AI-Predicted mTOR Inhibitor Reduces Cancer Cell Proliferation and Extends the Lifespan of C. elegans

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Article

AI-Predicted mTOR Inhibitor Reduces Cancer Cell Proliferation and Extends the Lifespan of *C. elegans*

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Abstract: The mechanistic target of rapamycin (mTOR) kinase is one of the top drug targets for promoting health and lifespan extension. Besides rapamycin, only a few other mTOR inhibitors have been developed and shown their ability to slow aging. We used machine learning to predict novel small molecules targeting mTOR. We selected one small molecule, TKA001, based on in-silico predictions of a high on-target probability, low toxicity, favorable physicochemical properties, and preferable ADMET profile. We confirmed TKA001 binding in silico by molecular docking. TKA001 potently inhibits both TOR complex 1 and 2 downstream signaling in vitro. Furthermore, TKA001 inhibits human cancer cell proliferation *in vitro* and extended the lifespan of *C. elegans*, suggesting that TKA001 can slow aging in vivo.

Keywords: AI drug discovery; mTOR, rapalog; *C. elegans*; cancer; longevity

Introduction

One of the most robust interventions to increase healthspan and lifespan in preclinical models is the inhibition of the growth-regulating serine/threonine kinase **m**echanistic **T**arget **o**f **R**apamycin (mTOR). This effect has been demonstrated using genetics in multiple species, including flies, worms, and mice. In *Drosophila,* suppression of TOR signaling by overexpression of the negative regulators TSC1 or TSC2, or knock-in of dominant negative forms of TOR or S6K significantly extended lifespan [1]. In *C. elegans*, rapamycin or RNAi-mediated knockdown in adulthood of the conserved TOR pathway components *daf-15* (RAPTOR), *rheb-1*, *raga-1,* or *ragc-1* all robustly extended lifespan [2–6]. In mice, while complete knockout of most mTOR components is embryonically lethal, knock-in of a hypomorphic mTOR allele extends lifespan by ~25% [7].

Pharmacologic inhibition of mTOR has also been proven to be effective in extending lifespan in mice. The mTOR inhibitor rapamycin has repeatedly shown lifespan-extending effects across sex, strain, and dosing regimens. Effective regimens include dosing continuously throughout life, intermittently throughout life, transiently early in life, transiently in midlife, and continuously starting late in life [8–12]. These striking preclinical effects are mediated both directly through suppression of tumorigenesis and indirectly via modulation of lifespan-regulating processes, including insulin and ATF4 signaling pathways [4,13,14].

Rapamycin and structurally related "rapalogs" have also shown promise in human trials against age-associated pathologies. In randomized clinical trials, pharmacological inhibition of mTOR ameliorated the age-related decline of the immune system and reduced skin senescence in elderly humans [15,16].

Despite the clear preclinical promise of rapamycin and related rapalogs, challenges of drug discovery and development have been well-described, with cost estimates of over $100 million over 10 years or more [17]. However, the use of artificial intelligence (AI) assisted methods for early drug discovery can fundamentally transform this process, cutting off years and tens of millions of dollars.

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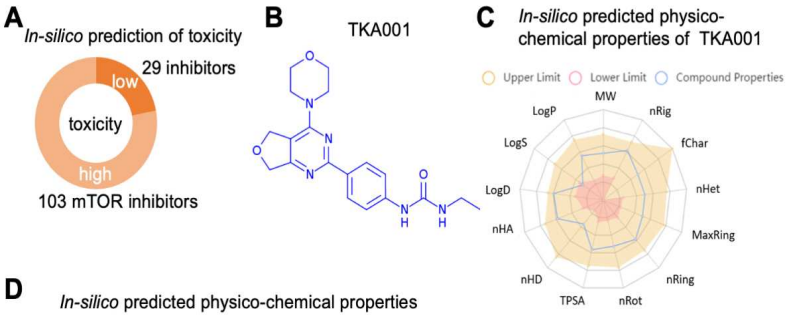
We employed an AI method to identify a potent and selective mTOR inhibitor and validated this compound's anti-cancer and pro-longevity effects in cell culture and *C. elegans* models.

