A769662, a novel activator of AMP-activated protein kinase, inhibits non-proteolytic components of the 26S proteasome by an AMPK-independent mechanism

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ABSTRACT

In this work we present evidence that A769662, a novel activator of AMP-activated protein kinase (AMPK), is able to inhibit the function of the 26S proteasome by an AMPK-independent mechanism. Contrary to the mechanism of action of most proteasome inhibitors, A769662 does not affect the proteolytic activities of the 20S core subunit, defining in this way a novel mechanism of inhibition of 26S proteasome activity. Inhibition of proteasome activity by A769662 is reversible and leads to an arrest of cell cycle progression. These side effects of this new activator of AMPK should be taken into account when this compound is used as an alternative activator of the kinase.

INTRODUCTION

Proteasomes are one of the major pathways of intracellular protein degradation in mammalian cells ([1], [2], [3], [4]). Although proteasomes can degrade proteins by ubiquitin-independent processes, they are mostly involved in the ATP- and ubiquitin-dependent pathway of protein degradation [5]. The 26S proteasome itself is also an ATP-dependent complex. This complex consists of the 20S catalytic core, where the proteins are degraded, plus one or two 19S regulatory complexes. The 19S complex is composed of at least 19 different subunits that form a lid- and a base-like structure; the lid provides the binding sites for poly-ubiquitinated substrates and a deubiquitinating activity involved in recycling of ubiquitin moieties upon substrate degradation; the base includes six ATPases that interact with the 20S proteolytic core; these ATPases have chaperone function and are required for the unfolding of substrates and their translocation to the 20S proteolytic chamber ([6], [7], [8]). Therefore, intracellular protein degradation by the proteasome is a highly energy-demanding process and, thus, it is expected that under conditions of energy depletion this process should be tightly regulated.

AMP-activated protein kinase (AMPK) is a conserved sensor of cellular energy. AMPK is a heterotrimer composed of three different subunits, i.e. α, β and γ. AMPKα is the catalytic subunit, whereas AMPKβ and AMPKγ play regulatory roles (see [9], for review). Activity of AMPK complex is regulated by the phosphorylation of the catalytic α-subunit at Thr172 by an upstream kinase, sharing LKB1 and CaMKKβ this role (see [9], for review). The activated kinase switches on ATP-generating catabolic pathways while turning off many ATP-requiring processes. Since AMPK is a sensor of cellular energy, it seems plausible that AMPK plays a role in the regulation of proteasomal protein degradation. In fact, we have recently described that AMPK activation (produced by AICAR or metformin) results in an inhibition of proteasomal activity [10]. Since AICAR has side effects in cell metabolism that

are not related to AMPK activation, we decided to confirm our results by the use of the A769662 compound, a novel activator of AMPK ([11], [12], [13]).

In this work we describe that A769662 is an inhibitor of proteasomal function that acts by an AMPK-independent mechanism. Inhibition of proteasome activity by A769662 is reversible and leads to an arrest of cell cycle progression. To our knowledge, this is the first time that side effects of this compound are described and they should be taken into account when this compound is used as an alternative activator of AMPK.

MATERIALS AND METHODS

Materials

A769662 was kindly provided by Dr. Kei Sakamoto (MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee) and dissolved in DMSO. Lactacystin and epoxomicin were from Biomol Research Labs (Exeter, UK). AMPKα subunit double knockout mouse embryonic fibroblasts (MEFs) (α1-/- and α2-/-) and wild type controls were grown as described previously [14]. HEK-GFPu-1 cells [15] were from the American Type Culture Collection.

In vivo determination of proteasome activity.

Wild type and AMPKα subunit double knockout (α1-/- and α2-/-) MEFs (35,000 cells) were grown in complete DMEM medium (Lonza Bioscience, Barcelona). Then, they were incubated for 1h in Krebs-Henseleit (KH) medium [10], containing lactacystin (10 μM), different amounts of A769662 (from 10 to 300 μM) or the corresponding vehicle (DMSO). Finally, the chymotrypsin-like activity of the 26S proteasome was measured using the Proteasome-Glo Cell-Based Assay with N-Suc-LLVY-aminoluciferin as substrate (Promega, Madison, WI), following the manufacturer's instructions. Luminiscence was measured in a

Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA). Results were referred to the activity found in wild type MEFs cells growing in KH medium with DMSO.

HEK-GFPu-1 cells were cultured in DMEM medium and then incubated for 1h in KH medium containing lactacystin (10 μM), different amounts of A769662 (50 μM or 300 μM) or the corresponding vehicle (DMSO). Fluorescence was determined by flow cytometry using a FACScanto flow cytometer (Becton Dickinson) using an excitation laser at 488 nm and GFP fluorescence detection at 520 nm.

