Aus dem Max-Planck-Institut für Psychiatrie München
Direktor der Klinik und Chefarzt: Prof. Dr. Dr. Martin E. Keck

24 drugs as potential SKP2 Inhibitors: a novel approach to finding new antidepressants

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der medizinischen Fakultät der
Ludwig-Maximilians-Universität München

vorgelegt von
Marie Louise Frevert
aus
Bielefeld

2017

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter: Prof. Dr. med. Günter Karl Stalla

Mitberichterstatter: PD Dr. med. Cornelius Schüle
Prof. Dr. Peter Zwanzger

Mitbetreuung durch den
promovierten Mitarbeiter: Dr. rer. nat. Nils Gassen
PD Dr. Theo Rein

Dekan: Prof. Dr. med. dent. Reinhard Hickel

Tag der mündlichen Prüfung: 30.03.2017

Table of Contents

1 Zusammenfassung

Major Depression ist eine belastende, weit verbreitete Krankheit. Derzeit wird sie meist mit Antidepressiva behandelt, welche die Verfügbarkeit von Serotonin und/oder Noradrenalin im synaptischen Spalt erhöhen. Eine hohe Anzahl von depressiven Patienten reagiert jedoch entweder gar nicht oder in insuffizientem Maße auf diese Erstlinientherapie: Niedrige Remissions- und hohe Chronifizierungsraten sowie der verzögerte Wirkeintritt von bis zu 6 Wochen deuten darauf hin, dass die Monoaminmangelhypothese unzulänglich ist. Daher bedarf es der Entwicklung neuer – mehr spezifischer, potenterer und schneller wirkender - Antidepressiva . In der Pathogenese von verschiedenen Krankheiten wie zum Beispiel diverser Krebsarten und neurodegenerativen Erkrankungen wie Parkinson, Alzheimer und Huntington scheint die Autophagie, ein intrazellulärer Prozess eine große Rolle zu spielen, bei dem Zellen Makromoleküle und Zellbestandteile abbauen und wiederverwerten. Neue Studien konnten zeigen, dass Marker der Autophagie sowie in den Prozess eingebundene Moleküle wie z.B. Beclin1 auch nach einer antidepressiven Therapie mit Serotonin Wiederaufnahmehemmern erhöhte Werte aufweisen. Darüber hinaus scheint eine Stabilisierung von Beclin1 (und somit eine Autophagieinduktion) einen antidepressiven Effekt auszulösen. In der hier vorliegenden Arbeit wurden 24 Medikamente, die in einem Hochdurchsatz-Screen als SKP2 E3 ligase Hemmer identifiziert worden sind, als potentielle neue Antidepressiva in primären Rattenastrozyten in nichttoxischer Dosis gestestet. Marker/Moleküle wie Beclin1, LC3 und Vps34 sowie der Zellzyklus Inhibitor p27 dienten der Western Blot Analyse. Die Applikation von Bafilomycin A1 und die Quantifizierung des p62 Abbaus ermöglichten die Beobachtung des autophagischen Flusses. Im Ergebnis hat sich gezeigt, dass Medikamente wie Salinomycin, Celecoxibe, Etoposide, Xanthohumol und Perphenazine einen

positiven Effekt auf die Autophagie hatten und somit für eine antidepressive Therapie erfolgsversprechend sein könnten. Salinomycin wurde ferner im Forced Swim Test in Mäusen untersucht. Mäuse, die damit injiziert worden waren, strampelten signifikant mehr und ließen sich weniger treiben als die Kontrollgruppe der Tiere, die mit Sodiumchlorid behandelt worden waren. Dies deutet auf einen antidepressiven Effekt von Salinomycin hin. Die Erhöhung der Autophagie scheint ein großes Potential für die Behandlung von Depressionen zu besitzen. In weiteren Studien muss jedoch exploriert werden, ob wirklich die Autophagie, der Umsatz von Proteinen an sich, oder die durch die Initatorkomplexe angestoßenen Membranprozesse der antidepressiven Wirkung zugrunde liegen. Bevor entsprechende Medikamente in klinischen Studien getestet werden können, bedarf es außerdem weiterer Experimente mit einer größeren Palette von autophagischen Markern und einer Erforschung ihrer Bluthirnschrankengängigkeit.

2 Abstract

Major depression is a debilitating and highly prevalent disease that up to now has been treated predominantly with antidepressants that increase the availability of serotonin and/or noradrenaline in the synaptic cleft. High rates of non-responders to first line therapy and high levels of chronicity within patients, however, demonstrate the limits of the monoamine hypothesis of depression. Thus the search for novel, more-rapidly acting and more effective antidepressants has become the focus of much research during the past years. Autophagy, a process whereby cells salvage and recycle cellular macromolecules or organelles involving dynamic reorganization and fusion of membranous structures in response to stress, has been implicated as playing a role in several diseases, namedly different cancers and neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's. It has recently been observed that autophagic markers such

as Beclin1 were increased following treatment with serotonin reuptake inhibitors and conversely, a stabilisation of Beclin1 (and therefore autophagic pathways) might lead to an antidepressant-like effect. 24 substances that were found to be inhibitors of the SKP2 E3 ligase via a high throughput screen were tested as potential autophagy inducers and thus novel antidepressants and primary rat astrocytes were treated with them in non-toxic concentrations. Effects on autophagic markers or molecules involved in autophagy such as Beclin1, LC3, and Vps34, as well as the cyclin dependent kinase p27, were investigated via Western Blot. In addition, effects on autophagic flux were determined with Bafilomycin A1 and p62 degradation assays. As a result, I could show that drugs such as Salinomycin, Celecoxibe, Etoposide, Xanthohumol and Perphenazine increased autophagic markers, making them promising candidates for the treatment of major depression. The effects of Salinomycin were further tested on mice subjected to a forced swim test. Here, Salinomycin was found to increase struggling and to decrease the time spent floating, thus exhibiting an antidepressant-like effect. An increase in autophagy seems to harbor a large psychopharmacological potential. Yet it still needs to be seen whether autophagy, the recycling of proteins per se, or the membrane processes kicked off by the initiator complexes underly the antidepressant action. Hence, before proceeding to test the respective substances in clinical trials, further research is needed to expand the range of autophagic markers examined, check the drugs' blood-brain-barrier crossing potentials, and test the substances in further animal experiments.

3 Introduction

3.1 Epidemiology and Definition of Major Depression

Major Depressive Disorder (MDD) is a medical condition that affects about 350 million people worldwide.[1] Unipolar depressive disorders are the third leading cause of the

total global burden of disease[2], taking into account premature mortality, disability and loss of health. The life-time prevalence of MDD is 20% in men and 30% in women[3]. MDD is an "affective" disorder, predominantly concerning mood, but also affecting sleep patterns and cognition. The core signs and symptoms are outlined in Box 1.

Box 1: Clinical diagnosis of major depression

According to the Fifth Edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), Major Depression[120], also referred to as Major Depressive Disorder, is defined as:

A. Five (or more)of the following symptoms have be present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.

Note: Do not include symptoms that are clearly attributable to another medical condition.

1) Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g. feels sad, empty, hopeless) or observation made by others (e.g. appears tearful)
2) Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation)
3) Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day
4) Insomnia or hypersomnia nearly every day
5) Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or beings slowed down)
6) Fatigue or loss of energy nearly every day
7) Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)
8) Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)
9) Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicidal attempt or a specific plan for committing suicide

B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

C. The episode is not attributable to the physiological effects of a substance or to another medical condition.

Note: Criteria A-C represent a major depressive episode.

Note: Responses to a significant loss (e.g. bereavement, financial ruin, losses from a natural disaster, a serious medical illness or disability) may include the feelings of intense sadness, rumination about the loss, insomnia, poor appetite, and weight loss noted in Criterion A, which may resemble a depressive episode. Although such symptoms may be understandable or considered appropriate to the loss, the presence of a major depressive episode in addition to the normal response to a significant loss should also be carefully considered. This decision inevitably requires the exercise of clinical judgment based on the individual's history and the cultural norms for the expression of distress in the context of loss.

D. The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.

E. There has never been a manic episode or a hypomanic episode.

3.2 Pharmacological treatment options of MDD

Despite a myriad of treatment options for MDD, including pharmacological antidepressants, psychotherapies and neuromodulation techniques, many patients with MDD demonstrate high rates of non-recovery, relapse, recurrence or chronification of the illness[4,5]. It has been shown that fewer than 50% of patients reached remission after an initial antidepressant treatment and that a third of patients could not reach remission even after four successive treatment steps with various antidepressants[6,7]. The well known fact that a specific drug that had brought relief to a patient in a previous depressive episode may not be equally as effective during a future episode again highlights the big variability in treatment response and efficacy between individuals. Since the Monoamine Theory of Depression, postulating that MDD orginates from a functional deficit of monoamine neurotransmitters in certain brain regions, has been proposed by Joseph Schildkraut in 1965[8], antidepressant development has been striving towards more specificity towards monoamines, thus resulting in fewer side effects. Existing antidepressants like Serotonin-Reuptake-Inhibitors (SSRIs) prolong the action of monoamines by increasing their duration in the synaptic cleft and thus rapidly

ameliorate the proposed imbalance of neurotransmitters in the brain. Their delayed onset of action with regard to behavioural changes as well as their poor effectiveness accentuate the need to understand their underlying mechanism of action.
Since the discovery of tricyclic antidepressants in the 1950s by serendipity, no radically new antidepressant drug therapies have been developed based on an *a prori* approach to the pathophysiology of depression[9].

3.3 The Pathophysiology of MDD

As to this date, many different studies trying to elucidate the pathogenesis of MDD have been carried out and several different theories have been formulated[10]. Abnormalities in the Hypothalamic-Pituitary- Axis (HPA) involved in the acute stress response as well as its several subsidiary parts have been implicated as a potential target for antidepressant action- such as the adrenocorticotropic hormone (ACTH), corticotropin releasing hormone (CRH) and cortisol itself, as well as various receptors such as the Glucocorticoid Receptor (GR)[11], the CRH Receptors, or the vasopressin receptor[12]. Other studies have presented inflammatory cytokines as contributors to the development of depression[13–16] thus hinting at cytokine antagonists or anti-inflammatory drugs as potential therapeutic strategies. Postmortem studies that have shown that the cell densities-of neurons and glia cells- as well as neuronal size, synaptic proteins and synapses in MDD patients were decreased in frontal and subcortical brain regions[17–19], taken together with MRI studies that document smaller hippocampal volumes in depressed patients[20], lead to the hypothesis that antidepressants might have a possible role in neurogenesis[21], mainly through the expression of Brain Derived Neurotrophic Factor (BDNF)[22]. As can be seen from the list above, there are many different approaches that are being tested for their efficacy in treating MDD. Recently, it has also been shown that

classic tricyclic antidepressants induce autophagy. The exact role of autophagy in this scenario, however, still has to be further investigated.

3.4 Autophagy

Autophagy, coming from the Greek "auto"-onself and "phagy"-to eat, is a conserved cellular degradation process that ensures the quality of the cytoplasm, the intactness of cells and therefore tissues, by controlling the elimination of waste- damaged macromolecules such as protein aggregates and organelles - out of the cell. Different forms of autophagy exist: chaperone-mediated, micro- and macroautophagy. In this research, the focus was placed on macroautophagy as that seems to be the most important one in ensuring cell homeostasis. Through the stepwise formation of an autophagosome, double-membrane vesicles, cytoplasmic to-be-degraded cargo are engulfed and shuttled along microtubules towards the lysosome, with which the autophagosome fuses to yield an autolysosome. Through the acidic components of the autolysosome, the captured material together with the inner membrane is then broken down and nutrients and membranes are recycled. The whole process is governed and regulated by more than 15 autophagy-related genes (ATG) as well as the mammalian target of rapamycine (mTOR), which integrates many upstream signals such as energy status, growth factors and amino acids, just to name a few. Other proteins important in the pathways of autophagy include class III phosphoinositide 3-kinases (PI3Ks or Vps34) and Beclin 1, which initiate the process, microtubule-associated protein 1 light chain 3 II (LC3-II results from the lipidation of LC3-I) and Atg13, involved in formation and expansion of the phagosome membrane, p62 which is an autophagy receptor that recognises cellular waste, and p27 that positively regulates autophagy, preventing the induction of apoptosis[23,24].
Autophagy is vital in regulating cell homeostasis, development, differentiation and survival. It has been found that alterations in autophagy are associated with a wide variety

of pathological conditions and diseases, including neurodegeneration, liver disease, cancers, infectious and metabolic diseases, like diabetes. Thus, recent research has focused on agents that might induce autophagy for therapeutic use[25].

3.5 Autophagy in Depression

A potential role of autophagy in depression has been discussed. Clomipramine, a tricyclic antidepressant, was shown to increase autophagosomal markers and obstruct autophagic flux[26], whereas Lithium and Sertraline, a SSRI, triggered autophagy[27–30]. The induction of autophagy thus seemed to contribute to the increased resilience of cells. Approaching the problem from a different angle, it was found that the mTOR inhibitor rapamycin, an inducer of autophagy, had a mild antidepressant effect in mice and rats that underwent testing with the Forced Swim Test and the Tail Suspension test[31].

3.6 FKBP51

The Hsp90 cochaperone FKBP5 seems to correspond with the treatment response to antidepressant. It has recently been postulated that this is not only due to its regulation of the glucocorticoid receptor and therefore the physiological stress response within the Hypothalamic-Pituitary-Adrenal (HPA) Axis[32,33], but also due to its association with Beclin 1, a regulator of autophagy, and other factors involved in autophagy, such as AKT 1. FKBP5 not only induces autophagy and enhances autophagic flux; its presence in cells also seems to be crucial for the effects of antidepressants to take place[29,34,35]. FKBP51 also seems to scaffold the binding of the E3 ligase SKP2, which binds to Beclin1, AKT and PHLPP. Inhibition of SKP2 in turn was shown to stabilize Beclin1, thus inducing autophagy.

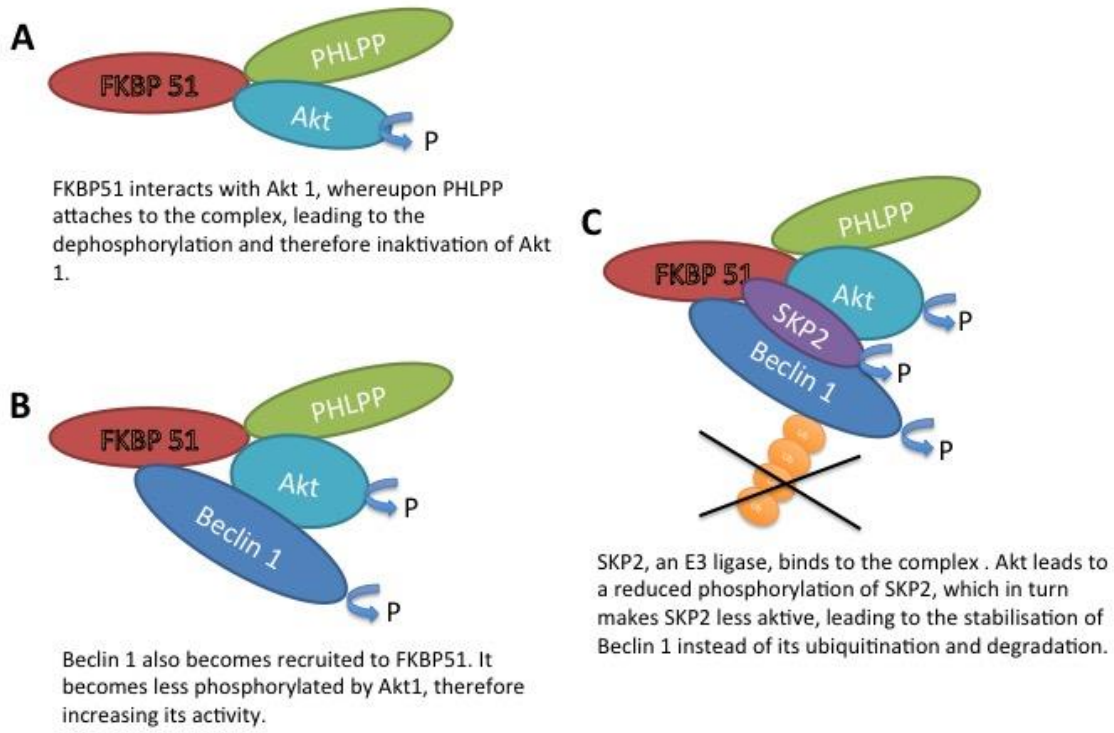

Figure 1: Model of FKBP51's effect on the Akt-Beclin1 heterocomplex and its pharmacological modulation. (A, B) FKBP51 interacts with PHLPP, Beclin1, and Akt, thus recruiting inactive Akt to Beclin1. Beclin1 becomes dephosphorylated and is therefore more active, inducing autophagy. (C) The inhibition of Akt by PHLPP results in the inactivation of the SKP2 E3 ligase, which in turn suppresses the ubiquination and hence the degradation of Beclin1.

