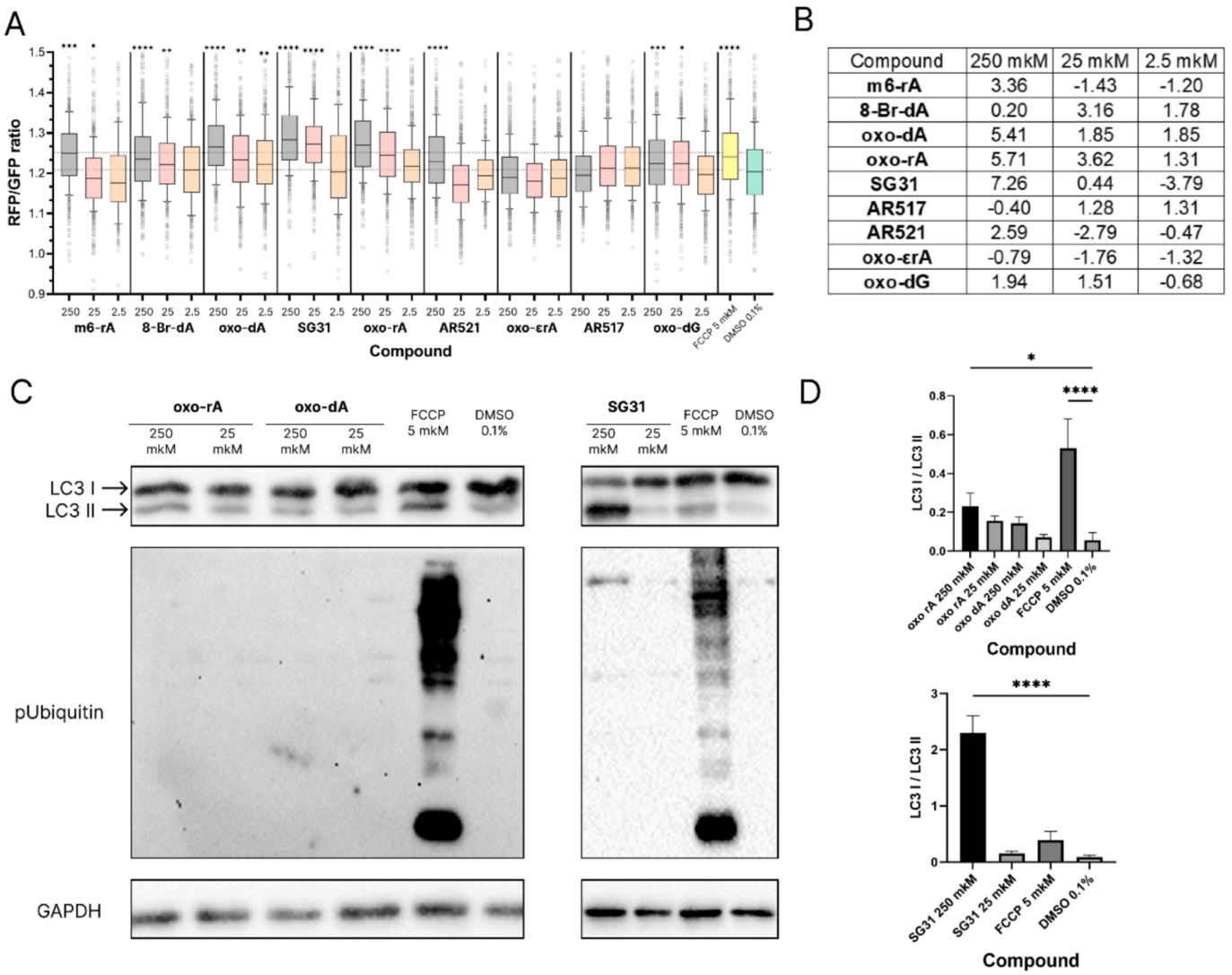
The activity of mTOR is regulated through the AMPK- and Akt-dependent pathways. The AMPK-dependent pathway senses energy starvation. In its active state, the NAD+-dependent deacetylase sirtuin 1 (SIRT1) deacetylates liver kinase B1 (LKB1), which further activates AMPK.[6]