In vitro determination of proteasome activity

To measure the *in vitro* activity of the 26S proteasome we used a commercial 26S proteasome preparation (from human erythrocytes; Biomol Research Labs; Exeter, UK) combined with a fluorescent detection procedure (based on the degradation of FITC-casein) from Sigma (St. Louis, Mo), as described in [16]. The *in vitro* activity of the 20S proteasome was measured using the commercial 20S Proteasome Assay Kit from Biomol Research Labs (Exeter, UK) containing a purified preparation of the 20S proteasome from human erythrocytes and Suc-LLVY-AMC as substrate. The *in vitro* effect of A769662 on both proteasome preparations was analyzed following the corresponding manufacturer's instructions. Fluorescence at 535 nm (excitation at 485 nm) in the case of the 26S proteasome reaction, and at 460 nm (excitation at 360 nm) in the case of the 20S proteasome reaction, was recorded in a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA).

In-gel peptidase overlay assays with purified 26S and 20S proteasomes were carried out using native gel electrophoresis and Suc-LLVY-AMC as substrate as in [17]. Three μg of commercial purified 26S and 20S proteasomes (see above) were used in these assays. After visualization of the reaction, the gels were stained with Coomassie blue.

Immunoblot analysis.

Cell lysates were prepared as described in [10]. Sixty μg of total protein from cell lysates were analyzed by SDS-PAGE and western blotting using anti-ubiquitin, anti-S1 (19S regulatory particle) or anti-β2 (20S proteasome) antibodies (Biomol Research Labs; Exeter, UK).

Flow cytometry and microscopic analysis

Cell viability of MEF cells treated or not with A769662 was performed as follows: cells were harvested by trypsinization and incubated with 0.5 mg/ml RNase and 50 μg/ml propidium iodine at room temperature in the dark; cell viability was analyzed by flow cytometry using a FACScanto flow cytometer (Becton Dickinson), using an excitation laser at 488 nm and a propidium iodine fluorescence detection at 600 nm. To determine the proportion of cells in each phase of the cell cycle, cells were harvested by trypsinization, collected by centrifugation, washed in PBS and fixed overnight in 80% ethanol at -20ºC. Subsequently these fixed cells were centrifuged to remove the fixative and incubated for 20 min in the dark at room temperature in PBS containing 0.5 mg/ml RNase and 50 μg/ml propidium iodine. Flow cytometry analysis was performed as above. The proportion of cells in G1, S, and G2 was determined using the MODFIT program.

Cell culture pictures were taken at the indicated times using a camera (Olympus Camedia C-5060 wide zoom) coupled to an inverted microscope (Zeiss Axiovert 40C) with a 20X objective.

Statistical data analysis.

Data are expressed as mean values ± standard error (SE). Statistical significance of differences between the groups was evaluated by a paired Student's t test with two-tailed distribution. The significance has been considered at ** $p<0.01$ or *** $p<0.001$, as indicated.

RESULTS AND DISCUSSION

1.- A769662 inhibits proteasomal activity by an AMPK-independent mechanism.

We have recently described that activation of AMPK, either by AICAR or by metformin, affects negatively proteasomal function [10]. To confirm these results we have used a novel activator of AMPK named A769662 (see Introduction). In agreement with previous results, treatment of wild type mouse embryonic fibroblast (MEF) cells with A769662 resulted in an increase in the phosphorylation of the AMPKα catalytic subunit at Thr172 residue (data not shown). This new compound was also able to inhibit *in vivo* the proteasomal function in MEF cells, with an IC50 of 62 μM (Fig. 1A). However, the compound was also able to inhibit *in vivo* the proteasomal function in AMPKα subunit double knock-out (α1-/-, α2-/-) MEFs, with similar IC50 (47 μM; no statistically significant differences were found respect to wild type MEFs), indicating that A769662 was able to inhibit proteasomal function in an AMPK-independent manner (Fig. 1A). A769662 was not as potent as other already known proteasomal inhibitors such as lactacystin, which has an IC50 in the nanomolar range [18]; in fact, 10 μM of lactacystin was able to inhibit almost completely proteasomal function (Fig. 1).