3.7 My research

Here is the point where my own research comes in. Rico-Bautista et al. conducted a high-throughput screen of 7368 chemical compounds to measure their effects on the levels of p27, a cyclin-dependent kinase inhibitor and tumor suppressor that was found to be downregulated in different human carcinomas[36]. 176 small molecule inhibitors of p27 depletion (SMIPs) were identified, with SMIP004, a SKP2 downregulator having the most cancer selective antiproliferative activity. SMIP004 had already been established as a potent stabilising agent of Beclin 1 and seemed to elicit antidepressant like effects similar to paroxetine in mice undergoing the tail suspension test. In my research, I hence

looked at the list of 176 molecules and searched the literature to see whether any of them had been described as having an effect on autophagy, with an eye on a potential antidepressant effect of these molecules. Ultimately, I focused on 24 substances, some of which are already used in clinical medicine today, some of which are currently undergoing clinical trials, to test their effects on autophagy in primary cortical rat astrocytes. My strategy has been elucidated in Box 2 below.

Box 2: Step-by-step course of action

1) Literature research to determine at which concentrations the 24 substances had been used previously
2) Cytotoxicity assays: MTT Assays were run to establish at which concentration of the drug administered 80% of cells were still viable
3) Primary cortical rat astrocytes were then treated with the ascertained concentrations and harvested
4) BCA Assays were carried out to determine the total protein concentration within each sample
5) Western Blots: Gel electrophoresis, followed by detection of markers of autophagy such as Beclin1, Vps34, LC3, and cell cycle inhibitor p27 via antibodies
6) Bafilomycin A1 Assays and p62 degradation assays were run for substances, in which LC3 levels increased following stimulation, to determine autophagic flux
7) Behavioural Model in Mice: The Forced Swim Test was carried out with substances bearing the most promise of having an antidepressant effect

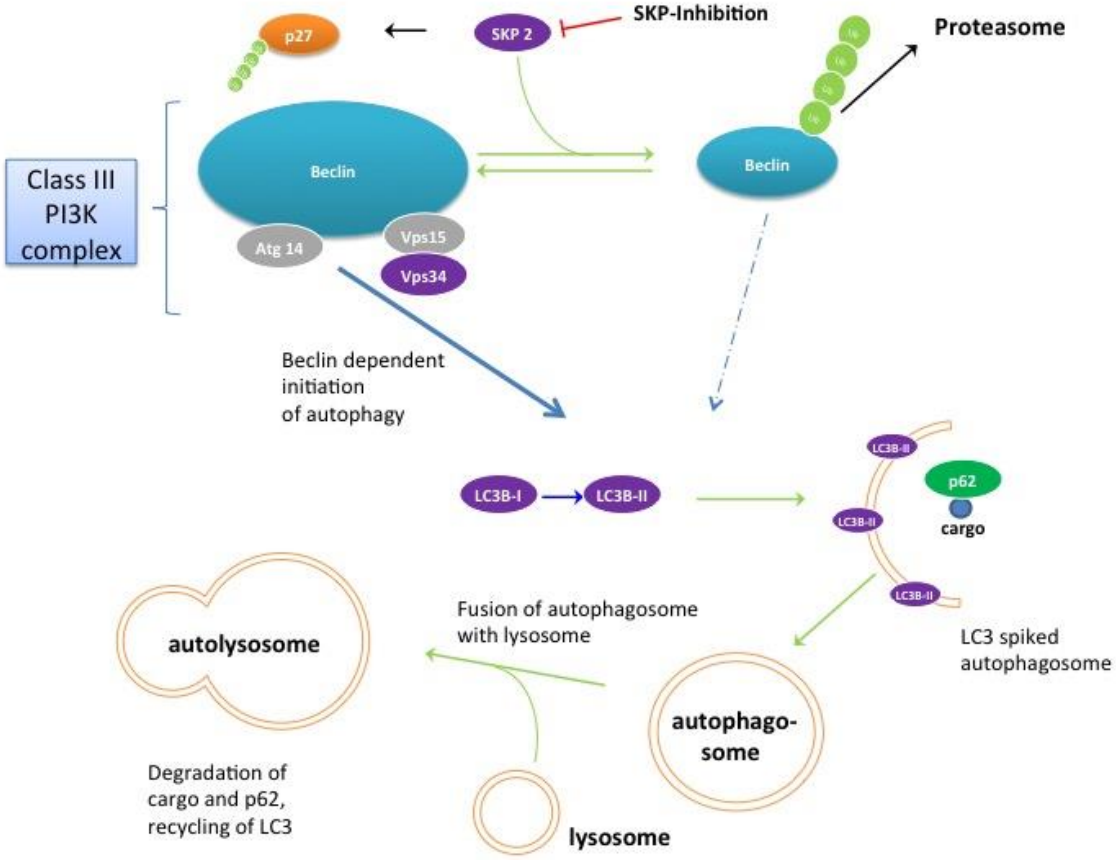

Figure 2: Model of Autophagy and some of the factors involved. p27 is a cyclin-dependent kinase inhibitor and tumor suppressor, controlling cell motility and proliferation as well as apoptosis. SKP2 leads to the ubiquitylation and therefore degradation in the proteasome of p27 as well as the that of Beclin1. It is inhibited by SMIP004, which thus stabilizes p27 and Beclin1. Beclin1, together with several other molecules such as Vps34, a class III PI3 Kinase, form the core complex of the autophagy initators, stabilising the conversion of LC3I (cytoplasmic form) via proteolysis and lipidation to LC3II. LC3II in turn gets incorporated into the membranes of autophagosomes, which envelop p62 bound to toxic cellular waste. When the autophagosome fuse with the lysosome, an autolysosome is formed. p62 and its sequestrated cargo are degraded, whereas LC3 is recycled.

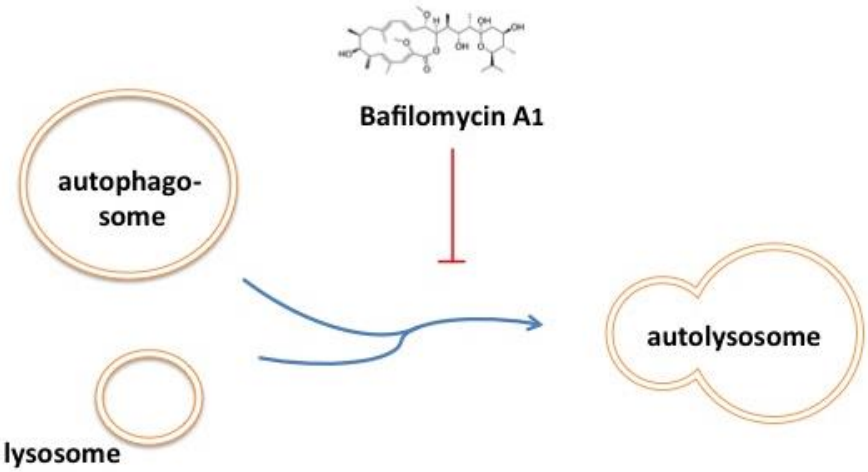

Figure 3: Bafilomycin A1 is a macrolide antibiotic that prevents the formation of an autolysosome by inhibiting the fusion of the autophagosome with the lysosome[37]. It also inhibits the degradation of the autolysosome content due to inhibiting the vacuolar Na^+H^+ ATPase, thus increasing lysosomal pH and impeding the lysosomal proteases[38]. By blocking the fusion and thus the aforementioned degradation and recycling step of LC3, Bafilomycin halts autophagic flux, leading to an increase in LC3II.

Drug	Abbreviation	action	Mechanism of action
Etoposide	**ETO**	chemotherapeutic	topoisomerase II inhibitor
Xanthohumol	XAN	chemotherapeutic, free radical scavenger	not known, COX-I and II inhibitor, found in hop
ABT-737	*ABT*	chemotherapeutic	Bcl-2 Inhibitor (pro-apoptotic)
Topotecan	**TOP**	chemotherapeutic	topoisomerase I inhibitor
17-AAG (derivative of geldanamycin)	AAG	antibiotic, antineoplastic	inhibits Hsp90
Gossypol	*GOS*	chemotherapy, antimalarial, contraceptive	Protein Kinase C inhibitor, proapoptotic properties (Bcl2, Bax)
Securinine	*SEC*	CNS stimulant, chemotherapeutic	GABA receptor antagonist
Tamoxifen	**TAM**	chemotherapeutic (antiestrogen), esp. Breast cancer, neuroprotective	SERM (selective estrogen receptor modulator)
Valinomycin	**VAL**	antibiotic, coronavirus	macrolide- bacteriostatic, inhibits bacterial protein synthesis by not allowing the translocation of tRNA from the donor-to the acceptor position in the ribosome, potassium ionophore, offsets membrane potential, cell death
Minocycline	**MIN**	antibiotic, neuroprotective	tetracycline- bacteriostatic, inhibits bacterial protein synthesis by not allowing the t-RNA to bind to the 30S subunit of ribosomes
Salinomycin	SAL	antibacterial, coccidiostat, possibly chemotherapeutic	polyether ionophore, generates reactive oxygen species, induces apoptosis, exact mechanism still to be determined
Parthenolide	PAR	antineoplastic/antimycobacterial (feverfew used in herbal medicine)	suppresses or modulates NF-κB- activity, induces apoptosis
Pimozide	**PIM**	antipsychotic, tranquilizer	D2-dopamine receptor agonist
Haloperidol	**HAL**	antipsychotic	D2-dopamine receptor antagonist, binds to α1 Receptor at low doses, to 5-HT2 receptors at a higher dosage
Perphenazine	**PEP**	antipsychotic, tranquilizer	D1- and D2-dopamine receptor antagonist,some anti-α-adrenergic and anticholinergic effects
Clomipramine	**CLO**	antidepressant	tricyclic antidepressant- inhibits reuptake of serotonin and noradrenaline by blocking their transporters
Bromocriptin mesylate	**BROM**	antiparkinsonian, diabetes mellitus type II, hyperprolactinaemia	ergoline derivative, Dopamin D2-receptor agonist, Serotonin receptor agonist
Rasagiline	**RAS**	antiparkinsonian, neuroprotective	irreversible inhibitor of monoamine oxidase (MAO)
Pyrvinium pamoate	**PYR**	anthelmintic (pinworms), newly discussed as an chemotherapeutic drug	suppresses NADH-fumarate reductase system, which in turn inhibits the mitochondrial, electron-transport chain complex II, thus inhibiting efficient ATP production, androgen receptor inhibitor
Niclosamide	**NIC**	anthelmintic (tapeworms)	uncouples oxidative phosphorylation- no more production of ATP
Perhexiline maleate	**PER**	prophylactic anti-anginal agent in Australia and New Zealand	inihibits mitochondrial carnitine palmitoyltransferase-1 => more glucose utilisation => more ATP, increased myocardial efficiency
Luteolin	LUT	antioxidant	not known, type of flavonoid, present in plants
Celecoxibe	**CEL**	NSAID	COX-2 selective inhibitor
Amiloride	**AMI**	potassium sparing diuretic	blocks epithelial Sodium channel (ENaC), thus inhibiting sodium reabsorption in the kidney
xxx		**used in clinical medicine**	
xxx		*currently in clinical trial*	

Table 1: Substances and their known effects

4 Material and Methods

4.1 Materials

4.1.1 Technical Equipment

Light Microscope Olympus CK30

Cell Culture Incubator (Binder)

Biofuge pico Heraeus Sigma 2-6 Centrifuge

Vortex peqlab peqtwist

Water Bath GFL

Herasafe, Heraeus Instruments Germany laminar flow hood

Biorda iMark Microplate Reader

Eppendorf Thermomixer comfort 1.5mL heat block

Heidolph Duomax 1030 shaker

BioRad Power Pac 200 Western Blot

Deckworks Low Binding Barrier Pipet Tips (Corning)

4.1.2 Chemicals, Reagents and Buffers

Skim Milk powder, Sigma Aldrich # 70166-500G

Thermo Scientific Pierce BCA Protein Assay Kit # 23228

ECL Detection Reagent (Millipore, Billerica, MA, USA, WBKL 0500)

Whatman® Gel Blotting Paper, Sigma Aldrich # WHA10427810

Nitrocellulose Membrane Amersham™ Protran™ 0.2µM NC GE Healthcare Life Sciences #10600001

Ponceau Solution for electrophoresis (0,2%) Serva #33427.01

Roti® Stock 20% SDS Roth # 1057.1

Acrylamide/bis solution 37.5:1 (30% w/V), 2.6% C Serva # 10688.01

Tris PUFFERAN® >99,9%, Tris-(hydroxymethyl-)aminomethan p.a. Carl Roth # 4855.2

TEMED <99,9%, N,N,N',N'-Tetramethylethylendiamin, 1,2-Bis(dimethylamino)-ethan p.a. für die Elektrophorese Carl Roth # 2367.1

Glycin ≥99 % zur Synthese, α-Aminoessigsäure, Glykokoll Carl Roth # 3790.2

Natriumchlorid >99,8 % Carl Roth # 9265.1

Ammonium persulfate (APS), Sigma Aldrich # A3678-25G

Tween® 20 Polyoxyethylen-20-sorbitanmonolaurat, Polysorbat 20 Carl Roth #9127.2

Methanol ROTIPURAN® ≥99,9 %, p.a., ACS, ISO Carl Roth #4627.6

Bafilomycin A1 ≥90%, Sigma Aldrich, #B1793-2UG

Etoposide ≥98%, Sigma Aldrich, # E1383-25MG

Xanthohumol, ROTICHROM®, Carl Roth #NC35.1

ABT-737, Bcl-2 Inhibitor, APExBIO #A8193

Topotecan hydrochloride hydrate ≥98%, Sigma Aldrich #T2705-10MG

Valinomycin ≥98%, Sigma Aldrich #94675-10MG

Minocycline hydrochlorine, Sigma Aldrich # M9511-25MG

Salinomycin ≥98%, Sigma Aldrich # S4526-5MG

Parthenolide ≥98%, Sigma Aldrich # P0667-5MG

Pyrvinium Pamoate salt hydrate ≥98%, Sigma Aldrich # P0027-10MG

Niclosamide, Sigma Aldrich # N3510-50G

Pimozide, Sigma Aldrich # P1793-500MG

Gossypol, Santa Cruz Biotechnology # sc-200501

Clomipramine hydrochloride, Sigma Aldrich # C7291-1G

Securinine ≥98%, Sigma Aldrich # SML0055-5MG

Tamoxifen ≥99%, Sigma Aldrich # T5648-1G

Amiloride hydrochorate hydrate ≥98%, Sigma Aldrich # A7410-1G

Rasagiline mesylate ≥98%, Sigma Aldrich # SML0124-10MG

Haloperidol, Sigma Aldrich # H1512-5G

Perphenazine, Sigma Aldrich # P6402-1G

Bromocriptin mesylate, Sigma Aldrich # 1076501-150MG

Perhexilene maleate ≥98%, Sigma Aldrich # SML0120-10MG

Luteolin ≥98%, Sigma Aldrich # L9283-10MG

Celecoxib ≥98%, Sigma Aldrich #PZ0008-5MG

17-AAG, 17-(Allyamino)-17-demethoxygeldanamycin, Sigma Aldrich #A8476-500UG

SKP2 E3 Ligase Inhibitor II, SMIP004, Merck Millipore #500517

4.1.3 Antibodies

4.1.3.1 Primary Antibodies

PI3K Kinase Class III (D9AS) Rabbit mAB Cell Signalling Technology # 4263S

P27 Kip1 (D69 C12) XP(R) Rabbit mAB Cell Signalling Technology # 3686S

Beclin-1 Rabbit Ab Cell Signalling # 3738S

SQSTM1/p62 Rabbit Ab Cell Signalling # 5114S

Akt(pan) (C67E7) Rabbit mAB Cell Signalling # 46912L

P-Akt (S473) (193H12) Rabbit mAb Cell Signalling # 4058L

Hsc70 (B-6) mouse monoclonal IgG Santa Cruz Biotechnology # sc-7298

Aktin (I-19) goat polyclonal IgG Santa Cruz Biotechnology # sc-1616

4.1.3.2 Secondary Antibodies

Anti-rabbit IgG, HRP-linked AB Cell Signalling Technology # 7074S

Alexa Fluor 488 donkey anti-goat IgG (H+L) Life Technologies # A11055

Alexa Fluor 488 donkey anti-mouse IgG (H+L) Invitrogen molecular probes # 898250

1xMEM Minimal Essential Medium gibco # 31095029

Gentamycin (10mg/mL) gibco by Life Technologies # 15710-049

Sodium pyruvate 100mM (100X) gibco # 11360-039

Horse Serum, Sigma Aldrich # H0146-5ML

Antibiotic Antimycotic Solution (100x), Stabilized, Sigma Aldrich # A5955-100ML

Phosphatase Inhibitor Cocktail Tablets, Phospho Stop EASYpack, Roche # 04906837001

Protease Inhibitor Sigma Aldrich #P2714

peqGOLD Protein Marker IV 10-170kDa, 10 bands # 27-2110, 27-2111

DMSO Santa Cruz Biotechnology # sc 202581

4.1.4 Sofware

BioRad Chemi Doc MP Imaging System

Sigma Plot 12.5

Image Lab 5.0

Adobe Illustrator CC

Audymaze 4.20

4.2 Methods

4.2.1 Primary cultures of rat astrocytes (already provided)

Enriched astroglial cell cultures were derived from postnatal day 1 Sprague-Dawley rat pups (Charles River, Suzfeld, Germany) and handled as described previously[39,40]. The dissected cortical hemispheres as well as the hippocampuses were trypsinized. To stop the trypsinization, Hank's balanced salt solution and 10% fetal calf serum were added. The mixture containing the tissue was then passed through a serological pipette, centrifuged and after the supernatant had been poured off, resuspended in Minimal Essential Medium (MEM) complemented with 10% horse serum.