Results

To identify and generate compounds that inhibit mTOR, we used generative adversarial networks and reinforcement learning methods. We generated more than 1000 small molecules predicted to target mTOR. We performed an independent validation using PASS software [18] to select which of the generated molecules have indeed a high probability of inhibiting mTOR. We narrowed down the candidate list to 132 compounds with a high probability of targeting mTOR (Figure 1A). Since PASS software can be used to predict toxic and adverse effects, we filtered these candidate compounds for their likelihood of low toxicity, of which 29 compounds remained (i.e., 22% of all 132 compounds; Figure 1A). Next, we assessed *in-silico* these 29 compounds with preferable ADMET profiles (absorption, distribution, metabolism, excretion, toxicity) and found one strong candidate 1-ethyl-3-(4-(4-morpholino-5,7-dihydrofuro[3,4-d]pyrimidin-2-yl)phenyl)urea, which we named TKA001 (Figure 1B-D).



D *In-silico* predicted physico-chemical properties

Property	Value	Comment
Molecular Weight	369.18	Contain hydrogen atoms. Optimal:100~600
Volume	365.854	Van der Waals volume
Density	1.009	Density = MW / Volume
nHA	8	Number of hydrogen bond acceptors. Optimal:0~12
nHD	2	Number of hydrogen bond donors. Optimal:0~7
nRot	6	Number of rotatable bonds. Optimal:0~11
nRing	4	Number of rings. Optimal:0~6
MaxRing	9	Number of atoms in the biggest ring. Optimal:0~18
nHet	8	Number of heteroatoms. Optimal:1~15
fChar	0	Formal charge. Optimal:-4 ~4
nRig	23	Number of rigid bonds. Optimal:0~30
Flexibility	0.261	Flexibility = nRot /nRig
Stereo Centers	0	Optimal: < 2
TPSA	88.61	Topological Polar Surface Area. Optimal:0~140
logS	-4.606	Log of the aqueous solubility. Optimal: -4~0.5 log mol/L
logP	1.817	Log of the octanol/water partition coefficient. Optimal: 0~3
logD	2.583	logP at physiological pH 7.4. Optimal: 1~3

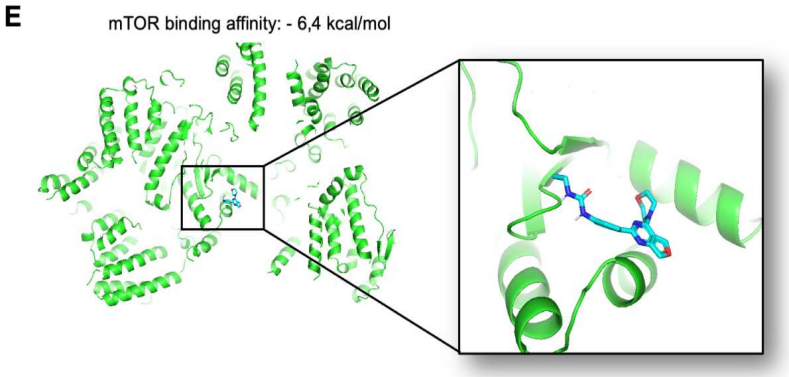


Figure 1. Characterization of mTOR inhibitor TKA001. (**A**) *In-silico* prediction of toxicity of mTOR inhibitor candidate compounds. (**B**) Lead candidate TKA001 ($C_{19}H_{23}N_5O_3$). (**C**,**D**) *In-silico* predicted physiochemical properties of TKA001. (**E**) Molecular docking of TKA001 and mTOR kinase.

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To validate TKA001, we first performed molecular docking of TKA001 to mTOR kinase and found a binding affinity of TKA001 to mTOR of -6.4 kcal/mol (Figure 1E). Next, we assessed mTOR downstream signaling. The mTOR kinase is found in two complexes (mTORC1 and mTORC2; [19]). The mTOR complex 1 (mTORC1) phosphorylates ribosomal protein S6 kinase (S6K; [20]), whereas the mTOR complex 2 (mTORC2) phosphorylates AKT [20]. At 1 µM of TKA001, phosphorylation of S6K and AKT were reduced in HT1080 cells (Figure 2A), suggesting that TKA001 inhibits mTOR kinase in both complexes. To stimulate mTOR signaling, we UV-B irradiated HT1080 cells and still found a strong reduction of S6K and AKT phosphorylation, suggesting that TKA001 efficiently reduces mTOR signaling (Figure 2A).