We then confirmed that A769662 was an inhibitor of proteasomal function by alternative methods. As it is shown in Fig. 1B, MEF cells treated with 300 μM A769662 for 1h were able to accumulate poly-ubiquitinated proteins, an indication of proteasomal impairment, as in the case of cells treated with the proteasomal inhibitor lactacystin (10 μM)

(similar results were obtained in double α1α2 -/- KO MEF cells; not shown). In addition, we used an alternative assay based on the degradation of a fusion protein of GFP with a short degron (CL1) fused to the C-terminus (GFPu) [15]. This fusion protein is unstable and is rapidly degraded by the 26S proteasome under regular conditions. If the function of the proteasome is impaired, then the GFPu protein is accumulated, what leads to an increase in the amount of GFP-dependent fluorescence. As shown in Fig. 1C, treatment of HEK-GFPu-1 cells with lactacystin (10 μM) resulted in an increase in the GFP-dependent fluorescence of the cells, assessed by flow cytometry. When the cells were treated with A769662, we also observed an increase in GFP-dependent fluorescence that was dose-dependent (Fig. 1C).

We also studied the possibility that A769662 could affect indirectly proteasome function, i.e. by preventing transcription or translation of proteasomal genes. However, we ruled out this possibility when we observed similar amounts of two characteristic endogenous subunits of the proteasome, such as the S1 subunit from the 19S regulatory particle and the β2 subunit from the 20S proteasome catalytic core, in extracts from cells treated or not with 300 μM A769662 for different periods of time (Fig. 1D).

All these results indicated that A769662 was an inhibitor of the proteasomal function and that the mechanism of its inhibition was AMPK-independent.

2.- Inhibition of proteasomal activity by A769662 is reversible.

In all our experiments we grew the cells in complete medium (DMEM) and then replaced this medium by a poor medium (KH) to increase intracellular protein degradation. In order to determine whether A769662 inhibited the activation of the proteasomal function upon shift from the complete to the KH medium or whether this compound was able to inhibit a pre-existing proteasomal activity, we subjected MEF cells to a shift from complete to KH medium for 1h and then, treated the cells with A769662 or with vehicle alone (DMSO). We

observed that A769662 was able to reduce the activity of pre-existing proteasomal activity (Fig. 2A), suggesting a direct effect of the compound on the proteasome. Additionally, we checked whether the inhibition was reversible. With this aim, we treated MEF cells with A769662 for 1h and then, we removed the culture medium and replaced it with fresh KH medium for an extra hour. As observed in Fig. 2B, a clear increase in the activity of the proteasome was observed upon removal of A769662, indicating a reversible mechanism of inhibition.

3.- A769662 affects *in vitro* the activity of purified 26S but not the activity of 20S proteasomes.

The 26S proteasome is a high molecular weight proteolytic complex consisting of the 20S core, where proteins are degraded, plus one or two 19S regulatory particles (RP), composed of six ATPases and other components necessary for binding, unfolding and translocation of the protein substrates into the 20S core for hydrolysis (see Introduction). In order to determine whether A769662 was able to inhibit *in vitro* the activity of either the 26S or the 20S proteasomes, we used commercial preparations of both complexes. As shown in Fig. 3A, the activity of the 26S preparation was inhibited by epoxomicin (0.5 μM), a known proteasomal inhibitor [18]. Similarly, A769662 was also able to inhibit the activity of the 26S proteasomes, [values were similar to those found in the sample without 26S proteasomes (blank), even at low concentrations of the compound (10 μM)], confirming the *in vivo* inhibition of proteasomal function described above. However, we were not able to detect any inhibition of the activity of the 20S proteasome preparation when we used A769662, at any of the tested concentrations (from 5 to 100 μM) (Fig. 3B). This result could indicate that A769662 does not affect the proteolytic activity present in the 20S proteasome core. Additionally, we observed that treatment of the 26S proteasomes with A769662 did not

disrupt the association of the 19S regulatory particle with the 20S proteasomes (Fig. 3C). Therefore, we suggest that A769662 inhibits 26S proteasomes by affecting the function of the 19S regulatory particle, preventing in this way the entry of the poly-ubiquitinated substrates into the 20S proteolytic chamber.

4.- A769662 has toxic effects on MEF cells.