4.2.2 MTT Assay

Cell viability of rat astrocytes was measured by the 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. MTT Assays were performed with staggered concentrations of the different substances that had previously been solubilised in DMSO. Cells were seeded in 96- well plates and grown in 1xMEM supplemented with Horse Serum, Gentamycin, and Sodium Pyruvate. Cells were stimulated for 48h at 37°C,

after which MTT solution (5mg/ml) was added and left for 4h, followed by solubilizing solution (100 μM), which was left overnight in the dark. The absorption was measured at 570nm and 655nm wavelength on the next day. Triplicates were performed.

4.2.3 Cell culture

Primary astrocytes were grown in MEM supplemented with Horse Serum, Gentamycin and Sodium Pyruvate. After stimulation and incubation overnight, cells were trypsinized, spun down, washed with 1xPBS, centrifuged again, and resuspended in 1x Lysis Buffer (62,5mM Tris, 2% SDS, 10% sucrose) containining Phosphatase Inhibitor and Protease Inhibitor. All manipulations were carried out with sterile reagents under a laminar flow hood. Samples were then incubated in a heating block at 95° C for 5 minutes and further analyzed. Stimulation was carried out with Xanthohumol, Topotecan, Securinine, Bromocriptin mesylate, Perhexilene maleate, Pyrvinium pamoate, Valinomycin, Rasagiline, Parthenolide, Minocyclin, Pimozide, Luteolin, ABT-737, Salinomycin, Etoposide, Gossypol, Niclosamide, Clomipramine, Tamoxifen, Perphenazine, Haloperidol, Celecoxibe, 17-AAG, Amiloride, and SKP-Inhibitor. DMSO, which served as the solvent for the different drugs, was used as a vehicle control, being set at 100% as a reference in further analyses.

4.2.4 Bafilomycin Assays

Cells were stimulated as described above. Two hours before harvesting the cells, the medium was exchanged and Bafilomycin A1 at a concentration of 100nM[35] was added. Two hours later cells were harvested as usual.

4.2.5 BCA Assay, SDS gel, Western Blotting

Protein concentration of the lysed cell probes was determined by BCA Assay using the Thermo Scientific Pierce BCA Protein Assay Kit. Samples were mixed with 1x Lysis Buffer with Phospho Stop and Protease inhibitor and LAP and incubated at 95°C for 5

minutes. 20 µl of each sample were loaded onto a 15% SDS-PAGE gel. The assays were performed in triplicates. 5 µl Protein Marker IV (peqGOLD) were loaded as a reference band marker. The gel was run at 100V for 10 minutes and then at 170V for 60 minutes. Proteins were then electrotransferred onto a nitrocellulose membrane using the Biorad in 1x Wetblot Buffer. The transfer was performed at 4°C using 100V for 90 minutes. Blots were then blocked by means of 5% low-fat milk in Tris-buffered saline and Tween 20 for 1 hour at room temperature and incubated with respective primary antibody diluted in TBS/ 0,05% Tween overnight at 4°C whilst gently shaking. The following primary antibodies were used: LC3B-I/II (1:1000), PI3K Kinase Class III (Vps34, 1:1000), Beclin-1 (1:1000), SQSTM1/p62 (1:1000), p27 Kip 1 (1:1000), Akt (pan) (1:1000), UVRAG (1:1000), P-Akt (1:1000), Atg3 (1:1000), Hsc-70 (1:4000), Aktin (1:4000). Subsequently, the blots were washed thrice for 10 minutes with TBS-T and probed with the appropiate secondary antibody- Horseradish-Peroxidase- linked (HRP- linked anti-rabbit IgG antibody) or fluorophore-conjugated- for 2 hours at RT. After washing the blots four times for 10 minutes with TBS-T, the immunoreactive bands were visualised using either ECL detection reagent according to the manufacturer's instructions or directly by the excitation of the respective fluorophore. Determination of the relative optical density and quantification of bands was performed using a BioRad ChemiDoc MP and Imagelab 5.0. Actin or Hsc70 were used as a loading controls as they exhibits constitutive high-level expression patterns, ensuring that samples have been loaded equally across all wells.

4.2.6 Animals and animal housing

24 male mice, 10-16 weeks old and weighing 280-320 g were used for the experiments. The animals were housed individullay and held under standard conditions in a temperature-controlled room (23±2°C) on a 12h/12h light/dark cylce (lights on at 8:00 am) with access to food (Altromin 1314) and water *ad libitum*. All of the animals were housed in polyprolene cages (41x32x16.5cm) with wood shavings as bedding.

All experiments took place at the animal facility of the Max-Planck-Institute of Psychiatry, Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 86/609/EEC and utmost care was applied to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

4.2.7 Animal treatment and behavioural experiments (conducted by Georgia Balsevich)

All mice were injected either with a single dose of saline (vehicle) solution or Salinomycin. The drug was administered intraperitoneally by syringe in a constant volume of 5.0ml/kg body weight. 45 minutes after the injection, each mouse was subjected to the Forced Swim Test (FST) for 6 minutes. The FST was carried out between 9:00 and 10:30 am in the same room that the mice were housed in. During the FST, each mouse was lowered into a 2L glass beaker (diameter: 13cm, height: 24cm) that was filled with tap water (21±1°C) up to a height of 15cm. The mouse could thus not reach the bottom with either hind paws or tail. The mice were videotaped by an automated video-tracking system (Audymaze 4.20) for the subsequent quantification of the following parameters: immobility (absence of motion of the entire body and only small movements necessary to keep the animal's head above water), climbing (lively movements of the forepaws in and out of the water, often directed towards the walls of the tank), and swimming (considerable forepaw motions that displaced water and moved the mouse's body around the cylinder, more than necessary to keep the head above water). After six minutes, the mouse was taken out of the water, dried off, and sacrificed. Quantification of the FST was carried out by an experienced observer, blind to the treatment of the animals.

4.2.8 Statistical Analysis

All statistical analyses were performed with the help of Sigma Plot. Drugs were placed

into groups of four depending on their medical use, and compared via one- or two-way analysis of variance (ANOVA). Otherwise, variables in groups were compared to controls pairwise via the Tukey Test, Dunn's Method or the Bonferroni t-test. Significant results of the comparison test are emphasized by asterisks in the graphs and will be discussed in the results section. P values of less than 0.05 were considered significant.

5 Results

5.1 Identification of appropriate drug concentrations

To determine the maximal concentrations of the drug at which 80% of cells would still survive (LD_{20}), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assays were carried out[41]. MTT Assays have been found to be suitable for the initial stage of in vitro drug screening[42] as the MTT (yellow) is reduced by metabolically active cells, resulting in a colour change (purple formazan) that can be quantified by spectrometric means. Hence, cell proliferation rate and activation or conversely, a reduction in cell viability (through apoptosis or necrosis), can be measured with a high degree of precision[41,43,44]. The amount of signal generated generally depends on the concentration of MTT used, the number of viable cells and their respective metabolic activity, as well as the length of incubation with MTT. Below, in Table 2, is the list of substances tested with their respective concentrations. Whereas the addition of several substances such as Bromocriptin mesylate, Clomipramine or Perphenazine reduced cell viability in a dose-dependent manner, other substances such as Luteolin or Salinomycin seemed to activate cell proliferation or metabolism, with the percentage of cell viability increasing, sometimes well beyond 100%. Due to unexplained results in the MTT assays, the concentrations for Luteolin[45,46] (Fig.9B), Celecoxibe[47] (Fig.9C), Niclosamide[48,49] (Fig. 8D),Haloperidol[50,51] (Fig. 7A), Salimomycin[52] (Fig. 6C) and ABT-737[53,54] (Fig. 4C) were derived from the literature as their cell viability percentage never passed below the 80%

cutoff. In one study, Celecoxibe was even described as not having any effect at all on cell viability in vitro when administered in a dose below 10 µM[55]. In the cases of Clomipramine[56,57] (Fig. 7D), Topotecan[58,59] (Fig. 4D), and Pimozide [60,61](Fig. 7A), cell viability percentage values oscillated quite a lot, so common concentrations cited in the literature were chosen. Lastly, Xanthohumol (Fig. 4B) showed a graph of cell viability, which was decreasing consistently with higher concentrations of Xanthohumol administered and seemed to suddenly increase at a concentration of 100 µM. Since Xanthohumol itself has a yellow colour, it thus seems that at such a high concentration, its absorption mirrors that of a high viable cell count. I therefore decided to elimit this point from the graph.

Drug	Abbreviation	Concentration
Etoposide	**ETO**	6 µM
Xanthohumol	XAN	10 µM
ABT-737	*ABT*	10 µM
Topotecan	**TOP**	30 nM
17-AAG (derivative of geldanamycin)	AAG	10 nM
Gossypol	*GOS*	10 µM
Securinine	*SEC*	100 µM
Tamoxifen	**TAM**	10 µM
Valinomycin	**VAL**	10 µM
Minocycline	**MIN**	3 µM
Salinomycin	SAL	5 µM
Parthenolide	PAR	10 µM
Pimozide	**PIM**	10 µM
Haloperidol	**HAL**	10 µM
Perphenazine	**PEP**	20 µM
Clomipramine	**CLO**	10 µM
Bromocriptin mesylate	**BRO**	100 µM
Rasagiline	**RAS**	100 µM
Pyrvinium pamoate	**PYR**	3 µM
Niclosamide	**NIC**	1 µM
Perhexiline maleate	**PER**	10 µM
Luteolin	LUT	50 µM
Celecoxibe	**CEL**	1 µM
Amiloride	**AMI**	10 µM
SKP-Inhibitor (SMIP 004)	SMI	10 µM
xxx	**used in clinical medicine**	
xxx	*currently in clinical trial*	

Table 2: Concentrations of drugs at which cell viability lay at or above 80%.

To evaluate the molecular effects that the different drugs had on autophagy, I analysed autophagic markers, such as Beclin1 and Vps34 (both part of the initiation complex), as well as LC3I and II and p27, a cell cycle inhibitor, in protein extracts from primary rat astrocytes that had been treated with the drugs for a period of 24 hours. As a control, DMSO was used to treat the cells. Groups of four substances were formed in order to ease statistical analysis.

5.2 Western Blot Analyses

5.2.1 Etoposide, Xanthohumol, ABT-737 and Topotecan

The chemotherapeutic drugs Etoposide, Xanthohumol, ABT-737 and Topotecan all increased the LC3II/I ratio of cells (Fig. 4E), with Etoposide, Xanthohumol and ABT-737 all showing a significant increase ($p<0.05$). The difference in Beclin1 expression was not nearly as stark (Fig. 4F), with only Topotecan leading to an increase of 100%. Since Beclin1 and p27 are both substrates of the E3 ligase SKP2, which had been shown to be inhibited by the drugs selected by Rico-Bautista et al., one would expect p27, which is autophagy independent, to have a similar expression pattern as Beclin 1. However, this is not always the case, as in response to treatment with all four drugs, expression levels of p27 decrease (Fig. 4G), in the case of Topotecan significantly so ($p=0.023$). Levels of Vps34 (Fig. 4H), which forms a complex with Beclin1[62,63], increased only upon treatment with Topotecan ($p<0.05$).

5.2.2 17-AAG, Gossypol, Securinine and Tamoxifen

Out of the next group of antineoplastic and antibiotic drugs consisting of 17-AAG, Gossypol, Securinine and Tamoxifen, only 17-AAG, Gossypol and Tamoxifen seemed to boost the lipidation of LC3 (Fig. 5E). Tamoxifen, a known activator of autophagy [64], also enhances expression of Beclin1 (Fig. 5F) and p27 (Fig. 5G), whereas 17-AAG elevates all four markers (Fig. 5 E-H), p27 significantly so ($p<0.05$). Securinine does not seem to

influence protein expression much, except in the case of Beclin1 (Fig. 5F), where it rises to about 400%, and in the case of Vps34 (Fig. 5H), which decreases significantly ($p<0.05$).

5.2.3 Valinomycin, Minocycline, Salinomycin and Parthenolide

The antibiotic group of drugs showed a big variation in expression levels. Salinomycin significantly ($p<0.05$) increased the expression of LC3II/I (Fig. 6E) and of Beclin1 (Fig. 6F), whereas Parthenolide enhanced the expression of Vps34 (Fig. 6H) significantly ($p=0.002$) and Valinomycin downregulated it ($p=0.011$).

5.2.4 Pimozide, Haloperidol, Perphenazide and Clomipramine

The psychoactive drugs Pimozide, Haloperidol, Perphenazide and Clomipramine showed no significant effect on Beclin1 expression (Fig. 7F). Pimozide, a potent D-2 Receptor antagonist has been shown to induce autophagic degradation[65]. It increased Vps34 expression (Fig. 7H) and the ratio of LC3II/I (Fig. 7E) significantly ($p<0.05$). Perphenazine had a significant effect on both LC3II/I (Fig. 7E, $p<0.05$) and p27 (Fig. 7G, $p<0.032$), increasing the expression of the first, and decreasing that of the later. Conversely, Clomipramine enhances the expression of p27 (Fig. 7G) drastically ($p<0.001$) and increases LC3 lipidation (Fig. 7E, $p<0.05$). Haloperidol also exerts a significant p27 stabilizing effect (Fig. 7G, $p=0.038$).

5.2.5 Rasagiline, Bromocriptin mesylate, Pyrvinium Pamoate and Niclosamide

Rasagiline and Bromocriptin mesylate, drugs used in the treatment of Parkinson disease did not affect LC3II/I or Beclin1 expression. Nevertheless, Rasagiline treatment reduced the expression of p27 significantly (Fig. 8G, $p<0.05$) and Bromocriptin mesylate increased the expression of Vps34 (Fig. 8H). The anthelminthic drugs Pyrvinium pamoate and Niclosamide did not exhibit an overall effect. Pyrvinium pamoate significantly induced LC3II/I ratio (Fig. 8E, $p<0.05$), however.

5.2.6 Perhexilene maleate, Luteolin, Celecoxibe and Amiloride

The prophylactic antianginal agent Perhexilene maleate[66] has markedly positive effects on expression levels of LC3II/I (Fig. 9E, $p<0.05$) and p27(Fig. 9G, $p<0.05$). Celecoxibe also had an overall pronounced effect on the upregulation of autophagy on all four markers: LC3II/I (Fig. 9E, $p<0.05$), Beclin1 (Fig. 9F, $p<0.05$), p27 (Fig. 9G, $p<0.05$) and Vps34 (Fig. 9H, $p<0.05$). Lastly, Luteolin, increased the levels of p27 significantly (Fig. 9G, $p<0.05$) without having an effect on the other markers of autophagy.

5.2.7 SMIP004, a SKP2 E3 ligase Inhibitor

As a control the SKP2 inhibitor, SMIP004, led to a significant increase in p27 (Fig. 10E, $p=0.017$) and Beclin1 (Fig. 10D, $p=0.039$) as expected. LC3II/I levels also rose significantly (Fig. 10C, $p=0.013$).

5.3 Autophagic Flux Assays

Assays assessing the autophagic flux, in which Bafilomycin A1 was added to the culture medium of astrocytes two hours before harvesting them, were carried out only with some substances that seemed to increase the LC3II/I signal. In addition, the autophagic marker p62 was tested in response to treatment with these substances. p62 is a protein that itself is degraded by autophagy; it thus accumulates when autophagy is inhibited and its levels decrease when autophagy is induced. Therefore, it, together with LC3II/I serves as a marker for autophagic flux[67].

5.3.1 Chemotherapeutic drugs on autophagic flux

When 100 nM of Bafilomycin A1 were added to cells previously treated with chemotherapeutics such as Etoposide or Xanthohumol, the increase of expression in LC3II/I, signifying the inhibition of autophagic flux, was much more pronounced than in the control cells (Fig. 11A, $p<0.001$), only having been treated with DMSO. This further supports

the idea that these two substances usually increase flux as LC3II/I drastically accumulates. Etoposide also increases the levels of p62, but not significantly (Fig. 11B).

5.3.2 Antibiotics on autophagic flux

Amongst the antibiotic drugs tested, only Minocyclin, Salinomycin and Parthenolide initially increased LC3II/I. When treated with Bafilomycin A1, Salinomycin had a pronounced effect on the accumulation of LC3BII/I (Fig. 11G, $p=0.002$). Both Minocyclin and Salinomycin led to an increase in p62 levels (Fig. 11H, $p=0.001$ and $p<0.001$, respectively).