Mitophagy is a subtype of autophagy that is responsible for the degradation of damaged mitochondria. Disruptions in mitophagy can lead to the development of cancer, cardiovascular and neurodegenerative diseases.[7] Studies on rodents have shown that activating mitophagy and autophagy through pharmacological drugs can improve the condition of animals with myocardial infarction, various types of cardiomyopathy, and atherosclerosis.[8]

Inducers of autophagy and mitophagy are widely found among natural compounds[9] and synthetic molecules.[10] One of the most commonly used pharmacological modulators of autophagy through the AMPK-dependent pathway is 5-aminoimidazole-4-carboxamide ribonucleoside (AICAr). After phosphorylation, its monophosphate metabolite acts as an allosteric activator of AMPK by binding to its γ



Scheme.1. Synthesis of (2′-deoxy)adenosine derivatives **oxo-εrA** and **SG31** and acyclic analogs **AR517** and **AR521**. Reagents and conditions: (a) NBS, DMF, rt, 20 h, 64 %; (b) 2-mercaptoethanol, TEA, H_2O, 100 °C, 3 h, 81 %; (c) chloroacetaldehyde, aq. sodium acetate buffer (pH 4.6), rt, 48 h, 79 %; (d) *t*-BuMgBr in THF, rt, 30 min, then chiral pentafluoro-phosphoramidate, 60 °C, 2 h; (e) TFA, H_2O, 18 h, 72 % over two steps.



Compound	250 mkM	25 mkM	2.5 mkM
m6-rA	3.36	-1.43	-1.20
8-Br-dA	0.20	3.16	1.78
oxo-dA	5.41	1.85	1.85
oxo-rA	5.71	3.62	1.31
SG31	7.26	0.44	-3.79
AR517	-0.40	1.28	1.31
AR521	2.59	-2.79	-0.47
oxo-εrA	-0.79	-1.76	-1.32
oxo-dG	1.94	1.51	-0.68

Fig. 2. Autophagy induction rate of (2′-deoxy)adenosine derivatives and analogs. (A) Changes of the RFP/GFP ratio in the cell line SH-SY5Y pMXs GFP-LC3-RFP when treated with compounds under study. ANOVA test results are indicated on the graph: * – p-value < 0.05, ** – p-value < 0.01, *** – p-value < 0.001, **** – p-value < 0.0001. (B) Changes in signal relative to DMSO, in percentage. Positive values indicate an increase in the level of autophagy, while negative values indicate a decrease. (C) Results of western blotting on the common markers of autophagy and mitophagy. (D) Ratio of the LC3 II to the LC3 I forms in cells after treatment with leader compounds at concentrations of 250 and 25 mkM. Data represents mean ± SD (n = 3). ANOVA test results are indicated on the graph: * – p-value < 0.05, **** – p-value < 0.0001.

subunit.[11,12] This interaction stimulates liver kinase B1-mediated phosphorylation of Thr172 and protects against dephosphorylation.[11,13]. However, AICAr has been shown to accumulate in cells at millimolar concentrations, which causes side effects, such as the undesirable modulation of enzymes involved in gluconeogenesis.[14] In addition, inhibition of AICAr-induced autophagy due to reduced binding of PI3K to Beclin-1 makes it a less effective activator.[15] Several adenosine analogs, including 3-methyladenosine, N^6-mercaptopurine riboside and N^6,N^6-dimethyladenosine, exhibit inhibitory effect at concentrations ranging from high micromolar to low nanomolar,[16] whereas others, such as cyclic triazolic nucleoside analogs, 8-aminoadenosine, 8-chloroadenosine, 2-chloro-N^6-cyclopentyl-adenosine, activate autophagy at micromolar-to-submicromolar concentrations; however, accompanied with significant toxicity, thus reducing their therapeutic potential.[17–20] While less toxic and more active autophagy activators, such as N^6-(2-hydroxyethyl)adenosine and N^6-(3-hydroxyphenyl)adenosine, galidesivir, cordycepin, and trachycladine A derivatives, have been studied,[21–25] there is still a need for more potent inducers.