It has been described that inhibition of the proteasome function results in cell cycle arrest and apoptosis, probably because of the accumulation of pro-apoptotic proteins and the downregulation of anti-apoptotic factors ([8], [19]). In order to determine whether A769662 promoted similar effects, we treated MEF cells with A769662 and determined its effects on cell cycle and cell viability. Microscopic analysis of cell cultures indicated that after 12 h of treatment, there was an increase in the amount of round cells that were not attached to the culture plate surface. The amount of unattached cells increased dramatically after 24 h of treatment (Fig. 4A). Similar results were obtained in double α1α2 -/- KO MEF cells (Fig. 4A) indicating that the effect was not due to AMPK activation. Cells were assessed for viability by flow cytometry and the results indicated a huge increase in the amount of non-viable cells after 24 hours of treatment (24.0% of non-viable cells in the samples treated with A769662 versus 6.3% in cells treated with vehicle alone; Fig. 4B). Similar results were obtained in double α1α2 -/- KO MEF cells (not shown). These results are in contrast to those previously reported that indicated that A769662 showed no measurable cytotoxicity in rat hepatocytes [11]. Probably the different cell type may account for this difference, since several studies have demonstrated that proliferating cell lines are usually more sensitive to proteasome inhibitors than non proliferating ones ([20], [21]). Flow cytometry analysis of MEF cells indicated that whereas in samples treated with vehicle alone (DMSO) the proportion of cells in G1 increased after 24 h (with the corresponding decrease of cells in G2), samples treated

with A769662 did not show any significant variation in the amount of cells in G1 and G2 after 24 h. These results are consistent with a cell cycle arrest caused by the inhibition of the proteasome, as the proteasome is essential for the progression through many steps in the cell cycle ([18], [22]).

In conclusion, we have demonstrated that A769662 is an inhibitor of proteasomal function that acts by an AMPK-independent mechanism. We have also observed toxic effects of this compound on MEF cells, probably as a consequence of proteasome inhibition. To our knowledge, this is the first time that side effects of this compound are described and they should be taken into account when this compound is used as an alternative activator of AMPK. On the other hand, inhibition of proteasomal activity by A769662 does not involve inhibition of the proteolytic activity of the 20S proteasome, in contrast to most proteasomal inhibitors. Thus, the mechanism of action of A769662 opens a new way in the development of novel inhibitors of proteasomal activity in the cells.

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FIGURE LEGENDS

Fig. 1: A769662 inhibits proteasomal function by an AMPK-independent mechanism. A) Wild type (WT) and AMPKα subunit double knockout (α1-/- and α2-/-; KO) MEFs were cultivated in complete DMEM medium. Then, they were incubated for 1h in KH medium containing lactacystin (10 μM), different amounts of A769662 (from 10 to 300 μM) or the corresponding vehicle (DMSO; 0 μM). The *in vivo* activity of the proteasome was determined in these cells as described in Materials and Methods. Results were referred to the activity found in wild type MEFs cells growing in KH medium with DMSO. B) Sixty μg of total protein from cell lysates from treated MEF cells as above were analyzed by SDS-PAGE and western blotting using anti-ubiquitin antibodies; Lact: lactacystin (10 μM); A769662 (300 μM). C) HEK-GFPu-1 cells were cultivated in DMEM medium and then incubated for 1h in KH medium containing lactacystin (Lact; 10 μM), A769662 (A; 50 μM or 300 μM) or the corresponding vehicle (DMSO). Fluorescence was determined as described in Materials and Methods and referred to the fluorescence found in KH medium with DMSO. D) Sixty μg of total protein from cell lysates from MEF cells treated as above with A769662 (300 μM) or with vehicle (DMSO) for different times, as indicated, were analyzed by SDS-PAGE and western blotting using anti-S1 (19S regulatory particle) or anti-β2 (20S proteasomes) antibodies. Bars indicate standard error (SE).

Fig. 2: Inhibition of proteasomal activity by A769662 is reversible**.** A) Wild type MEF cells were grown in complete DMEM medium and then incubated for 1h in KH medium. Then the cells were treated with 50 μM A769662 or with DMSO in KH for an extra hour. The *in vivo* proteasomal activity was measured as described in Materials and Methods. B) Wild type MEF cells were grown in complete DMEM medium and then incubated for 1h in KH medium containing or not 50 μM A769662; then, the media were removed, replaced by fresh KH

medium and the cells were incubated for an extra hour. The *in vivo* proteasomal activity was measured as above. In A) and B), results are referred to the activity measured in the cells incubated for 1h in KH medium without A769662; bars indicate standard error (SE) and asterisks indicate differences which were found to be statistically significant at $p<0.01$ (n: 4).

Fig. 3: A769662 affects the *in vitro* activity of purified 26S proteasomes but not the *in vitro* activity of purified 20S proteasomes. Commercial purified preparations of the 26S proteasome (A) and the 20S proteasomes (B) were incubated or not with 0.5 μM epoxomicin (Epox) or the indicated concentrations of A769662. The activity of the mixtures was assayed as described in Materials and Methods. Blank: sample containing no 26S proteasome; in B), the activity was referred to the proteasome activity found in the control. Bars indicate standard error (SE) and asterisks indicate differences which were found to be statistically significant at $p<0.001$ (n: 4). C) In-gel peptidase overlay assays of proteasomes were carried out using Suc-LLVY-AMC as substrate (see Materials and Methods). Three μg of commercial purified 26S proteasomes were treated with 100 μM A769662 or vehicle (DMSO) for 30 min at 37ºC. Three μg of commercial purified 20S proteasomes were included as control (left panel). After visualization of the reaction, the gel was stained with ~~e~~Coomassie blue (right panel).