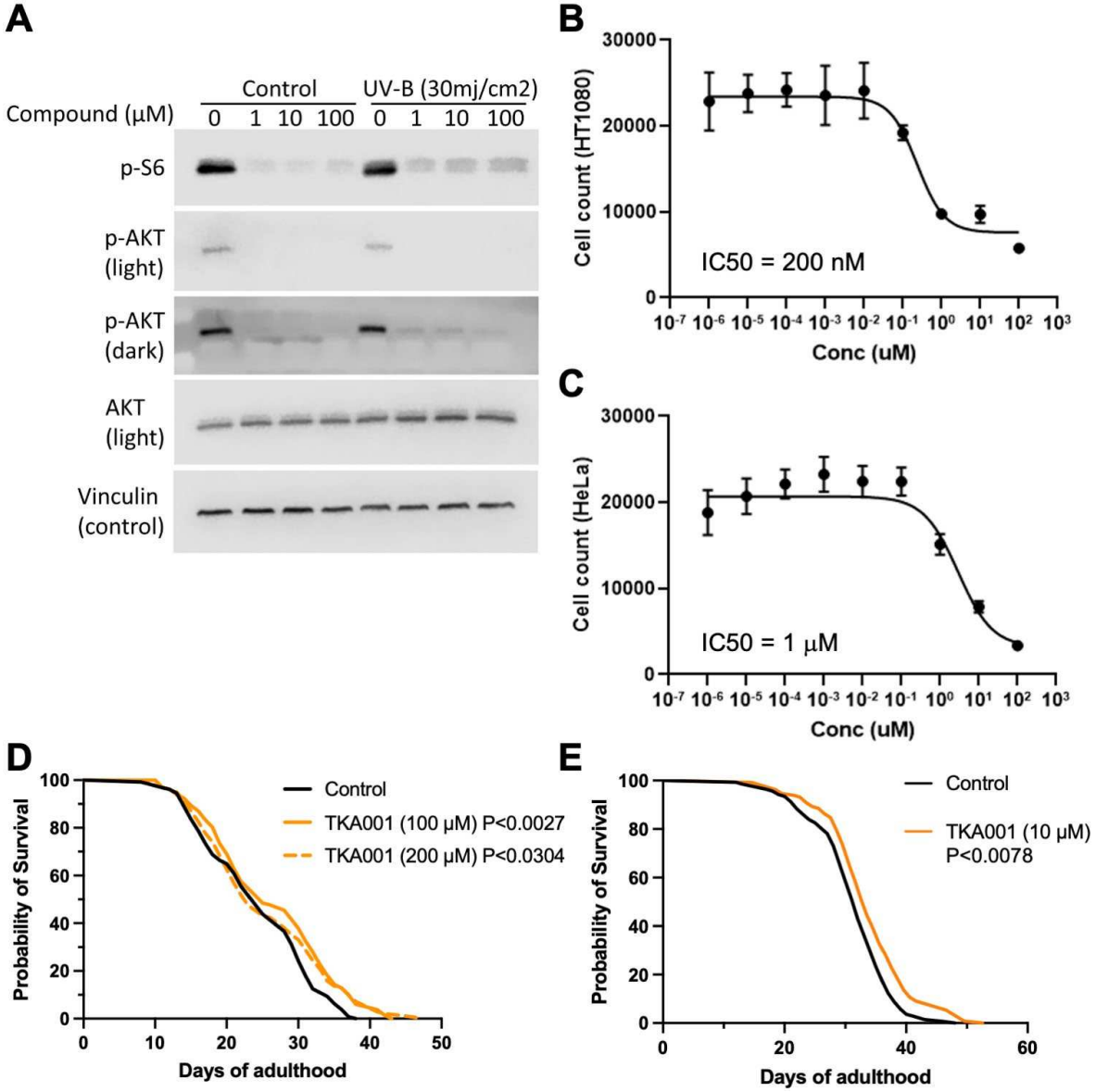

Figure 2. *In-vitro* and *in-vivo* validation of mTOR inhibitor TKA001. (**A**) Western blot of HT1080 cells pre-treated with increasing concentrations of TKA001 with or without UV-B irradiation. Phospho-S6 (S240/244), phospho-AKT (S473), total AKT, and vinculin loading control. (**B**) Proliferation assays of TKA001 treatment on HT-1080 cells. Half maximal inhibitory concentration (IC50) = 200 nM. (**C**) Proliferation assays of TKA001 treatment on HeLa cells. IC50 = 1 µM. (**D**,**E**) Feeding 10, 100, or 200 µM TKA001 increased the lifespan of *C. elegans*. Two independent biological trials. Control = 0.2% DMSO. *p*-value determined with Log Rank.