In this study, we examined several derivatives and analogs of (2′-deoxy)adenosine, assessed their ability to activate autophagy using a fluorescent cell reporter assay and immunoblotting analysis of the phosphorylation status for key participants involved in autophagy regulatory cascade. We also evaluated the ability of the leader compounds to induce mitophagy through the PINK/PARKIN-dependent pathway by immunoblotting analysis of the accumulation of phospho-ubiquitinated proteins in cells. Finally, we tested the specificity of the leader compounds' influence on an AMPK-dependent pathway using knockout cell lines for *AMPK (ΔAMPK)* and its upstream regulator *SIRT1 (ΔSIRT1)*.

For an initial assessment of the autophagy-activating ability, we selected a small set of (2′-deoxy)adenosine derivatives and analogs (Fig. 1). The set included 8-bromo-2′-deoxyadenosine (**Br-dA**),[26] 7,8-dihydro-8-oxo-2′-deoxyadenosine (**oxo-dA**),[27] 7,8-dihydro-8-oxoadenosine (**oxo-rA**),[27] 7,8-dihydro-8-oxo-2′-deoxyguanosine (**oxo-dG**),[28] N^6-methyladenosine (**m6-rA**),[29] as well as newly synthesized **AR517** and **AR521,** acyclic analogs of 2′-deoxyadenosine, **oxo-εrA,** a derivative of **oxo-dA** containing an additional 1,N^6-etheno bridge, and **SG31,** a derivative of **6m-rA** bearing a masked phosphate group.

Acyclic analog **AR517** was prepared by the treatment of **1**[30] with *N*-

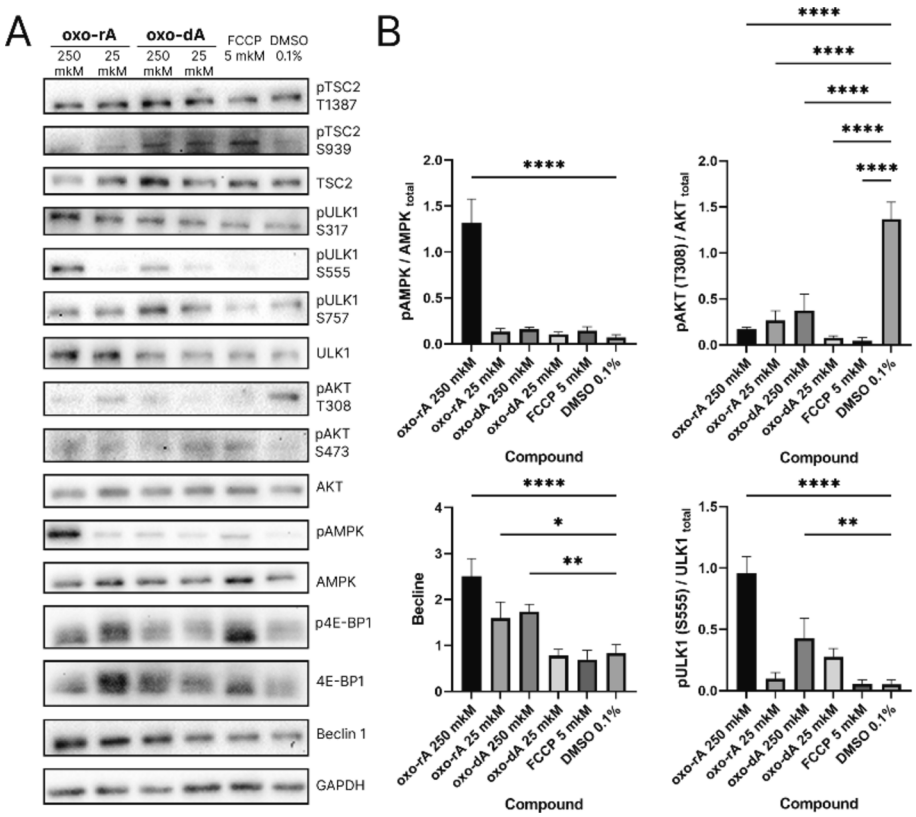


Fig. 3. Investigation of the mechanism of autophagy induction by **oxo-dA** and **oxo-rA**. (A) Western blotting on various protein regulators of the autophagy cascade. (B) Changes in phosphorylation status of main autophagy regulators. Data represents mean ± SD (n = 3). ANOVA test results are indicated on the graph: * – p-value < 0.05, ** – p-value < 0.01, **** – p-value < 0.0001.