5.3.3 Psychoactive Drugs on autophagic flux

Within the group of psychoactive drugs, only Perphenazide had a significant (Fig. 12A, $p=0.019$) effect on both LC3BI/II and p62 (Fig. 12B, $p<0.05$), but Clomipramine also slightly increased the LC3BII/I after Bafilomycin A1 treatment in comparison to the control.

5.3.4 Antibiotic and Antineoplastic drugs on autophagic flux

Gossypol increased levels of LC3B/II (Fig. 12F) after cells were treated with Bafilomycin A1 and also increased the p62 signal (Fig. 12G), but not significantly.

5.3.5 NSAID and Diuretic on autophagic flux

Both Celecoxibe and Amiloride further increased the levels of LC3BII/I levels in response to Bafilomycin A1 treatment (Fig. 13A), but only Amiloride led to a rise in p62 levels (Fig. 13B).

5.3.6 Anthelminthics on autophagic flux

Lastly, the anthelminthic Pyrvinium pamoate made LC3BII/I levels rise after Bafilomycin A1 treatment (Fig. 13E) and also increased the p62 signal (Fig. 13F).

5.4 Forced Swim Test (carried out by Georgia Balsevich)

The forced swim test is a behavioral paradigm to assess depressive-like behavior in rodents, which is used to screen drugs for their potential antidepressant activity by comparing the time an animal struggles to that it spends floating or being immobile in response to intraperitoneal injection with a drug[68,69]. A significant difference in behavior between animals having been injected with saline (control) and those injected with 5mg/kg Salinomycin 45 minutes prior to the forced swim test was observed. Salinomycin led to an increase in struggling (Fig. 14A, $p=0.027$) as well as a decrease in floating (Fig. 14B, $p=0.034$), thus confirming my hypothesis that Salinomycin, an upregulator of autophagy, acts antidepressant-like in mice.

6 Discussion

6.1 The need for new antidepressants

Many antidepressants treatments are currently available, but these only insufficiently address the high rates of recurrence, non-recovery, and chronicity of major depression. Moreover, clinical improvement tends to only set in after several weeks or months of treatment. The monoamine hypothesis of depression, according to which the augmentation of synaptic availability of serotonin and/or noradrenaline would suffice in treating major depression, has been challenged not only after observing that selective serotonin reuptake inhibitors and selective serotonin and noradrenaline reuptake inhibitors do not ameliorate response and remission rates of major depression as quickly and sufficiently as hoped, but also by studies showing that a selective serotonin reuptake enhancer, Tianeptine, which enhances the presynaptical uptake of serotonin, thereby decreasing its concentration in the synaptic cleft, was clinically effective in treating major depression despite having the opposite effect as SSRIs[70]. In the search of novel psychopharmacological therapies to combat major depression, autophagy, a repair mechanism,

that has been implicated in many different diseases, including neurodegenerative ones like Huntington, Parkinson's and Alzheimer's[71–74], has recently come into the focus of antidepressant research. As current antidepressants seem to act not only on neurons as primary targets, but also directly on astrocytes by affecting intracellular signalling pathways and gene expression[39,75,76], because major depression is associated with compromised glial function[77] and because astrocytes are vitally important for recycling of substances, especially glutamate, at the synapse[78], I tested the 24 chosen substances on primary rat astrocytes.

6.2 MTT Assays

As mentioned in the Results section and as presented in Figure 1-6, many substances did not elicit a decreased cell viability at high concentrations, but instead led to a higher rate of reduction of MTT than the vehicle control did. In the case of Luteolin, this could be due to the finding that Luteolin even seems to reduce MTT even in the absence of cells[79,80], feigning higher rates of viability, thus showing no correlation to mitochondrial activity and rendering my MTT assay void. Ultimately, Luteolin was used at a concentration of 50 μM as described in the literature[46]. Other substances might have similar effects. However, a viability of above that of the vehicle control (above 100% in the graphs) could also signify that a substance, instead of having a toxic effect, leads to cell proliferation and therefore a higher total mitochondrial activity (and thus a higher reduction rate, measured as higher absorbance rates). A drug could also lead to higher mitochondrial activity per se in individual cells, hence also increasing absorbance values[44]. In order to better assess drug toxicity on cell viability, it might be advisable to also run other cytotoxicity assays like LDH release assays to evaluate necrosis, Trypan blue staining assays to assess membrane damage, caspase activity to assess apoptosis or several others.

6.3 Levels of p27

All of the 24 drugs were selected from the high throughput screen carried out by Rico-Bautista et al. that tested different substances with their regard to SKP2 inhibition. As a readout of this inhibition, the stabilization of p27, well-characterized as a SKP2 substrate, was used. Theoretically, all 24 drugs thus should have increased the levels of p27 due to a decreased ubiquitination and degradation of p27 upon SKP2 inhibition. With the vehicle set to be at 100% as reference, however, p27 did not seem to increase in response to many drugs. This might be explained by the fact that the screen done by Rico-Bautista et al. was carried out by using higher concentrations of the drugs, whereas I titrated them down to low-toxic concentrations by means of LD_{20}. By basically eliminating toxic effects of the substances on cells, autophagic processes, like the stabilization of Beclin 1, that were elicited in response to an SKP2 inhibition could be focused upon. Since p27 is a cell cycle inhibitor that regulates cell differentiation and proliferation, it only makes sense that its level increased in response to toxic stimuli, however, thus trying to induce cell cycle arrest and enhancing expression of DNA repair proteins.

6.4 Levels of p27 in accordance with Beclin1

The E3 ligase SKP2 catalyses the ubiquitination of both Beclin1 and p27 as can be seen in Figure 1, thus targeting the two proteins for degradation by the proteasome. When this E3 ligase is inhibited by the small molecule inhibitor SMIP004 or other substances, it would only be logical that both, levels of p27 and Beclin1, would increase as seen in Fig. 7D and E. For certain substances like 17-AAG, Clomipramine and Celecoxibe this holds true (Fig. 2F and G, Fig. 4F and G, Fig. 6F and G, respectively). However, this does not seem to be the case in all instances as other drugs, like Topotecan, Salinomycin or Securinine increase Beclin1 levels whilst decreasing p27 levels (Fig. 1F and G, Fig. 3F and G, and Fig. 2F and G, respectively), whereas some drugs like Perphenazine and Luteolin decrease the p27 signal significantly whilst the Beclin1 signal stays nearly the same

(Fig. 4F and G). Despite both Beclin1 and p27 being substrates of SKP2, this phenomenon could be explained by a difference in affinity for the E3 ligase. Since the substances used were administered at different concentrations, depending on the results of the MTT assays performed, this is a possible explanation. Binding affinities are affected by many different parameters, including allosteric regulation which might very well play a role in the differing response of p27 and Beclin1 levels in response to SKP2 inhibition, but it would go beyond the scope of this study to test them all.

6.5 Beclin1 and Vps34

Out of the four molecules that I looked at in my study, Beclin1 and Vps34 are part of a class III PI3 kinase-containing complex that is also termed the autophagy initiation complex, acting as a binding platform for other proteins such as Atg 14 (that leads to autophagy induction)[81], UVRAG (which promotes autophagosome maturation by fostering the fusion of autophagosome and late endosomes)[82–84], Rubicon (negative regulator of autophagy that inhibits the autophagosome maturation)[85,86,87] or others.
As Beclin1 plays a crucial role in the coordination of the cytoprotective role of autophagy[88] and Vps34, vacuolar protein sorting 34, a phosphoinositide 3-kinase, also seems important in regulating autophagy (as inhibition of it led to a blockade of autophagic degradation)[89], it makes sense to analyze the protein levels of Beclin1 and Vps34 in order to understand whether the applied drugs induce autophagy (as could be seen by increased levels of Beclin1 and Vps34) or downregulated it (in which case levels of Beclin1 and Vps34 would decrease).
In the case of levels of Beclin1 in comparison to those of Vps34, a similar issue as that of the relationship of levels of p27 and Beclin1 can be observed. Since Beclin1 and Vps34 form the above mentioned complex, it would only seem plausible that these two parameters would change in a related manner, i.e. to either both increase or both decrease in a perhaps even proportional fashion as could be observed in response to

treatment with Celecoxibe (Fig. 6F and H). Nevertheless, several drugs had opposite effects on the ratio of Beclin1 to Vps34 levels. Whereas Valinomycin decreased the levels of Vps34, the levels of Beclin1 stayed almost constant (Fig.3 F and H). Parthenolide, on the other hand, increased Vps34 significantly, but Beclin1 levels lingered at 100% (Fig. 3F and H). In the opposite direction, this could also be observed: Beclin1 levels increased in response to Clomipramine or Niclosamide, but Vps34 levels stagnated (Fig. 4F and H, Fig 5 F and H, respectively). These results could be explained by the finding that not the entire amount of Vps34 is bound to Beclin1[62,63] as part of the initiation complex, but that part of it is free. Which part is affected by the administration of the drug remains to be seen and would be subject to further research, like coimmunoprecipitation using crosslinks to identify physiological protein-protein interactions. Similarly, the experiments carried out for this thesis do not differentiate between expression vs. activity of a protein marker. Beclin1, for example, becomes phosphorylated by the ULK1/2 kinase, which leads to the activation of Vps34[90], whereas Rubicon, on the other hand, decreases the activation of Vps34 and therefore leads to a downregulation of autophagy[85]. It therefore would be important to make the distinction between activated vs. inactive Vps34, which cannot be deduced from my data, as that would provide more information about the effect of up- or downregulation of a marker on autophagy.

6.6 Bafilomycin Assays and assay of p62 degradation

The level of lipidated LC3, a downstream marker of autophagy that was tested, was affected by the addition of Bafilomycin A1 upon treatment with some of the different substances as well. Autophagic flux, defined as the dynamic process of autophagy, includes autophagy formation and maturation, lysosomal fusion and the following degradation and recycling of macromolecules. It is useful to look at autophagic flux instead of only

conducting experiments measuring the protein abundance of „static“ markers, because autophagic flux is a measure of autophagic degradation activity[91] and is therefore crucial in understanding the dynamics of autophagy in cells. Since an accumulation of autophagosomes, represented by an increased lipidated LC3, could be due to an increased rate auf autophagic induction or a block somewhere downstream, inhibiting autosomal maturation and degradation, it is important to distinguish between these two scenarios by means of visualizing autophagic flux. Bafilomycin A1, a macrolide antibiotic that as a vacuolar H(+) ATP-ase inhibitor[92] increases the pH of lysosomes[93] and inhibits the fusion of lysosomes and autophagosomes[37], thereby acting as a lysosomal degradation inhibitor. Bafilomycin A1 increases the level of LC3II when autophagic flux occurs, because the transit of autophagosomes (to whose membranes LC3II is linked) through the autophagic pathway is blocked[94]. Thus, the difference in the amount of LC3II/I in the samples without Bafilomycin A1 to the ones treated with Bafilomycin A1 account for the amount of LC3II/I that is degraded by lysosomes[95]. The fact that the effect of Bafilomycin A1, a marked increase in the signal of LC3II/I, was amplified even more upon the addition of the substance shows that more marker accumulates due to increase in autophagic flux in response to administration of the drug. This was the case for Etoposide, Xanthohumol, Perphenazine and Salinomycin as they increased the lipidated LC3 significantly (Fig. 11A and B, Fig. 12 A and B, Fig. 11 G and H, respectively). However, although LC3II usually correlates with the number of autophagosomes[96], not all LC3II is linked to autophagic membranes as some seems to be ectopically generated[97]. These LC3II populations being independent of autophagy makes it difficult to account changes in the expression of LC3II solely to autophagic flux. Therefore, it is important to also use other means of monitoring autophagic flux in combination with methods looking at LC3II turnover. As p62 is degraded by autophagy, p62 levels can also be implemented to serve this purpose. Thus, an activation of autophagy leads to decreased levels of p62, whereas

a suppression of it leads to an accumulation of the marker. Clomipramine and Celecoxibe increased the lipidated LC3 in response to Bafilomycin administration and also decreased the level of p62 (Fig. 12 A and B), but not significantly and therefore seem to increase autophagic flux. Salinomycin, which has been shown to induce autophagic flux at low concentrations[98,99], however, increases both the LC3II/I ratio and p62 significantly (Fig. 11 G and H). Similar effects can be seen in the case of Perphenazine (Fig. 12 A and B), Etoposide (where only LC3II/I, however, increases significantly) and Pyrvinium pamoate (where both LC3II/I and p62 increase, but neither does so significantly). These effects could be explained by pitfalls in this system of monitoring autophagic flux as p62 is degraded not only through autophagy, but also through a ubiquitin-proteasome system, leading to increased levels of p62 also when the proteasome is inhibited. Additionally, p62 seems to be linked to other molecular signalling pathways that affect autophagy[100], thus making the monitoring of autophagic flux based on p62 more difficult. Conversely, levels of p62 could have also increased due to a stimulatory effect of the applied substances as p62 by means of transcriptional upregulation, for example[101]. Despite the drawbacks of both Bafilomycin A1 and p62 degradation assays, taken in combination with each other, they should yield reliable results. However, because Bafilomycin A1 assays seem to have fewer flaws than p62 degradation assays, I decided to impart more importance to their results when p62 and LC3II/I values did not correlate as in several instances mentioned above. Nevertheless, it might be advisable to look at other ways of measuring autophagic flux through tandem-fluorescence tagged LC3[102] or lysomome-dependent long-lived protein degradation[97] or to even try to measure the rate of autophagic flux in further studies.

6.7 Forced Swim Test

The forced swim test is a behavioural test in rodents, used to test the efficacy of potential new antidepressant compounds by creating a situation in which "behavioural despair" is induced. Salinomycin, chosen to be tested by these means, exhibited a significant antidepressant-like effect by increasing the time of struggling and decreasing the time that the mouse spent floating (Fig. 14). The forced swim test was developed in the late 1970s as a animal behavioural paradigm for the evaluation of antidepressant drugs. The assumption is that animals will try to escape stressful stimuli (in this case, being placed into a cylinder of water), but if that is impossible, they would give up. Antidepressant-acting substances should increase the time an animal spends in escape attempts (measured by struggling), instead of giving up (floating, being immobile)[103]. Since minimal equipment is needed in order to carry out the test in mice, because high-throughput screening of drugs is possible via this method, yielding a wealth of data which can be used to compare different studies, the FST is an important tool in drug discovery and research[68]. Of course, there are drawbacks to this test as it does not necessarily represent the human condition[104]. To verify results, it would therefore be advisable to also carry out other models for depression-like behaviour, such as the open field test, the elevated plus maze or the tail suspension test. Since only 12 animals were tested in the drug and the control group of our study, more behavioural assessments should be carried out to verify the results.

6.8 Potential novel antidepressants

Taking into account all the things discussed above, the substances most promising of being potential novel antidepressants are Celecoxibe (in response to treatment with which all markers tested increased significantly and which increased autophagic flux, if not significantly), Salinomycin (which increased both Beclin1 and LC3II/I significantly and seems to increase autophagic flux), Etoposide, Xanthohumol and Perphenazine (all three increasing LC3II/I significantly and increasing autophagic flux). Pimozide, a potent

D-2 receptor antagonist which is often coprescribed with serotonin reuptake inhibitors to better treat depression in patients with Tourette Syndrom[60] , and Perhexilene maleate (which has been shown to rapidly induce autophagy[105]) should also be further evaluated in the future. Lastly, Clomipramine, a substance that has been used as tricyclic antidepressant for years has previously been shown to interfere with autophagic flux[26], in our study increased Beclin1, p27 and LC3II/I, LC3II/I and p27 even significantly so, and also increased autophagic flux, if not significantly. Of course, our study only used a sample size of three, hence further experiments should be carried out to validate these results. Salinomycin, which has been shown to induce autophagy especially in cancer cells[99,106], still looked promising after being tested in mice during the forced swim test, whereas Celecoxibe, a cyclooxygenase-2 inhibitor used as a non steroidal antiinflammatory drug in clinical medicine, has already been tested in rats[107] as well as in several placebo-controlled clinical trials[108,109], catering to the hypothesis that an inflammation underlies the pathophysiology of depression. It was found to have antidepressive effects. Interestingly enough, Luteolin, which was shown to have antidepressant-like effects, in part because of a suppression of endoplasmic reticulum stress[45], did not have an overwhelming effect on autophagic markers. Similarly, Gossypol and ABT-737, which as a Bcl-2 inhibitors seemed promising, did not convince either as they did increase LC3II/I (ABT-737 significantly so), but did not significantly affect flux at all. This was suprising, as through Bcl-2 inhibition, Bcl-2's interaction with the BH3 domain of Beclin1 was eliminated, thus reducing its inhibitive effect on autophagy[110,111], rendering Gossypol[112] and ABT-737[53,113] autophagic inducers. Lastly, Minocycline, which has been tested as a potential novel antidepressant in several instances and has had positive effects, did not affect expression patterns of autophagic markers very much at all and even seems to suppress autophagic flux (decreasing lipidated LC3 and increasing p62), again showing that there might be different cellular processes involved in the pathophysiology of major

depression. Since the drugs tested here were only administered for a period of 24 hours, after which the cells were harvested and protein effects were determined, it is impossible to say what the drugs' effects would be when used over an extended period of time, in chronic treatment. Different experiments, including those measuring the concentration of drugs in the brain to see what kind of gradients could be built up, as well as electrophysiological test, examining the reaction of hippocampal neurons in response to drug administration[114], would be useful in creating a more complete picture of the drugs' actions.