Fig. 4: A769662 has toxic effects on MEF cells. A) Cultured MEF cells from wild type and double α1-/- α2-/- KO mice were incubated at the indicated times in KH medium containing 300 μM A769662 or the corresponding vehicle (DMSO). B) Cell viability of wild type MEF cells was determined after 24 h of incubation in KH medium containing DMSO or 300 μM A769662, as described in Materials and Methods. C) The percentage of wild type MEF cells in each phase of the cell cycle in cultures incubated in the presence of DMSO (white bars) or

in the presence of 300 μM A769662 (grey bars), was determined at the indicated times, as described in Materials and Methods. Error bars indicate standard error (SE).

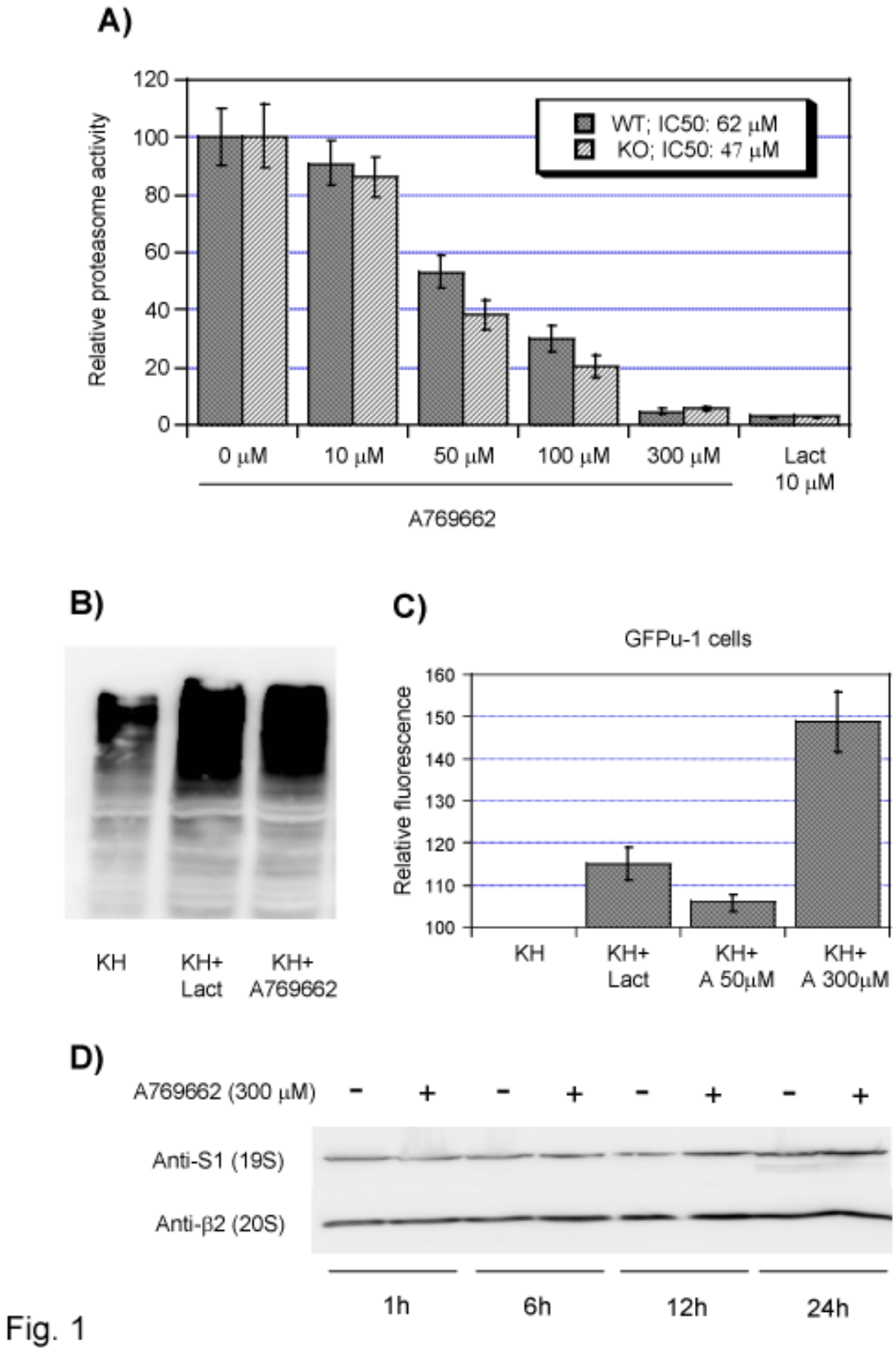

A)
Relative proteasome activity
120
100
80
60
40
20
0
WT; IC50: 62 μM
KO; IC50: 47 μM
0 μM
10 μM
50 μM
100 μM
300 μM
Lact
10 μM
A769662
B)
KH
KH+
Lact
KH+
A769662
C)
GFPu-1 cells
Relative fluorescence
160
150
140
130
120
110
100
KH
KH+
Lact
KH+
A 50μM
KH+
A 300μM
D)
A769662 (300 μM)
- + - + - + - +
Anti-S1 (19S)
Anti-β2 (20S)
1h
6h
12h
24h

Fig. 1

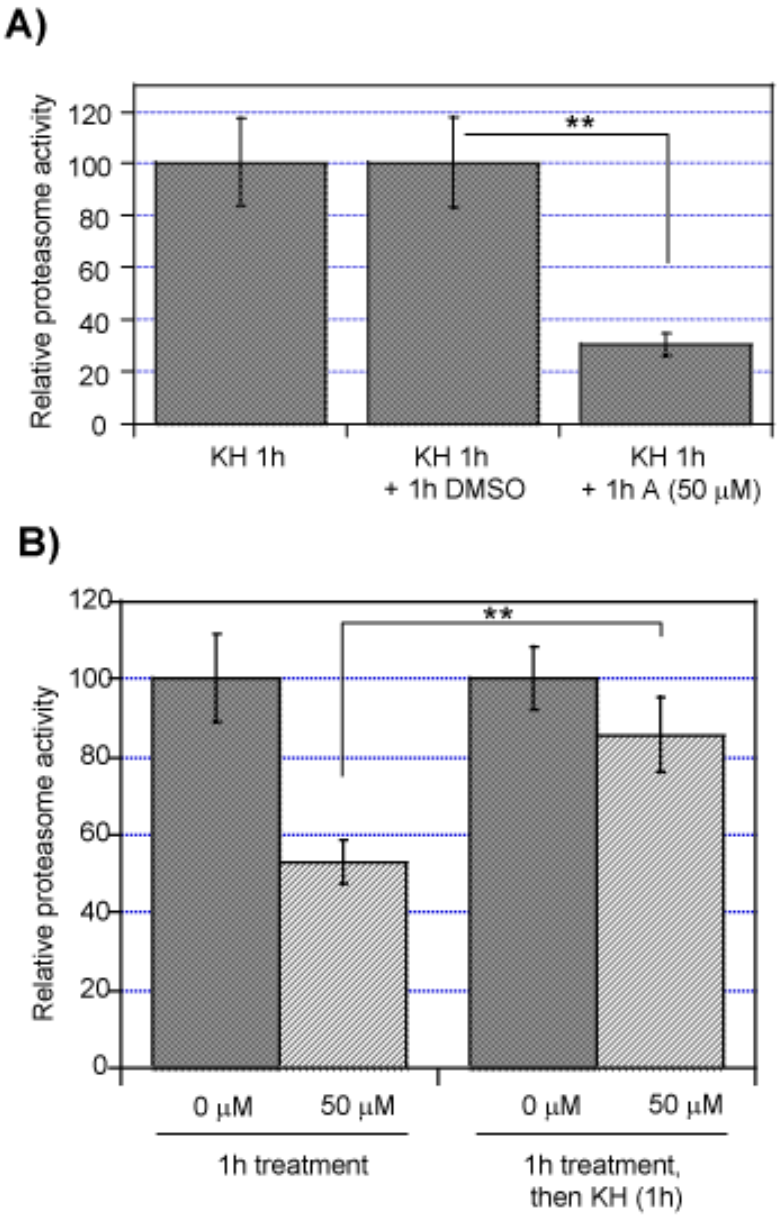

A)
Relative proteasome activity
120
100
80
60
40
20
0
**
KH 1h
KH 1h
+ 1h DMSO
KH 1h
+ 1h A (50 μM)
B)
Relative proteasome activity
120
100
80
60
40
20
0
**
0 μM
50 μM
0 μM
50 μM
1h treatment
1h treatment,
then KH (1h)