bromosuccinimide (NBS) in DMF at room temperature using the reported procedure for the bromination of N^9-alkyladenine[31] (Scheme 1). To obtain its 7,8-dihydro-8-oxo-derivative **AR521**, an aqueous solution of **AR517** was refluxed in the presence of 3 equiv. of 2-mercaptoethanol and 10 equiv. of TEA.[27] An etheno bridge was introduced by the treatment of **oxo-rA** with chloroacetyldehyde in aq. sodium acetate buffer (pH 4.6)[32], affording **oxo-εrA**. Finally, the coupling of 2′,3′-*O*-protected adenosine derivative **2**[33] with the chiral pentafluoro-phosphoramidate[34] in the presence of *t*-BuMgBr, followed by hydrolysis of the intermediate ketal, led to target **SG31**, the phosphate-masked **6m-rA** derivative.

In order to investigate the ability of the compounds to stimulate autophagy, a monoclonal cell culture of SH-SY5Y cells expressing GFP-LC3-RFP was established.[35] In brief, lentiviruses carrying pMX GFP-LC3-RFP were generated and used to infect SH-SY5Y-based cell lines (ΔAMPK, ΔSIRT1, and wild type). The fluorescent cells were sorted, and monoclonal cultures were obtained. The resulting reporter cell lines were analyzed using fluorescent microscopy in the presence of FCCP, a well characterized autophagy inducer.

In the obtained reporter cell line, endogenous ATG4 proteins cleave GFP-LC3-RFP to produce equal amounts of GFP-LC3 and RFP.[35] RFP is diffusely distributed throughout the cytoplasm, and autophagy does not significantly affect its fluorescence level. In contrast, the GFP-LC3 fusion is incorporated into autophagosomes upon the initiation of autophagy. A decrease in pH after the fusion of the autophagosome and lysosome results in a diminished fluorescent signal of GFP within the cell. The green-to-red signal ratio after exposure is indicative of the level of autophagy activity.

All obtained compounds were tested in three concentrations of 250, 25 and 2.5 mkM (Fig. 2A). Compounds **oxo-rA**, **oxo-dA** and **SG31** exhibited the highest autophagy-activating potential. Moreover, the level of autophagy activation under **SG31** treatment was 1.5 times higher than that of the other two compounds (Fig. 2A, B).

The obtained results were validated through immunoblotting for the common autophagy marker, the LC3 protein. The accumulation of a lipidated form of LC3 (LC3 II), which migrates faster, indicates active incorporation of the protein into the expanding autophagosome membrane, thereby indicating an increase in the level of autophagy in cells (Fig. 2C, D.). Among the compounds tested, **m6-rA** and **AR521** were the weakest autophagy inducers and demonstrated the activity only in the highest concentration (250 mkM). At the same time, **SG31**, a **6m-rA** derivative bearing a phosphate-masking group that was shown to be metabolized rapidly to phosphate within cells,[36,37] exhibited higher activity compared with the parent compound, suggesting the importance of the phosphate group for the manifestation of activity. Derivatives **oxo-rA** and **oxo-dA** exerted moderate autophagy induction, although the former appeared to be slightly more efficient. Thus, for further experiments we decided to use three most active molecules: **SG31**, **oxo-rA** and **oxo-dA.**

Immunoblotting analysis and fluorescent reporter results corroborated strongly. However, the latter represents a more sensitive method than immunoblotting, capable of detecting even lower amounts (2.5 mkM) in autophagy induction upon treatment with **oxo-rA** and **oxo-dA**. The difference lies in the way they evaluate the activation of autophagy. Immunoblotting is a method that averages the characteristics of all cells in a sample. In contrast, a fluorescent reporter system evaluates the activation of autophagy in each cell individually and then compares the characteristics of the two populations.

We also evaluated the ability of these compounds to activate mitophagy via the PINK/PARKIN-dependent pathway. For this purpose, immunoblotting with antibodies against phospho-ubiquitin, a key marker of the process, was conducted. However, none of the tested compounds demonstrated the ability to activate mitophagy (Fig. 2C).