7 Conclusion and Outlook

In conclusion, out of the 24 drugs that were tested with respect to their effect on autophagy, a few showed positive effects on the induction of autophagy and thus should be further investigated in light of potentially being novel antidepressants.

There is still much to be researched in the field of major depression as very little is yet known about the pathophysiology of this disease. Many more experiments will have to be carried out, and just in the field of autophagy, many processes will have to be more completely understood in order to properly evaluate the results. Until these mechanisms have been understoood, it would be important to look at even more different markers of autophagy, such as UVRAG, Rubicon or Atg14, that are also involved in the initiation complex, as well as sites of phosphorylation or the oligomerization behaviour of different molecules, in order to get a broader picture of a drug's action. In addition, the experiments should also be repeated in neurons, as both astrocytes and neurons are critical for the antidepressant response. Furthermore, a greater number of *in vivo* experiments still have to be carried out, including potentially ones of the drugs that I found out to be promising targets. In order to be able to undertake those, however, it would be wise to also look at the drug's ability to cross the blood brain barrier, as otherwise, it would

have to be discussed whether a peripheral effect of the drug would be great enough to show a strong antidepressant effect. Similarly, it would be important to consider the side effects of potential new antidepressants, especially in the view of taking them over an extended period of time as the antidepressant effect would have to warrant the side effects. Side effects were especially pronounced in some of the first antidepressant drugs, tricyclic antidepressants, which since then have almost been replaced in the treatment of depression by the more selective (and thus fewer side effect-inducing) SSRIs. It would be hoped that new antidepressants would also induce faster antidepressant responses in patient behaviour. Ketamine, an NMDA-Receptor antagonist used in clinical anesthesia, has recently been discovered to elicit a rapid and potent antidepressant effect, after having been administered intravenously only once[115,116]. Since then, the effect of the glutamatergic system on major depression has been the focus of much research. In response to NMDA stimulation, autophagy in neurons seems to be upregulated[117,118]. Since autophagy also seems to a play role in NMDA-Receptor modulated synaptic neurotransmission and synaptic plasticity[119], substances acting on autophagic pathways might have a faster onset of action than many of the antidepressant drugs used currently in clinical medicine, hence making the further study of autophagy in the context of the pharmacological therapy of major depression worthwhile.

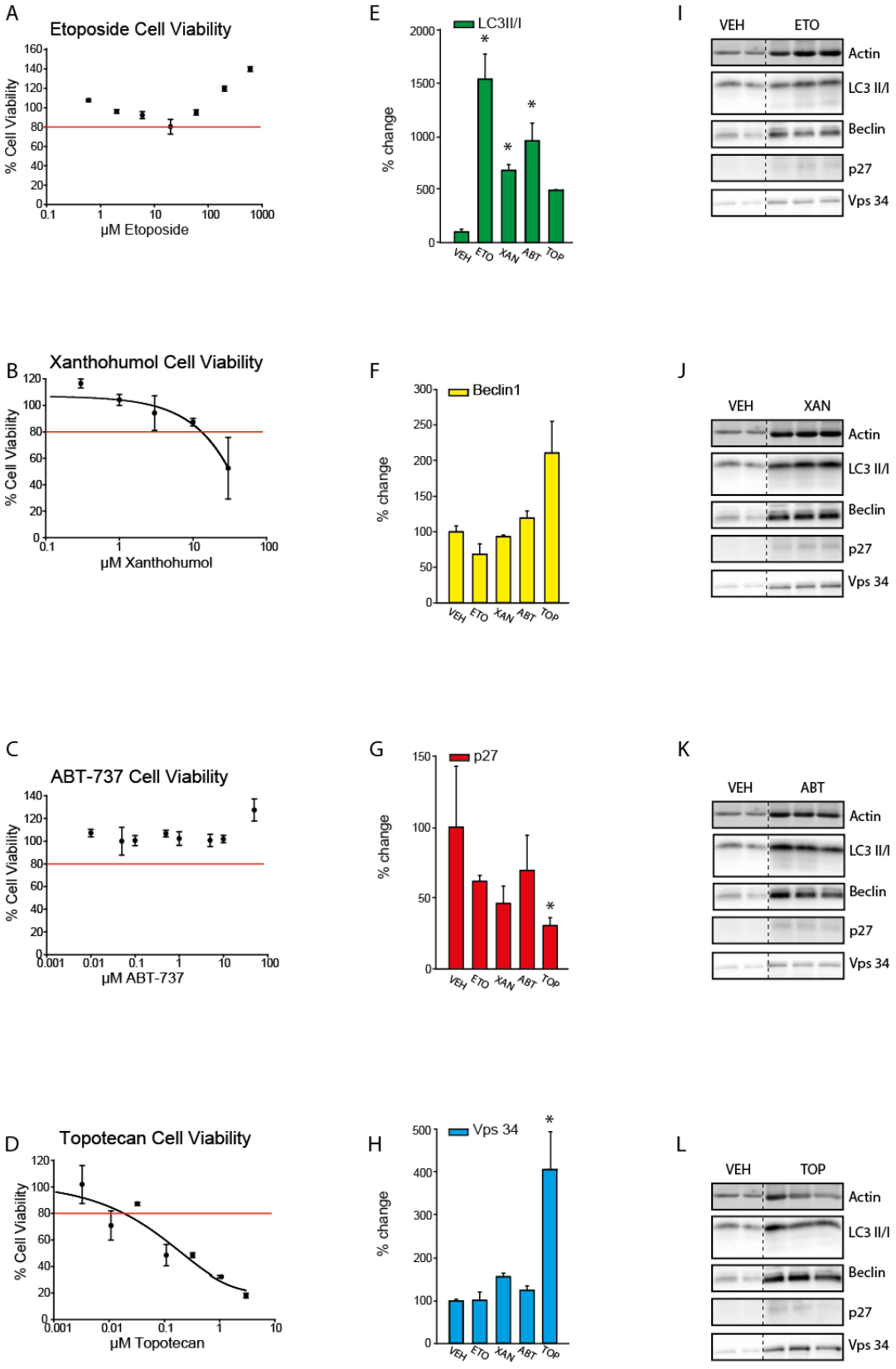

Figure 4. Group of chemotherapeutic substances inhibiting SKP2: cell viability and protein effects tested in primary rat cortical astrocytes. MTT assay graphs (A-D) display the mean ± SEM difference to control (vehicle) in triplicates. Vehicle was set to 100%. The red line indicates a 80% viability. Western Blot analyses (E-H) of cell lysates show effects of compounds on LC3II/I, Beclin1, p27 and Vps34 levels. Bars represent the mean + SEM (n=3). *p<0.05. (I-L) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

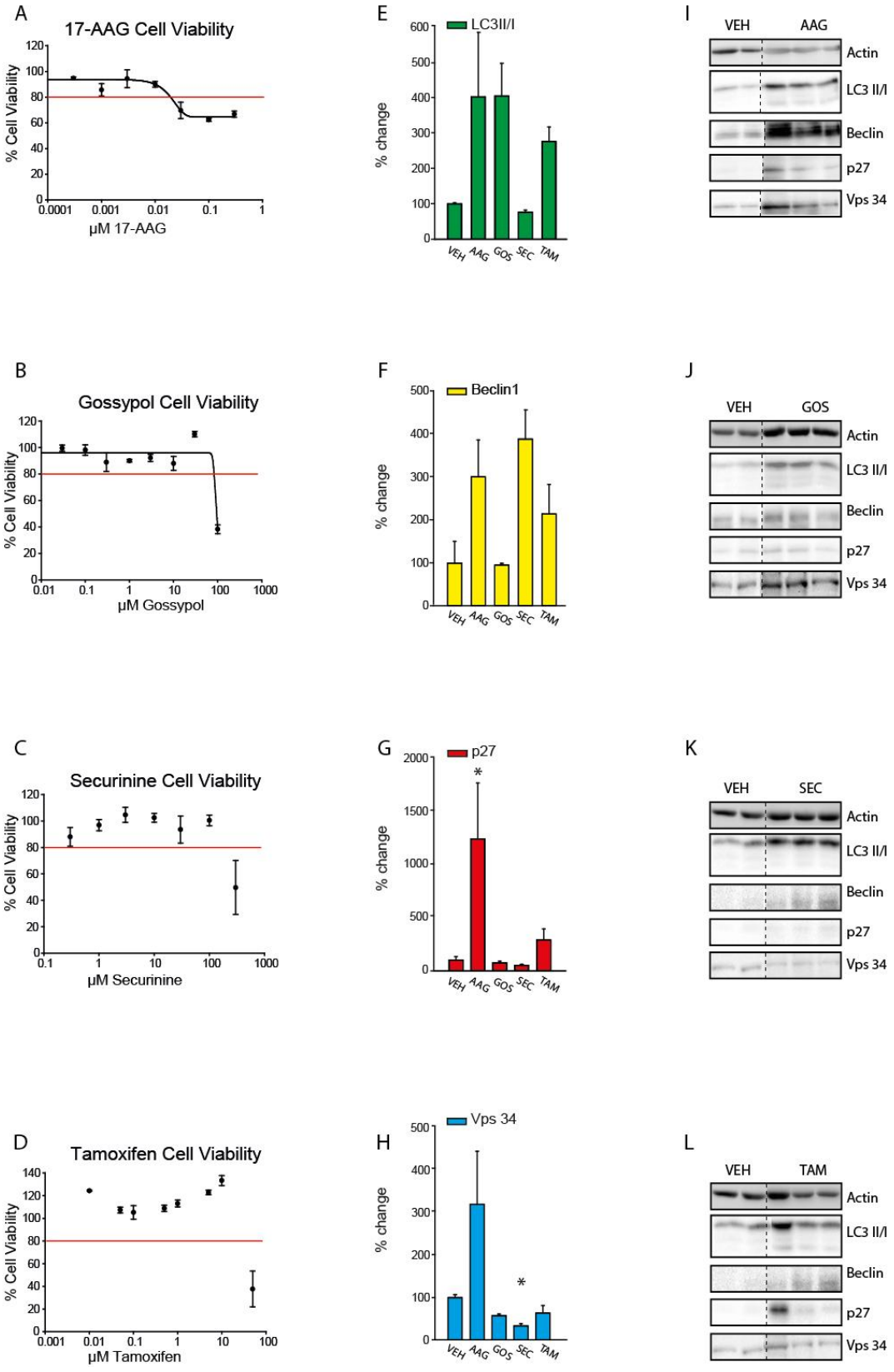

Figure 5. Group of substances inhibiting SKP2: cell viability and protein effects tested in primary rat cortical astrocytes. MTT assay graphs (A-D) display the mean ± SEM difference to control (vehicle) in triplicates. Vehicle was set to 100%. The red line indicates a 80% viability. Western Blot analyses (E-H) of cell lysates show effects of compounds on LC3II/I, Beclin1, p27 and Vps34 protein levels. Bars represent the mean + SEM (n=3). *p<0.05. (I-L) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

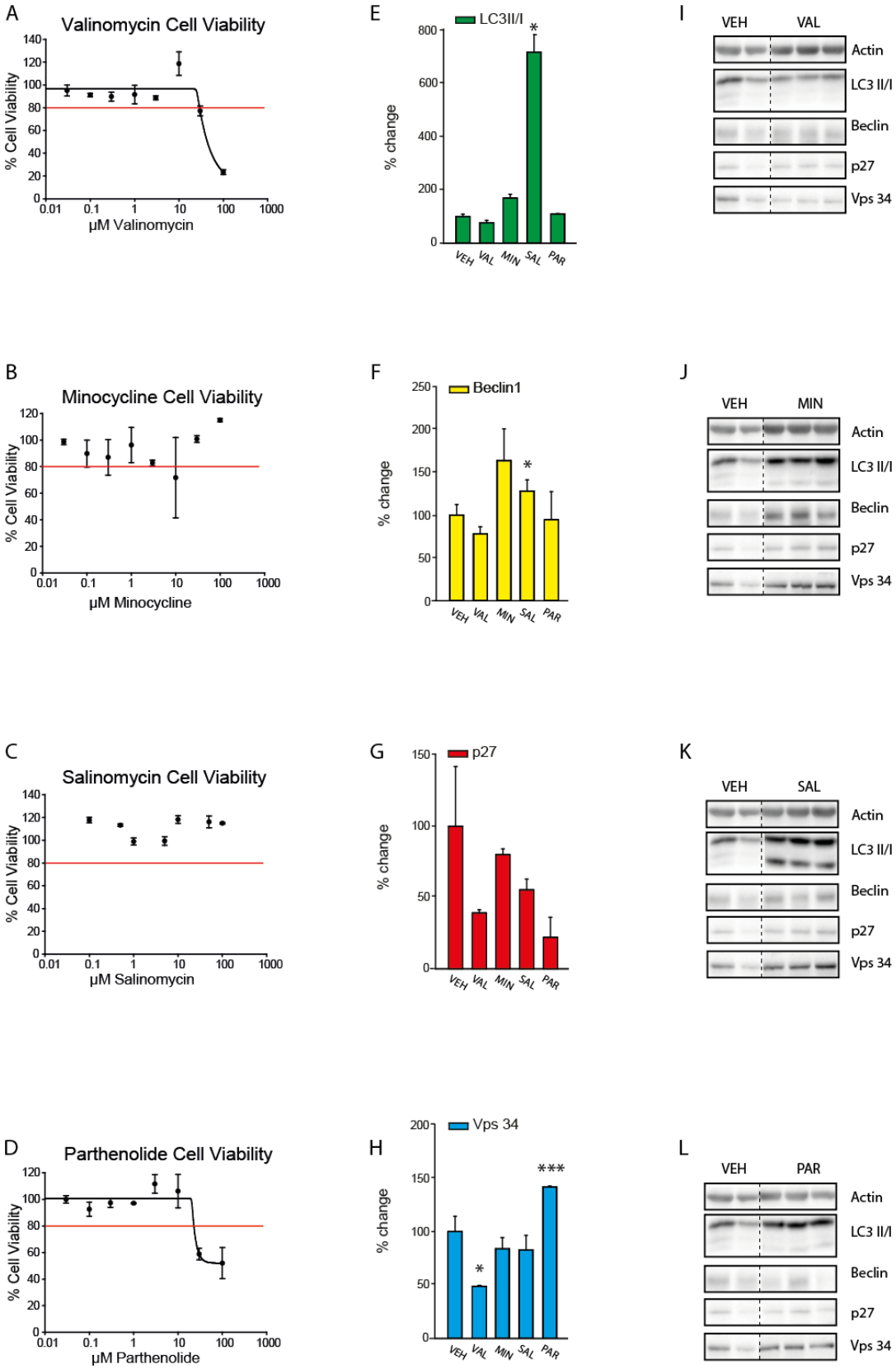

Figure 6. Group of antibiotic substances inhibiting SKP2: cell viability and protein effects tested in primary rat cortical astrocytes. MTT assay graphs (A-D) display the mean ± SEM difference to control (vehicle) in triplicates. Vehicle was set to 100%. The red line indicates a 80% viability. Western Blot analyses (E-H) of cell lysates show effects of compounds on LC3II/I, Beclin1, p27 and Vps34 protein levels. Bars represent the mean + SEM (n=3). *p<0.05; **p<0.01; ***p<0.001. (I-L) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