Our in-silico analysis of TKA001 using CLC-Pred [21] predicted an 85% likelihood as an effective treatment against prostate cancer. We found that TKA001 inhibits cancer cell proliferation of epithelial cancer cells from patients with a fibrosarcoma (HT-1080; half maximal inhibitory

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concentration (IC_{50}) = 200 nM; Figure 2B) and cervical cancer cells (HeLa; IC_{50} = 1 μM; Figure 2C). In comparison, IC_{50} of rapamycin on HT-1080 or HeLa is 1.8 μM and 0.25 μM, respectively [22]. This suggests that TKA001 is a potent inhibitor of cancer cell proliferation in vitro.

Since TKA001 performed well in silico and in vitro, we next wanted to assess the in-vivo efficacy of TKA001 on mTOR inhibition. Genetic inhibition of mTORC1 or mTORC2, knockdown of mTOR/LET-363, or rapamycin treatment increases the lifespan of *C. elegans* [2–6,23–25]. To determine whether TKA001 could also increase the lifespan of *C. elegans*, we used 100 μM and 200 μM of TKA001 because about 100 μM of rapamycin results in the most robust lifespan extension [2,4,6]. We found that adulthood-specific application of 200 μM of TKA001 only extended the maximal lifespan, whereas 100 μM of TKA001 resulted in both mean and maximum increase of lifespan (Figure 1H). Next, we assessed whether a lower dose would be sufficient to increase lifespan. Indeed, supplementing *C. elegans* starting from the young adult stage with 10 μM of TKA001 was sufficient to extend the lifespan.

Discussion

The nutrient-sensing mTOR kinase is a master growth regulator essential for development and tissue homeostasis [26]. However, mTOR activity becomes deregulated during aging, showing improper and sustained mTOR signaling in older animals [27,28]. Reducing the function of mTOR increases the lifespans from yeast to mammals [29]. Despite mTOR being the prime target against many age-related and chronic pathologies [30], relatively few mTOR inhibitors have been developed to slow the aging process.

Using deep neuronal artificial learning, we identified many potential mTOR inhibitors. Through in-silico analysis, we selected one mTOR inhibitor with predicted low toxicity and a preferable ADMET profile. We validated on-target by molecular docking to the mTOR kinase, confirming the inhibition of mTOR downstream signaling and cancer cell proliferation in vitro, and increasing lifespan in vivo.

In a series of rational design and classical medicinal chemistry approaches, structurally similar mTOR inhibitors were previously developed based on a quaternary-substituted dihydrofuropyrimidine [31]. The structurally most similar compounds, inhibited mTOR kinase signaling in vitro at 3–4.4 nM [31] and had an IC_{50} of 31-1700 nM in cancer cell proliferation assays (NCI-PC3, MCF7neo/Her2) [31] compared to our IC_{50} of 200 nM or 1000 nM in HT-1080 or HeLa, respectively. Given these comparable in-vitro results, reassuringly validates our machine-learning approach to identifying novel small molecules for mTOR inhibition. More importantly, we showed that our TKA001 mTOR inhibitor is able to slow aging in vivo. Further machine-learning approaches hold the potential to speed up drug discovery and facilitate the selection of compounds for future clinical candidates targeting the mTOR-mediated healthy longevity benefits.

Materials and Methods

Reagents

TKA001 is 1-ethyl-3-(4-(4-morpholino-5,7-dihydrofuro[3,4-d]pyrimidin-2-yl)phenyl)urea ($C_{19}H_{23}N_5O_3$). Molecular weight: 369.43, Melting point 213°C. TKA001 was synthesized by Otava Chemicals (ZINC71297044 (catalog number 27705871)).

Dimethyl sulphoxide (DMSO) CAS# 67-68-5 BDH Chemicals (VWR) 500 ml, analytical reagent.

In-silico prediction of the mechanism of action and toxicity of candidate compounds

The online web tool PASS [18] was used to predict the pharmacological activities of compounds, as well as their toxicity. PASS indicates the probable activity (Pa) and probable inactivity (Pi) of 'drug-like' substances. Using PASS, it is possible to obtain an estimated biological activity profile of a drug-like molecule using only structural formulas. Some of the predicted activities of PASS software are pharmacological effects, mechanism of action, as well as toxic and adverse effects.

In-silico predicted physicochemical properties of TKA001

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ADMETlab 2.0 [32] was used to predict the physicochemical properties, drug-likeness, and toxicity of TKA001.