The mechanism of action of the selected activators was investigated by means of immunoblotting, with a focus on proteins involved in the

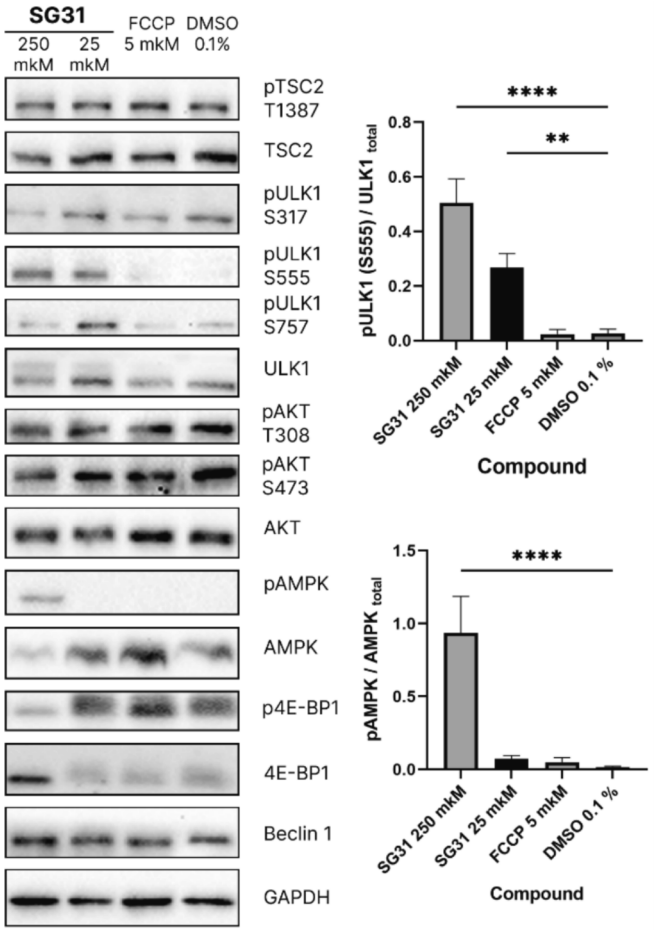


Fig. 4. Investigation of the mechanism of autophagy induction by **SG31**. (A) Western blotting on various protein regulators of the autophagy cascade. (B) Changes in phosphorylation status of main autophagy regulators. Data represents mean ± SD (n = 3). ANOVA test results are indicated on the graph: ** – p-value < 0.01, **** – p-value < 0.0001.