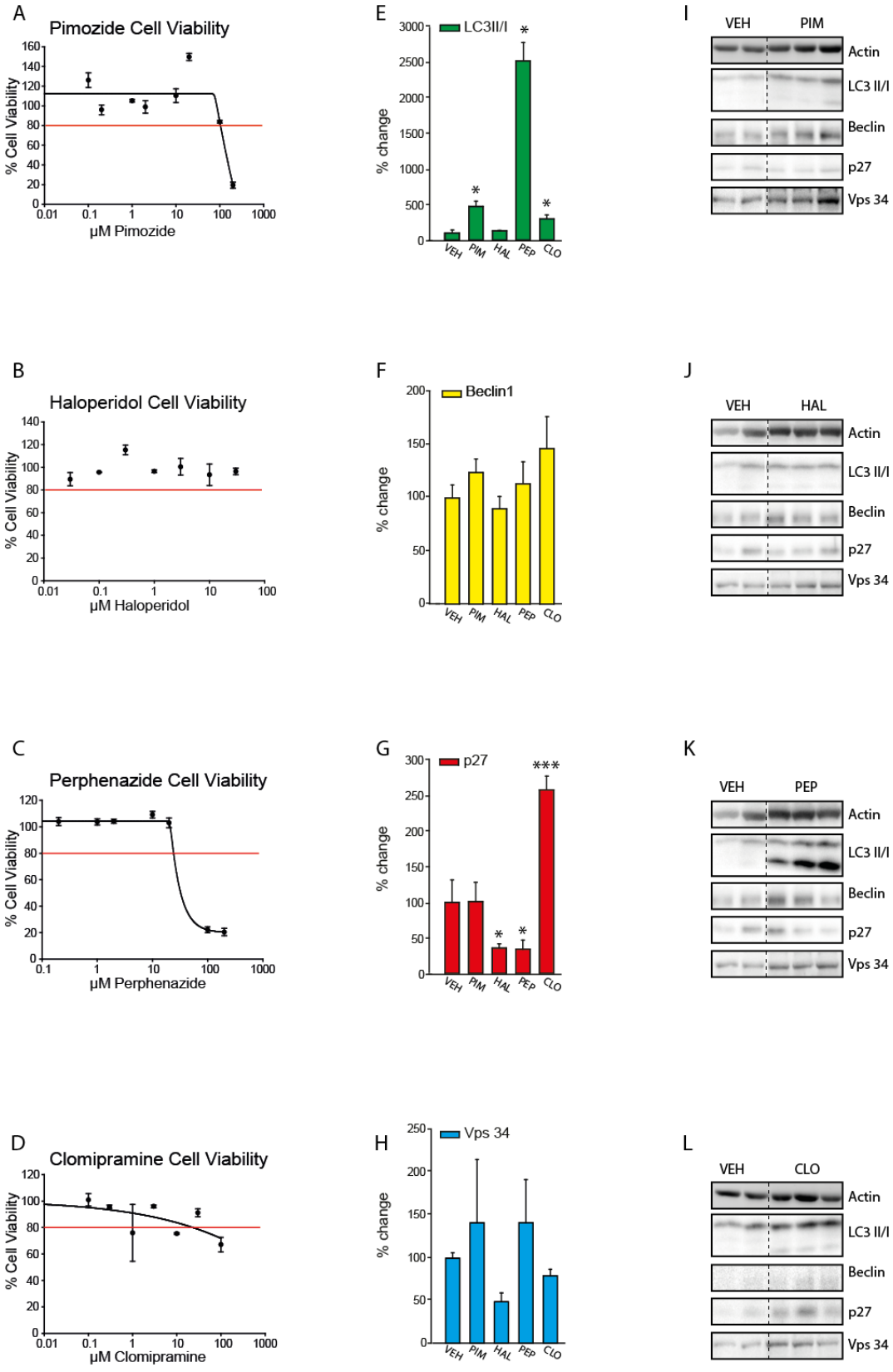

Figure 7. Group of psychoactive substances inhibiting SKP2: cell viability and protein effects tested in primary rat cortical astrocytes. MTT assay graphs (A-D) display the mean ± SEM difference to control (vehicle) in triplicates. Vehicle was set to 100%. The red line indicates a 80% viability. Western Blot analyses (E-H) of cell lysates show effects of compounds on LC3II/I, Beclin1, p27 and Vps34 protein levels. Bars represent the mean + SEM (n=3). *p<0.05; ***p<0.001. (I-L) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

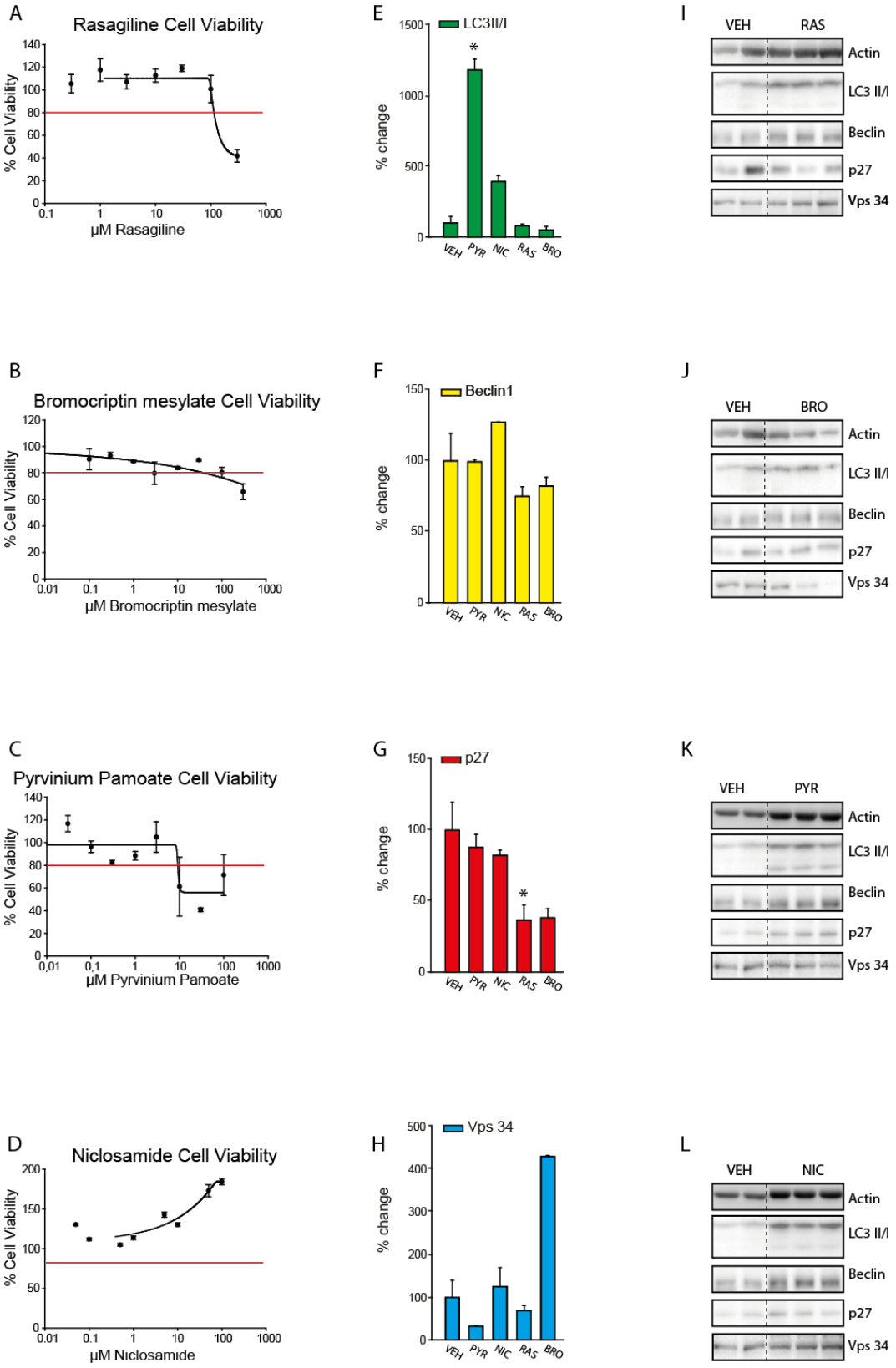

Figure 8. Group of substances inhibiting SKP2: cell viability and protein effects tested in primary rat cortical astrocytes. MTT assay graphs (A-D) display the mean ± SEM difference to control (vehicle) in triplicates. Vehicle was set to 100%. The red line indicates a 80% viability. Western Blot analyses (E-H) of cell lysates show effects of compounds on LC3II/I, Beclin1, p27 and Vps34 protein levels. Bars represent the mean + SEM (n=3). *p<0.05. (I-L) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

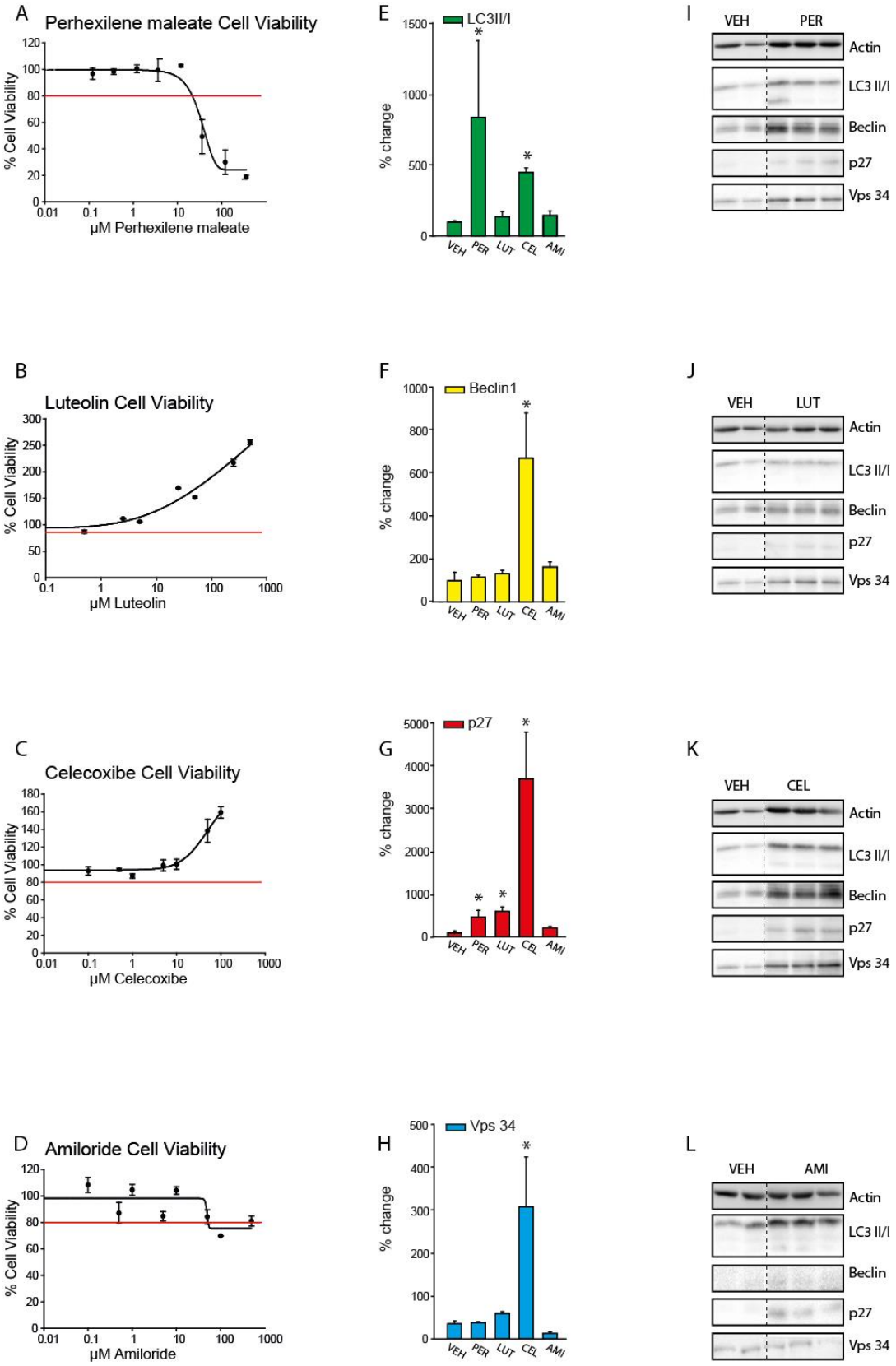

Figure 9. Group of substances inhibiting SKP2: cell viability and protein effects tested in primary rat cortical astrocytes. MTT assay graphs (A-D) display the mean ± SEM difference to control (vehicle) in triplicates. Vehicle was set to 100%. The red line indicates a 80% viability. Western Blot analyses (E-H) of cell lysates show effects of compounds on LC3II/I, Beclin1, p27 and Vps34 protein levels. Bars represent the mean + SEM (n=3). *p<0.05. (I-L) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

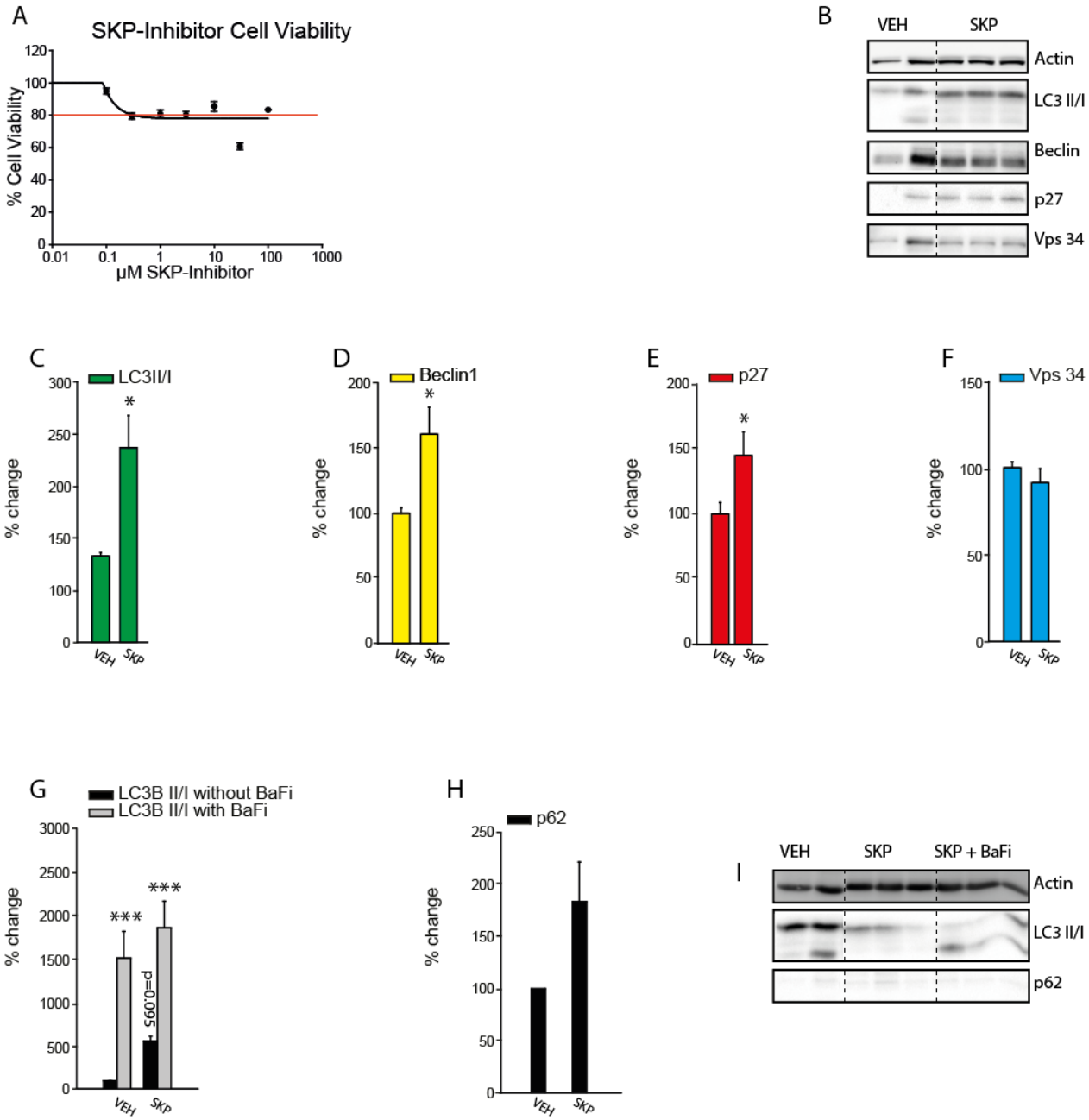

Figure 10. SMIP004, a SKP2/ E3 ligase Inhibitor: cell viability and protein effects tested in primary rat cortical astrocytes. MTT assay graph (A) displays the mean ± SEM difference to control (vehicle) in triplicates. Vehicle was set to 100%. The red line indicates a 80% viability. Western Blot analyses (C-F) of cell lysates show effects of compound on LC3II/I, Beclin1, p27 and Vps34 protein levels. Bars represent the mean + SEM (n=3). Autophagic flux was measured with Bafilomycin A1 assays (G) and p62 quantification (H). *p<0.05; ***p<0.001. (B,F) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