Fig. 2

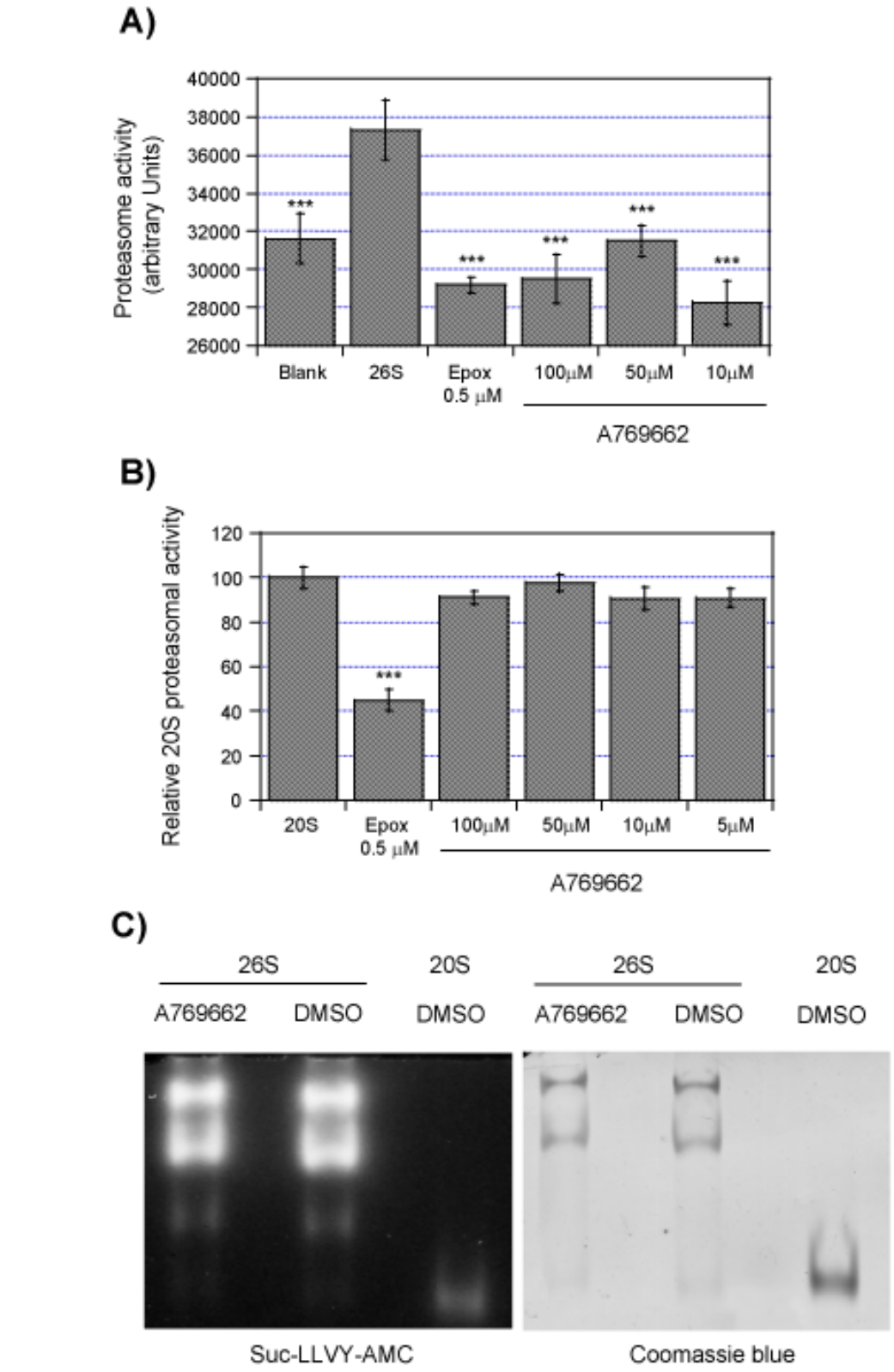
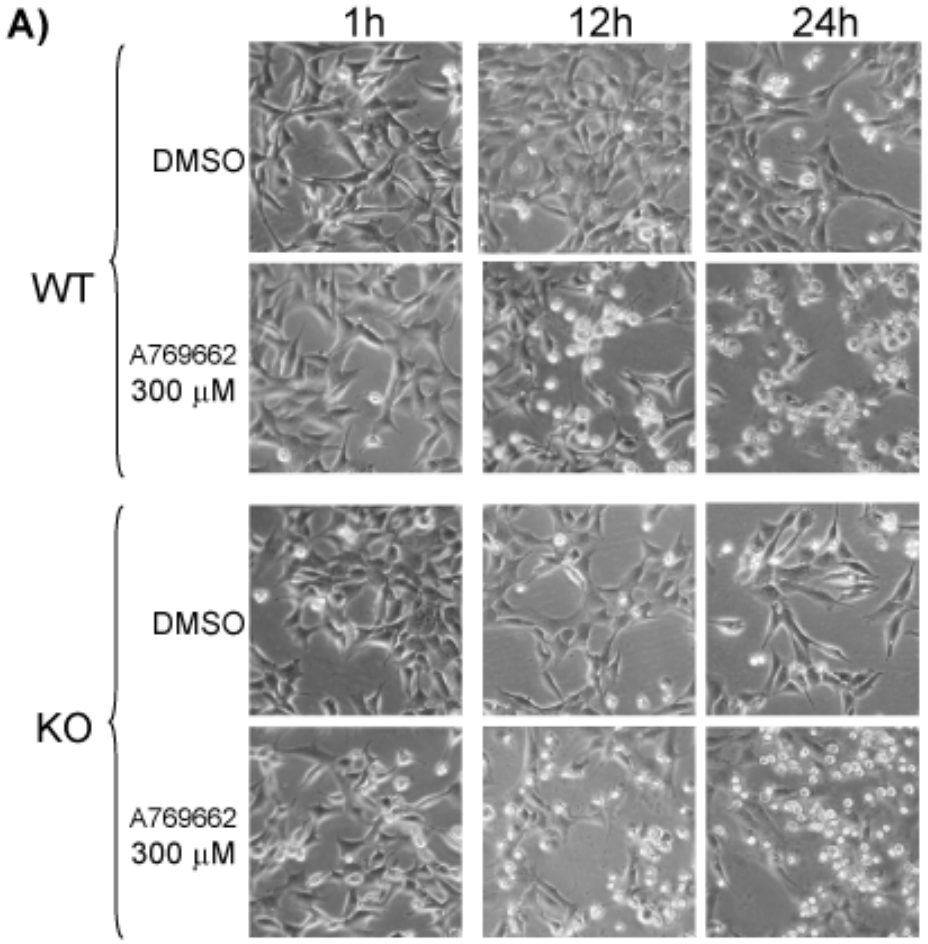
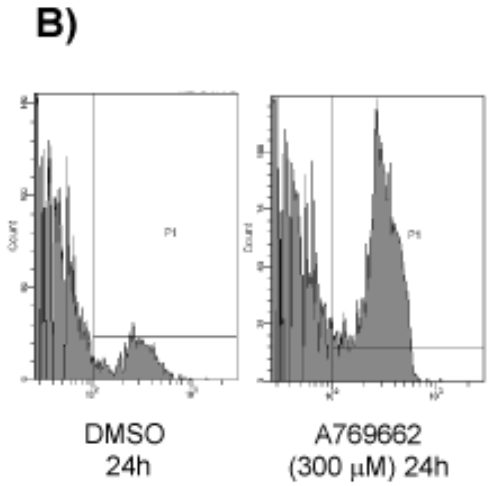