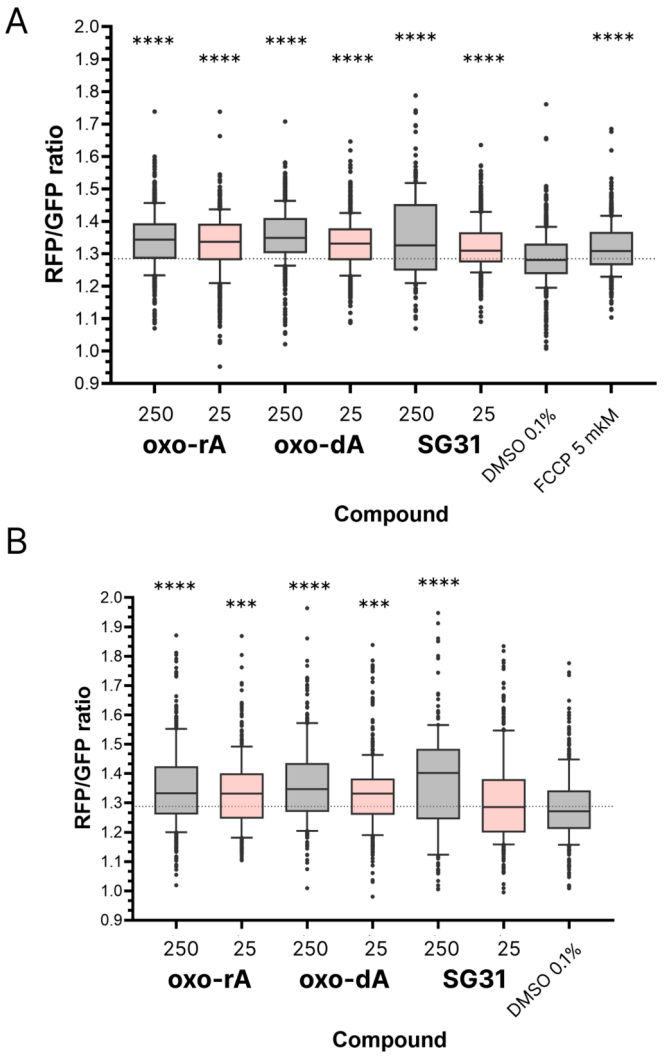


Fig. 5. Investigation of activity of **oxo-rA**, **oxo-dA** and **SG31** on cell lines with knockout *AMPK* (A) and *SIRT1* (B) genes. ANOVA test results are indicated on the graph: * – p-value < 0.05, ** – p-value < 0.01, *** – p-value < 0.001, **** – p-value < 0.0001.

autophagy activation cascade and their regulatory phosphorylation. Compounds **oxo-rA** and **oxo-dA** are similar in structure and activity, and thus should have a similar mechanism of action. Indeed, both compounds caused a decrease in activating phosphorylation of AKT (T308) (Fig. 3A, B), which is carried out by the kinase PDK1.[38] AKT has been demonstrated to function as a repressor of autophagy, as it inactivates tuberin (TSC2), which in turn inhibits mTOR.[39,40] Nevertheless, no discernible alteration in TSC2 phosphorylation was observed following the treatment with these compounds.

In addition, treatment with both compounds led to an increase in the level of AMPK phosphorylation, which appeared to be concentration-dependent. Furthermore, the accumulation of the phosphorylated (S555) form of ULK1 also serves as an indicator of increased AMPK activity. Additionally, an increase in intracellular Beclin 1 levels was observed, which suggested a general activation of autophagy. Thus, these compounds induce autophagy primarily through the AMPK/ULK1-dependent pathway. Moreover, the presence of 2′-OH in **oxo-rA** compared with **oxo-dA** is responsible for an additional increase in the activity.

Compound **SG31**, a derivative of **m6-rA** with metabolizable phosphate-masking group, also exhibited *in vitro* activation of autophagy via the AMPK/ULK1-dependent pathway (increased phosphorylation of AMPK and ULK1 at S555) (Fig. 4A, B). Nevertheless, in contrast to the effects observed in the case of **oxo-dA** and **oxo-rA**, **SG31** did not appear to influence the AKT phosphorylation status. Additionally, treatment with **SG31** has led to a reduction in the phosphorylation of ULK (Ser 757) and 4E-BP1, which provides indirect evidence of mTOR inhibition, which might be mediated by hyperactivated AMPK.

The leader compounds have the greatest effect on the activation of AMPK. In similar experiments, AICAr and other nucleoside-based autophagy activators exhibit such activity at concentrations of 500 mkM,[41] whereas compounds under study are active at 250 mkM. Furthermore, the ratio of phosphorylated to total forms of AMPK increases by more than 10-fold, while AICAr increases the ratio by 2-fold only.[41]

In all experiments, **SG31** exhibited the highest activity, which indicates that the approach of masking the phosphate group is fruitful for further search for nucleotide-based autophagy activators.

The studied compounds are thought to activate autophagy primarily through the AMPK/ULK1-dependent pathway. To address this possibility, we inactivated *AMPK* and *SIRT1* genes in the reporter cell line by a CRISPR/Cas9 system. After the verification of knockouts by amplicon sequencing, we tested their activity on cell lines with knockout of the regulators of this pathway, *AMPK* and *SIRT1* (Fig. 5). However, the activity of none of these compounds changed, indicating that all tested molecules have low specificity for these enzymes.