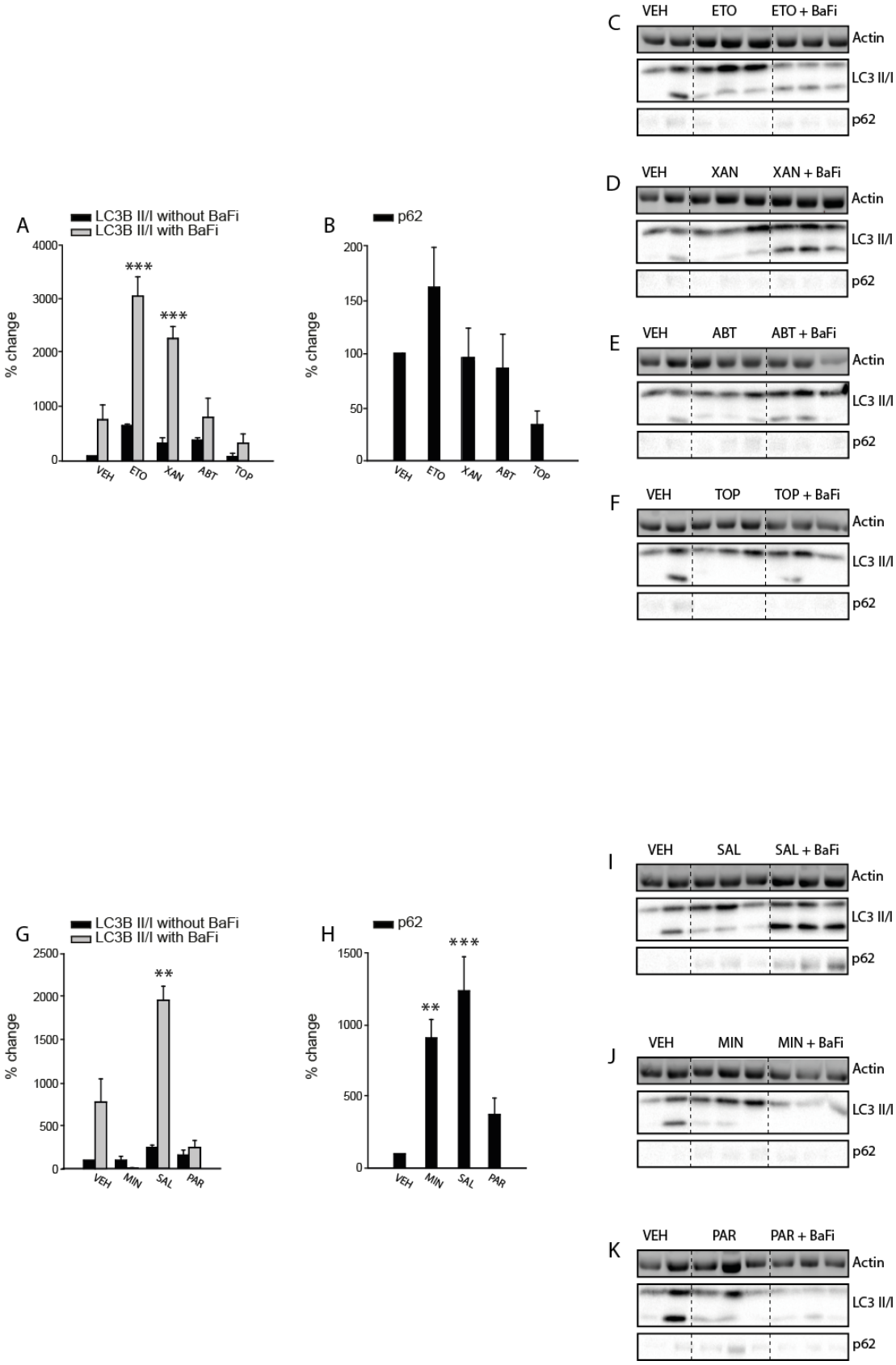

Figure 11. Bafilomycin assays and levels of p62. 100nM of Bafilomycin A1 were added to cells 2 hours before harvesting after cells had been treated with drugs for 24h. Western blot analyses (A-B, G-H) of cell lysates show effects of compound on LC3II/I as well as p62 protein levels. Bars represent the mean + SEM (n=3). **p<0.01; ***p<0.001. (I-K) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

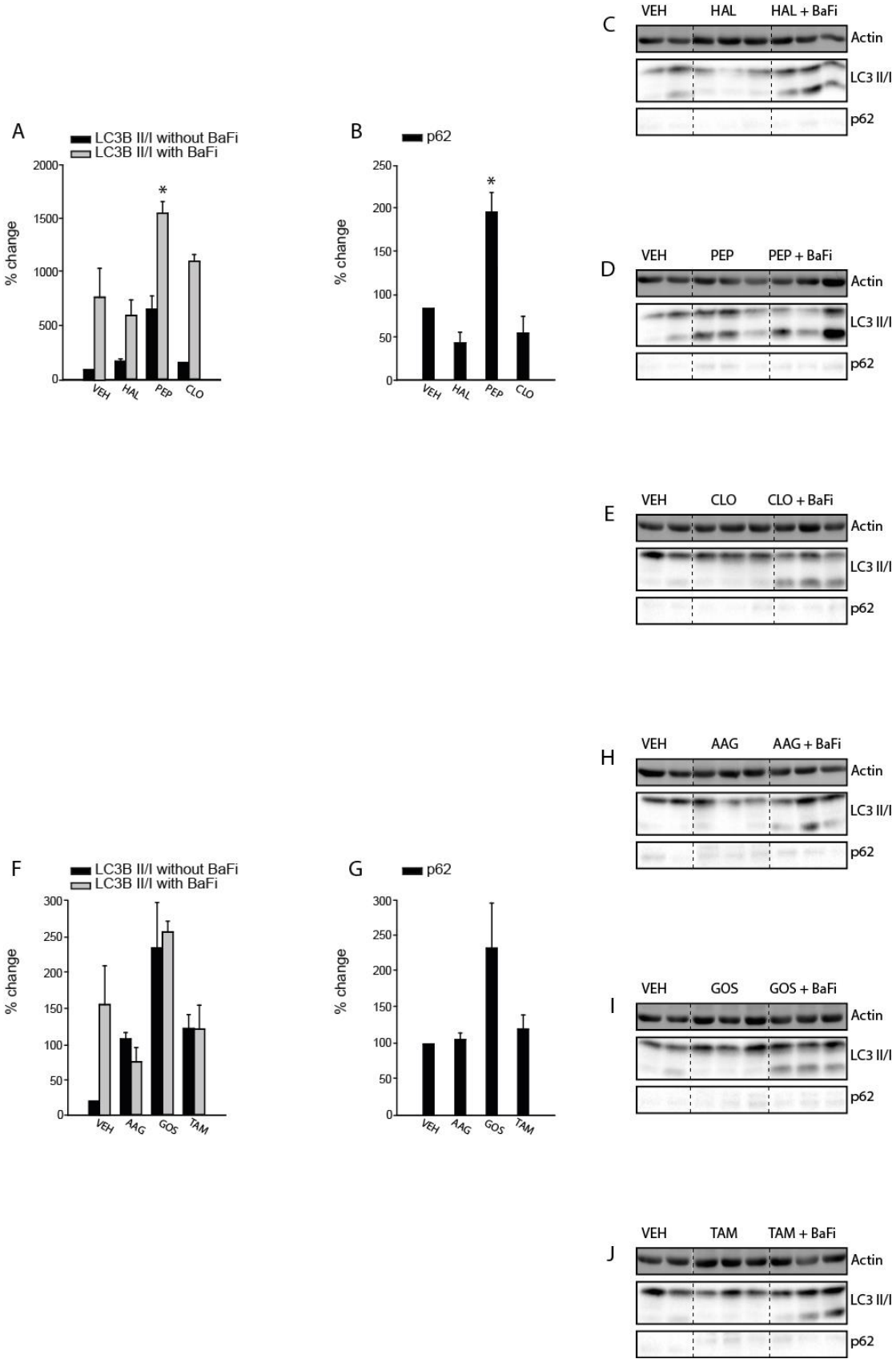

Figure 12. Bafilomycin assays and levels of p62. 100nM of Bafilomycin A1 were added to cells 2 hours before harvesting after cells had been treated with drugs for 24h. Western blot analyses (A-B, G-H) of cell lysates show effects of compound on LC3II/I as well as p62 protein levels. Bars represent the mean + SEM (n=3). *p<0.05. (C-J) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

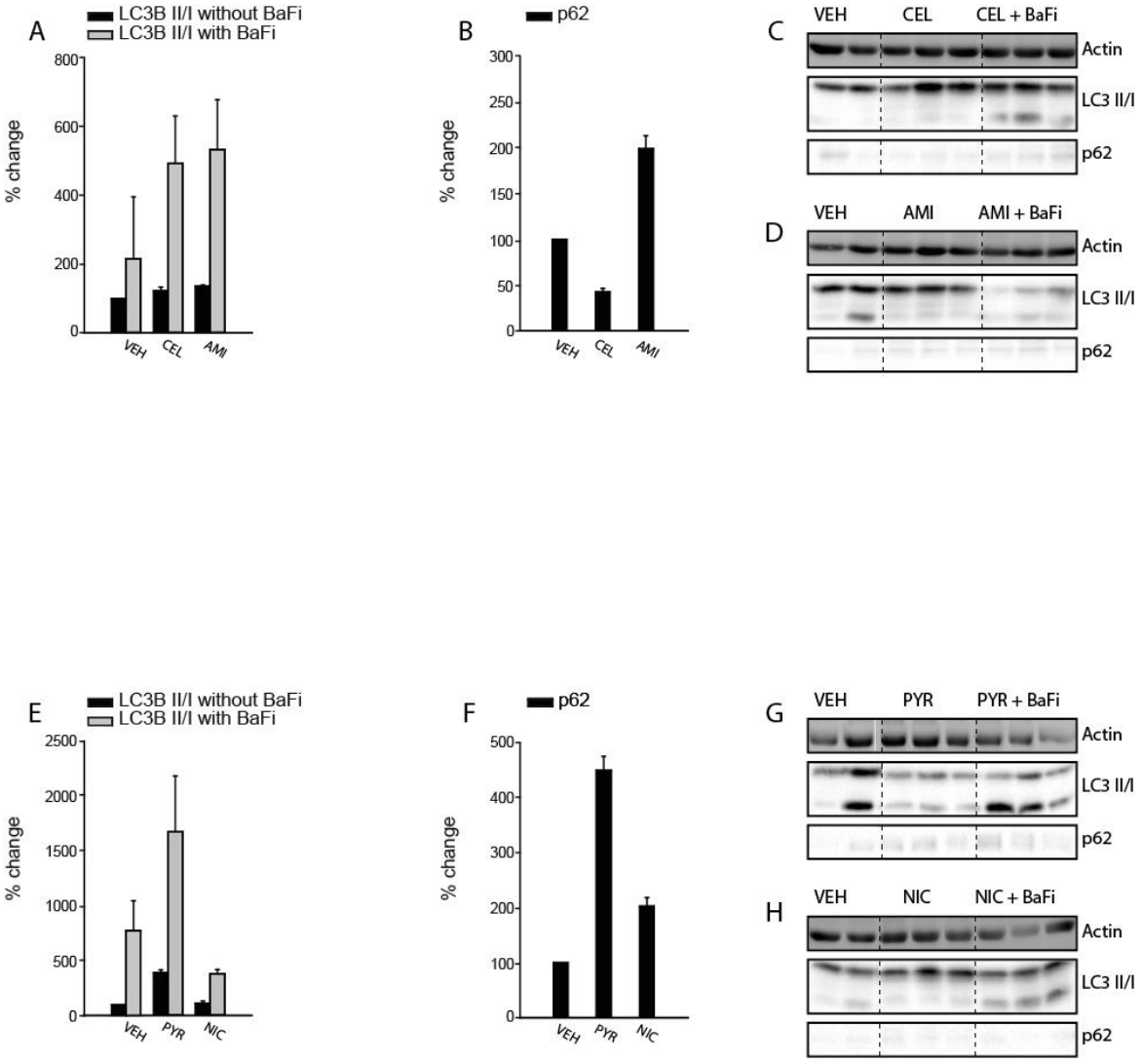

Figure 13. Bafilomycin assays and levels of p62. 100nM of Bafilomycin A1 were added to cells 2 hours before harvesting after cells had been treated with drugs for 24h. Western blot analyses (A-B, G-H) of cell lysates show effects of compound on LC3II/I as well as p62 protein levels. Bars represent the mean + SEM (n=3). (G,H) show cropped images of Western blots. The dotted line represents the separation between DMSO and drug-treated blots.

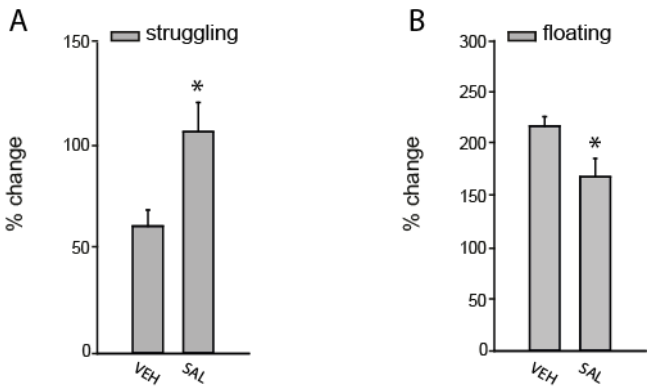

Figure 14. Effect of FDA- approved drug Salinomycin (antibiotic that inhibits E3 ligase SKP2) on Forced Swimming Behaviour. Mice received an intraperitoneal injection of 5mg/kg Salinomycin or equal quantity of saline (as vehicle, control) 45 minutes prior to testing (n=12). *p<0.05.

8 Bibliography

1. WHO | Depression. *WHO* Available at: http://www.who.int/mediacentre/factsheets/fs369/en/. (Accessed: 6th April 2016)

2. WHO | The global burden of disease: 2004 update. *WHO* Available at: http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/. (Accessed: 6th April 2016)

3. Kruijshaar, M. E. *et al.* Lifetime prevalence estimates of major depression: an indirect estimation method and a quantification of recall bias. *Eur. J. Epidemiol.* **20,** 103–111 (2005).

4. Yiend, J. *et al.* Long term outcome of primary care depression. *J. Affect. Disord.* **118,** 79–86 (2009).

5. Paykel, E. S. Partial remission, residual symptoms, and relapse in depression. *Dialogues Clin. Neurosci.* **10,** 431–437 (2008).

6. Rush, A. J. *et al.* Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. *Am. J. Psychiatry* **163,** 1905–1917 (2006).

7. Warden, D., Rush, A. J., Trivedi, M. H., Fava, M. & Wisniewski, S. R. The STAR*D Project results: a comprehensive review of findings. *Curr. Psychiatry Rep.* **9,** 449–459 (2007).

8. The catecholamine hypothesis of affective disorders: a review of supporting evidence. 1965 [classical article]. *J. Neuropsychiatry Clin. Neurosci.* **7,** 524–533 (1995).
9. Nestler, E. J. Antidepressant treatments in the 21st century. *Biol. Psychiatry* **44,** 526–533 (1998).
10. O'Leary, O. F., Dinan, T. G. & Cryan, J. F. Faster, better, stronger: towards new antidepressant therapeutic strategies. *Eur. J. Pharmacol.* **753,** 32–50 (2015).

11. Holsboer, F. Stress, hypercortisolism and corticosteroid receptors in depression: implications for therapy. *J. Affect. Disord.* **62,** 77–91 (2001).

12. Purba, J. S., Hoogendijk, W. J., Hofman, M. A. & Swaab, D. F. Increased number of vasopressin- and oxytocin-expressing neurons in the paraventricular nucleus of the hypothalamus in depression. *Arch. Gen. Psychiatry* **53,** 137–143 (1996).

13. Noto, C. *et al.* Targeting the inflammatory pathway as a therapeutic tool for major depression. *Neuroimmunomodulation* **21,** 131–139 (2014).

14. Miller, A. H., Maletic, V. & Raison, C. L. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol. Psychiatry* **65,** 732–741 (2009).

15. Felger, J. C. & Lotrich, F. E. Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. *Neuroscience* **246,** 199–229 (2013).

16. Han, Q.-Q. & Yu, J. Inflammation: a mechanism of depression? *Neurosci. Bull.* **30,** 515–523 (2014).

17. Hercher, C., Turecki, G. & Mechawar, N. Through the looking glass: examining neuroanatomical evidence for cellular alterations in major depression. *J. Psychiatr. Res.* **43,** 947–961 (2009).

18. Duman, R. S. Depression: a case of neuronal life and death? *Biol. Psychiatry* **56,** 140–145 (2004).

19. Drevets, W. C. Neuroplasticity in mood disorders. *Dialogues Clin. Neurosci.* **6,** 199–216 (2004).

20. Videbech, P. & Ravnkilde, B. Hippocampal volume and depression: a meta-analysis of MRI studies. *Am. J. Psychiatry* **161,** 1957–1966 (2004).

21. Nestler, E. J. *et al.* Neurobiology of depression. *Neuron* **34,** 13–25 (2002).

22. Shirayama, Y., Chen, A. C.-H., Nakagawa, S., Russell, D. S. & Duman, R. S. Brain-Derived Neurotrophic Factor Produces Antidepressant Effects in Behavioral Models of Depression. *J. Neurosci.* **22,** 3251–3261 (2002).

23. Nickeleit, I., Zender, S., Kossatz, U. & Malek, N. P. p27kip1: a target for tumor therapies? *Cell Div.* **2,** 13 (2007).

24. Hiromura, K., Pippin, J. W., Fero, M. L., Roberts, J. M. & Shankland, S. J. Modulation of apoptosis by the cyclin-dependent kinase inhibitor p27Kip1. *J. Clin. Invest.* **103,** 597–604 (1999).

25. Shoji-Kawata, S. *et al.* Identification of a candidate therapeutic autophagy-inducing peptide. *Nature* **494,** 201–206 (2013).

26. Rossi, M. *et al.* Desmethylclomipramine induces the accumulation of autophagy markers by blocking autophagic flux. *J. Cell Sci.* **122,** 3330–3339 (2009).