Molecular docking

The SMILES string of TKA001 was converted using the Open Babel tool into the PDBQT format [33]. Docking simulations were performed using PyRx [34]. The selected compound was subjected to docking with mTOR kinase, PDB: 4JSV [35–37]. A molecular docking simulation of protein and ligand was performed using PyRx to predict their preferential binding affinity in terms of binding energy (ΔG). Adequate spacing between the grids was ensured so that the ligands could move freely inside. One of the best conformational poses of ligand showing the least ΔG was selected for further analysis using PyMol (Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Human cell lines

Cell lines were maintained at 37°C in a 5% CO2 incubator. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Hyclone Cosmic Calf Serum (Cytiva) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were routinely passaged at 70% confluence. The following cell lines were used: HT1080: ATCC CCL-121, HeLa: ATCC CRM-CCL-2.

Human cell proliferation

For proliferation assays, cells were seeded in 96-well plates at 2,000 cells per well and treated with vehicle (0.1% DMSO) or TKA001. After 24 hours, cells were stained with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) at 10 μg/mL, followed by imaging using a Cytation 5 Cell Imaging Multimode Reader (BioTek, Winooski, VT, USA). Cell count was performed by automated nuclei counting using Gen5 Data Analysis Software (BioTek, Winooski, VT, USA).

Western blots

For western blots, cells were grown to 70% confluence in 6-well plates. Cells were then treated with vehicle (0.1% DMSO) or TKA001 for 2 hours, followed by UV-B irradiation (30 mj/cm2) or control (no irradiation). Thirty minutes after irradiation, cells were placed on ice, washed with cold phosphate-buffered saline (PBS) then harvested in 60 μL of cold RIPA buffer containing protease/phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated on ice for 20 minutes, then centrifuged at 20000× *g* for 10 minutes, and supernatants were collected. Supernatants were standardized to a concentration of 2 mg/mL and combined with 6x loading buffer containing SDS, glycerol, beta-mercaptoethanol, and bromophenol blue. Samples were boiled at 100°C for 10 minutes, and 15 μL was loaded on a Tris/glycine SDS-polyacrylamide gel. Proteins were separated by SDS-PAGE, transferred to a polyvinylidene diflouride (PVDF) membrane, and immunoblotted with indicated antibodies. Primary antibodies used were pS6 (S240/244): CST 5364, p-AKT (S473): CST 4060, ATK: CST 4691, and Vinculin: CST 13901. Secondary antibody was HRP-linked anti-rabbit IgG (CST 7074). Immunoblots were imaged using an enhanced chemiluminescent detection kit (ECL, Bio-Rad) and visualized on a LiCor Odyssey Fx Imaging System.

C. elegans lifespan

TJ1060 [*spe-9(hc88)*; *rrf-3(b26)*] *C. elegans* were grown until the gravid adult stage and synchronized by bleaching [38]. Hatched overnight cultures of L1 *C. elegans* were seeded onto plates covered with heat-inactivated OP50 bacteria and left at +25°C incubator for 2 days to induce temperature-sensitive *spe-9(hc88)* mutation to achieve sterility of eggs. Then young adult animals were transferred manually by picking 70-75 individuals onto two 6 cm plates (n ≥ 140 animals per condition) with corresponding TKA001 concentrations in both agar and heat-inactivated OP50. Further lifespan assay was performed at 20°C until death [39]. The TKA001 was dissolved in DMSO, resulting in a 0.2% DMSO final concentration in the NGM plates, and, thus, 0.2% DMSO was used as empty vehicle control. All the plates were normalized by 0.2% DMSO to exclude differences in solvent concentration. Death events were counted every second day and starting from adulthood day 12 (AD12) – on a daily basis. Worms with a vulval protrusion, matricide events, dried on the walls, and abnormally looking were censored. Kaplan-Meier estimator was used for analysis in GraphPad Prism 8 ® software. Log-Rank (Mantel-Cox) test was utilized for statistical analysis (n ≥ 100). Figure

2D: control = 128 death events, TKA001 (100 μM) = 132 death events [+ 8.9% mean lifespan increase], TKA001 (200 μM) = 132 death events [+ 4.7% mean lifespan increase]. Figure 2E: control = 137 death events, TKA001 (10 μM) = 131 death events [+ 6.3% mean lifespan increase].

Author Contributions: All authors participated in analyzing and interpreting the data. T.V. and M.R.M. performed machine learning. T.V. performed molecular dockings. M.R.M. and C.Y.E. designed the experiments. A.D. performed lifespan assays. M.R.M. performed western blots and cell survival assays. C.Y.E. and M.R.M. wrote the manuscript in consultation with the other authors. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. TV, MRM, and CYE are co-founders of Tinka Therapeutics. CYE is a co-founder and shareholder of Avea Life AG, and is on the Scientific Advisory Board of Maximon AG, Biotein, Longaevus Technologies LTD, and Galyan Bio, INC.

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