In conclusion, we tested a small set of nucleoside derivatives and analogs for autophagy activation. (2′-Deoxy)adenosine derivatives

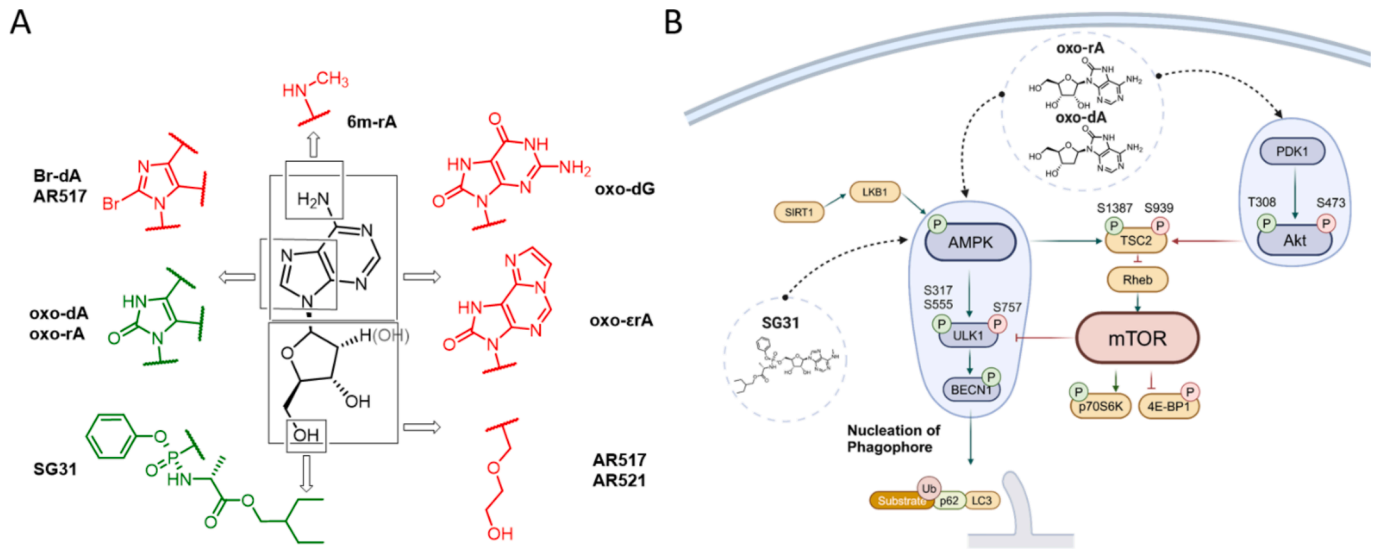


Fig. 6. Structure-activity relationship (SAR) of (2′-deoxy)adenosine derivatives and analogs (A) and possible mechanism of autophagy activation by **oxo-rA**, **oxo-dA** and **SG31** (B). (A) Modifications that increase activity are shown in green, while modifications with no or negligible effect are shown in red. (B) Ub – ubiquitination; P – phosphorylation (inhibitory phosphorylation is shown in pink, activating in light green), black arrows show the targets of the autophagy activators; activating effect of proteins in the autophagy activation cascade is shown by green arrows and suppressing effect by red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

containing 8-bromo, N^6-methyl or a combination of 8-oxo/1,N^6-etheno substituents, as well as adenine derivatives bearing an acyclic analog of a 2′-deoxyribose residue and 8-bromo or 8-oxo moiety, did not show any activity (Fig. 6A). Activity was observed for the nucleoside forms of natural DNA and RNA lesions **oxo-dA** and **oxo-rA**, while **oxo-dG** showed no activity. Surprisingly, introduction of a metabolizable phosphate-masking group into the almost inactive derivative **m6-rA** resulted in the highest activity in the series. Thus, the phosphate-masking strategy can be used in the further search for autophagy activators among nucleoside derivatives and their analogs.

The autophagy activators **oxo-rA**, **oxo-dA**, and **SG31** exhibited a degree of heterogeneity in their mechanism of action, although all of them primarily contributed to the activation of AMPK (Fig. 6B), as was demonstrated by the accumulation of the phosphorylated form of AMPK, in addition to an increased phosphorylation of the AMPK target ULK1. However, it is important to note that these compounds also act through alternative pathways, since all compounds retain their activity on *ΔAMPK* and *ΔSIRT1* cell lines.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2024.129980.

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