A)
Proteasome activity (arbitrary Units)
40000
38000
36000
34000
32000
30000
28000
26000
***
Blank
26S
Epox 0.5 μM
100μM
50μM
10μM
A769662
B)
Relative 20S proteasomal activity
120
100
80
60
40
20
0
***
20S
Epox 0.5 μM
100μM
50μM
10μM
5μM
A769662
C)
26S
20S
26S
20S
A769662
DMSO
DMSO
A769662
DMSO
DMSO
Suc-LLVY-AMC
Coomassie blue

Fig. 3


A)
1h
12h
24h
DMSO
WT
A769662
300 μM
DMSO
KO
A769662
300 μM


B)
DMSO
24h
A769662
(300 μM) 24h

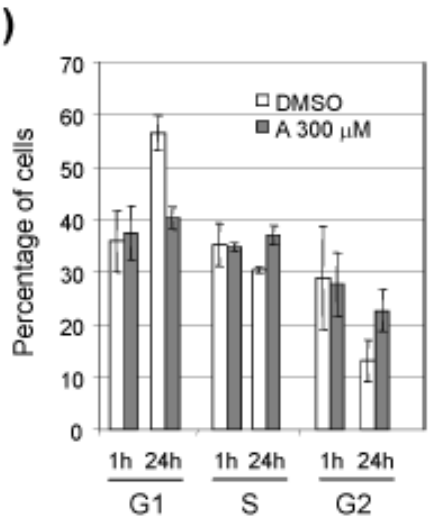

C)
Percentage of cells
DMSO
A 300 μM
70
60
50
40
30
20
10
0
1h 24h
1h 24h
1h 24h
G1
S
G2

Fig. 4