27. Chen, J., Korostyshevsky, D., Lee, S. & Perlstein, E. O. Accumulation of an Antidepressant in Vesiculogenic Membranes of Yeast Cells Triggers Autophagy. *PLOS ONE* **7,** e34024 (2012).

28. Sarkar, S. *et al.* Lithium induces autophagy by inhibiting inositol monophosphatase. *J. Cell Biol.* **170,** 1101–1111 (2005).

29. Gassen, N. C., Hartmann, J., Schmidt, M. V. & Rein, T. FKBP5/FKBP51 enhances autophagy to synergize with antidepressant action. *Autophagy* **11,** 578–580 (2015).

30. Zschocke, J. *et al.* Antidepressant Drugs Diversely Affect Autophagy Pathways in Astrocytes and Neurons—Dissociation from Cholesterol Homeostasis. *Neuropsychopharmacology* **36,** 1754–1768 (2011).

31. Cleary, C. *et al.* Antidepressive-like effects of rapamycin in animal models: Implications for mTOR inhibition as a new target for treatment of affective disorders. *Brain Res. Bull.* **76,** 469–473 (2008).

32. Touma, C. *et al.* FK506 binding protein 5 shapes stress responsiveness: modulation of neuroendocrine reactivity and coping behavior. *Biol. Psychiatry* **70,** 928–936 (2011).

33. Albu, S. *et al.* Deficiency of FK506-binding protein (FKBP) 51 alters sleep architecture and recovery sleep responses to stress in mice. *J. Sleep Res.* **23,** 176–185 (2014).

34. Gassen, N. C. *et al.* Chaperoning epigenetics: FKBP51 decreases the activity of DNMT1 and mediates epigenetic effects of the antidepressant paroxetine. *Sci Signal* **8,** ra119-ra119 (2015).

35. Gassen, N. C. *et al.* Association of FKBP51 with priming of autophagy pathways and mediation of antidepressant treatment response: evidence in cells, mice, and humans. *PLoS Med.* **11,** e1001755 (2014).

36. Rico-Bautista, E., Yang, C.-C., Lu, L., Roth, G. P. & Wolf, D. A. Chemical genetics approach to restoring p27Kip1 reveals novel compounds with antiproliferative activity in prostate cancer cells. *BMC Biol.* **8,** 153 (2010).

37. Yamamoto, A. *et al.* Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct. Funct.* **23,** 33–42 (1998).

38. Barth, S., Glick, D. & Macleod, K. F. Autophagy: assays and artifacts. *J. Pathol.* **221,** 117–124 (2010).

39. Perisic, T. *et al.* Valproate and amitriptyline exert common and divergent influences on global and gene promoter-specific chromatin modifications in rat primary astrocytes. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **35,** 792–805 (2010).

40. Franke, B., Figiel, M. & Engele, J. CNS glia are targets for GDNF and neurturin. *Histochem. Cell Biol.* **110,** 595–601 (1998).

41. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63 (1983).

42. Alley, M. C. *et al.* Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **48,** 589–601 (1988).

43. Gerlier, D. & Thomasset, N. Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods* **94,** 57–63 (1986).

44. Rexen, P. & Emborg, C. Investigation of different modifications to the tetrazolium based colorimetric viability assay. *Biotechnol. Tech.* **6,** 255–260 (1992).

45. Ishisaka, M. *et al.* Luteolin shows an antidepressant-like effect via suppressing endoplasmic reticulum stress. *Biol. Pharm. Bull.* **34,** 1481–1486 (2011).

46. Seelinger, G., Merfort, I., Wölfle, U. & Schempp, C. M. Anti-carcinogenic effects of the flavonoid luteolin. *Mol. Basel Switz.* **13,** 2628–2651 (2008).

47. Sakane, K. K. *et al.* Cellular and molecular studies of the effects of a selective COX-2 inhibitor celecoxib in the cardiac cell line H9c2 and their correlation with death mechanisms. *Braz. J. Med. Biol. Res.* **47,** 50–59 (2013).

48. Circu, M. L. *et al.* Correction: A Novel High Content Imaging-Based Screen Identifies the Anti-Helminthic Niclosamide as an Inhibitor of Lysosome Anterograde Trafficking and Prostate Cancer Cell Invasion. *PLoS ONE* **11,** (2016).

49. Sack, U. *et al.* Novel Effect of Antihelminthic Niclosamide on S100A4-Mediated Metastatic Progression in Colon Cancer. *J. Natl. Cancer Inst.* **103,** 1018–1036 (2011).

50. Masri, B. *et al.* Antagonism of dopamine D2 receptor/β-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc. Natl. Acad. Sci.* **105,** 13656–13661 (2008).

51. Canfrán-Duque, A. *et al.* Curcumin Mitigates the Intracellular Lipid Deposit Induced by Antipsychotics In Vitro. *PLOS ONE* **10,** e0141829 (2015).

52. Qin, L., Jia, P., Zhang, Z. & Zhang, S. ROS-p53-cyclophilin-D signaling mediates salinomycin-induced glioma cell necrosis. *J. Exp. Clin. Cancer Res.* **34,** 57 (2015).

53. van Delft, M. F. *et al.* The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10,** 389–399 (2006).

54. Konopleva, M. *et al.* Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* **10,** 375–388 (2006).

55. Williams, C. S. *et al.* Celecoxib prevents tumor growth in vivo without toxicity to normal gut: lack of correlation between in vitro and in vivo models. *Cancer Res.* **60,** 6045–6051 (2000).

56. Trzeciak, H. I. *et al.* Effect of antidepressants on ATP content, 3H-valine incorporation and cell morphometry of astrocytes cultured from rat brain. *Dev. Neurosci.* **17,** 292–299 (1995).

57. Tardy, M., Costa, M. F., Fages, C., Bardakdjian, J. & Gonnard, P. Uptake and binding of serotonin by primary cultures of mouse astrocytes. *Dev. Neurosci.* **5,** 19–26 (1982).

58. Cruz-Muñoz, W. *et al.* Analysis of acquired resistance to metronomic oral topotecan chemotherapy plus pazopanib after prolonged preclinical potent responsiveness in advanced ovarian cancer. *Angiogenesis* **17,** 661–673 (2014).

59. King, I. F. *et al.* Topoisomerases facilitate transcription of long genes linked to autism. *Nature* **501,** 58–62 (2013).

60. Desta, Z., Soukhova, N. & Flockhart, D. A. In vitro inhibition of pimozide N-dealkylation by selective serotonin reuptake inhibitors and azithromycin. *J. Clin. Psychopharmacol.* **22,** 162–168 (2002).

61. Desta, Z. *et al.* Identification and Characterization of Human Cytochrome P450 Isoforms Interacting with Pimozide. *J. Pharmacol. Exp. Ther.* **285,** 428–437 (1998).

62. Funderburk., S. F., Wang, Q. J. & Yue, Z. Beclin 1-VPS34 complex – At the Crossroads of Autophagy and Beyond. *Trends Cell Biol.* **20,** 355–362 (2010).

63. Morris, D. H., Yip, C. K., Shi, Y., Chait, B. T. & Wang, Q. J. BECLIN 1-VPS34 COMPLEX ARCHITECTURE: UNDERSTANDING THE NUTS AND BOLTS OF THERAPEUTIC TARGETS. *Front. Biol.* **10,** 398–426 (2015).

64. Bursch, W. *et al.* Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. *Carcinogenesis* **17,** 1595–1607 (1996).

65. Zhang, L. *et al.* Small molecule regulators of autophagy identified by an image-based high-throughput screen. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 19023–19028 (2007).

66. Horowitz, J. D., Sia, S. T., Macdonald, P. S., Goble, A. J. & Louis, W. J. Perhexiline maleate treatment for severe angina pectoris--correlations with pharmacokinetics. *Int. J. Cardiol.* **13,** 219–229 (1986).

67. Bjørkøy, G. *et al.* Monitoring autophagic degradation of p62/SQSTM1. *Methods Enzymol.* **452,** 181–197 (2009).

68. Can, A. *et al.* The Mouse Forced Swim Test. *J. Vis. Exp. JoVE* (2012). doi:10.3791/3638

69. Porsolt, R. D., Le Pichon, M. & Jalfre, M. Depression: a new animal model sensitive to antidepressant treatments. *Nature* **266,** 730–732 (1977).

70. Nickel, T. *et al.* Clinical and neurobiological effects of tianeptine and paroxetine in major depression. *J. Clin. Psychopharmacol.* **23,** 155–168 (2003).

71. Nixon, R. A. The role of autophagy in neurodegenerative disease. *Nat. Med.* **19,** 983–997 (2013).

72. Kesidou, E., Lagoudaki, R., Touloumi, O., Poulatsidou, K.-N. & Simeonidou, C. Autophagy and neurodegenerative disorders. *Neural Regen. Res.* **8,** 2275–2283 (2013).

73. Menzies, F. M., Fleming, A. & Rubinsztein, D. C. Compromised autophagy and neurodegenerative diseases. *Nat. Rev. Neurosci.* **16,** 345–357 (2015).

74. Ghavami, S. *et al.* Autophagy and apoptosis dysfunction in neurodegenerative disorders. *Prog. Neurobiol.* **112,** 24–49 (2014).

75. Czéh, B. & Benedetto, B. D. Antidepressants act directly on astrocytes: Evidences and functional consequences. *Eur. Neuropsychopharmacol.* **23,** 171–185 (2013).

76. Manev, H., Uz, T. & Manev, R. Glia as a putative target for antidepressant treatments. *J. Affect. Disord.* **75,** 59–64 (2003).

77. Cotter, D., Mackay, D., Landau, S., Kerwin, R. & Everall, I. Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. *Arch. Gen. Psychiatry* **58,** 545–553 (2001).

78. Rajkowska, G. & Miguel-Hidalgo, J. J. Gliogenesis and Glial Pathology in Depres-

sion. *CNS Neurol. Disord. Drug Targets* **6,** 219–233 (2007).

79. Peng, L., Wang, B. & Ren, P. Reduction of MTT by flavonoids in the absence of cells. *Colloids Surf. B Biointerfaces* **45,** 108–111 (2005).

80. Talorete, T. P. N., Bouaziz, M., Sayadi, S. & Isoda, H. Influence of medium type and serum on MTT reduction by flavonoids in the absence of cells. *Cytotechnology* **52,** 189–198 (2006).

81. He, C. & Levine, B. The Beclin 1 interactome. *Curr. Opin. Cell Biol.* **22,** 140–149 (2010).

82. Itakura, E., Kishi, C., Inoue, K. & Mizushima, N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol. Biol. Cell* **19,** 5360–5372 (2008).

83. Liang, C. *et al.* Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat. Cell Biol.* **10,** 776–787 (2008).

84. Kim, Y.-M. *et al.* mTORC1 phosphorylates UVRAG to negatively regulate autophagosome and endosome maturation. *Mol. Cell* **57,** 207–218 (2015).

85. Zhong, Y. *et al.* Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat. Cell Biol.* **11,** 468–476 (2009).

86. Matsunaga, K. *et al.* Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat. Cell Biol.* **11,** 385–396 (2009).

87. Lin, M. G. & Zhong, Q. Interaction between small GTPase Rab7 and PI3KC3 links autophagy and endocytosis. *Small GTPases* **2,** 85–88 (2011).

88. Moreau, K., Luo, S. & Rubinsztein, D. C. Cytoprotective roles for autophagy. *Curr. Opin. Cell Biol.* **22,** 206–211 (2010).

89. Jaber, N. *et al.* Class III PI3K Vps34 plays an essential role in autophagy and in heart and liver function. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 2003–2008 (2012).

90. Russell, R. C. *et al.* ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat. Cell Biol.* **15,** 741–750 (2013).

91. Loos, B., du Toit, A. & Hofmeyr, J.-H. S. Defining and measuring autophagosome flux—concept and reality. *Autophagy* **10,** 2087–2096 (2014).

92. Crider, B. P., Xie, X. S. & Stone, D. K. Bafilomycin inhibits proton flow through the H+ channel of vacuolar proton pumps. *J. Biol. Chem.* **269,** 17379–17381 (1994).

93. Mauvezin, C., Nagy, P., Juhász, G. & Neufeld, T. P. Autophagosome-lysosome fusion is independent of V-ATPase-mediated acidification. *Nat. Commun.* **6,** 7007 (2015).

94. Zhang, X., Chen, S., Huang, K. & Le, W. Why should autophagic flux be assessed?

Acta Pharmacol. Sin. **34,** 595–599 (2013).

95. Mizushima, N. & Yoshimori, T. How to interpret LC3 immunoblotting. *Autophagy* **3,** 542–545 (2007).

96. Kabeya, Y. *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19,** 5720–5728 (2000).

97. Mizushima, N., Yoshimorim, T. & Levine, B. Methods in Mammalian Autophagy Research. *Cell* **140,** 313–326 (2010).

98. Jangamreddy, J. R., Panigrahi, S. & Łos, M. J. Monitoring of autophagy is complicated—salinomycin as an example. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1853,** 604–610 (2015).

99. Jangamreddy, J. R. *et al.* Salinomycin induces activation of autophagy, mitophagy and affects mitochondrial polarity: differences between primary and cancer cells. *Biochim. Biophys. Acta* **1833,** 2057–2069 (2013).

100. Moscat, J. & Diaz-Meco, M. T. p62 at the crossroads of autophagy, apoptosis, and cancer. *Cell* **137,** 1001–1004 (2009).

101. Sahani, M. H., Itakura, E. & Mizushima, N. Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy* **10,** 431–441 (2014).

102. Zhou, C. *et al.* Monitoring autophagic flux by an improved tandem fluorescent-tagged LC3 (mTagRFP-mWasabi-LC3) reveals that high-dose rapamycin impairs autophagic flux in cancer cells. *Autophagy* **8,** 1215–1226 (2012).

103. Porsolt, R. D., Anton, G., Blavet, N. & Jalfre, M. Behavioural despair in rats: A new model sensitive to antidepressant treatments. *Eur. J. Pharmacol.* **47,** 379–391 (1978).

104. Yankelevitch-Yahav, R., Franko, M., Huly, A. & Doron, R. The forced swim test as a model of depressive-like behavior. *J. Vis. Exp. JoVE* (2015). doi:10.3791/52587

105. Balgi, A. D. *et al.* Screen for chemical modulators of autophagy reveals novel therapeutic inhibitors of mTORC1 signaling. *PloS One* **4,** e7124 (2009).

106. Li, T. *et al.* Salinomycin induces cell death with autophagy through activation of endoplasmic reticulum stress in human cancer cells. *Autophagy* **9,** 1057–1068 (2013).

107. Santiago, R. M. *et al.* Antidepressant-like effect of celecoxib piroxicam in rat models of depression. *J. Neural Transm. Vienna Austria 1996* **121,** 671–682 (2014).

108. Akhondzadeh, S. *et al.* Clinical trial of adjunctive celecoxib treatment in patients with major depression: a double blind and placebo controlled trial. *Depress. Anxiety* **26,** 607–611 (2009).

109. Müller, N. *et al.* The cyclooxygenase-2 inhibitor celecoxib has therapeutic effects in major depression: results of a double-blind, randomized, placebo controlled, add-on pilot study to reboxetine. *Mol. Psychiatry* **11,** 680–684 (2006).

110. Levine, B., Liu, R., Dong, X. & Zhong, Q. Beclin orthologs: integrative hubs of cell signaling, membrane trafficking, and physiology. *Trends Cell Biol.* **25,** 533–544 (2015).
111. Levine, B., Sinha, S. & Kroemer, G. Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy* **4,** 600–606 (2008).

112. Gao, P. *et al.* The Bcl-2 Homology Domain 3 Mimetic Gossypol Induces Both Beclin 1-dependent and Beclin 1-independent Cytoprotective Autophagy in Cancer Cells. *J. Biol. Chem.* **285,** 25570–25581 (2010).

113. Rooswinkel, R. W., van de Kooij, B., Verheij, M. & Borst, J. Bcl-2 is a better ABT-737 target than Bcl-xL or Bcl-w and only Noxa overcomes resistance mediated by Mcl-1, Bfl-1, or Bcl-B. *Cell Death Dis.* **3,** e366 (2012).

114. Gobbi, G. & Blier, P. Effect of neurokinin-1 receptor antagonists on serotoninergic, noradrenergic and hippocampal neurons: Comparison with antidepressant drugs. *Peptides* **26,** 1383–1393 (2005).

115. Berman, R. M. *et al.* Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry* **47,** 351–354 (2000).

116. Iadarola, N. D. *et al.* Ketamine and other N-methyl-D-aspartate receptor antagonists in the treatment of depression: a perspective review. *Ther. Adv. Chronic Dis.* **6,** 97–114 (2015).

117. Shehata, M., Matsumura, H., Okubo-Suzuki, R., Ohkawa, N. & Inokuchi, K. Neuronal stimulation induces autophagy in hippocampal neurons that is involved in AMPA receptor degradation after chemical long-term depression. *J. Neurosci. Off. J. Soc. Neurosci.* **32,** 10413–10422 (2012).

118. Wang, Y. *et al.* IGF-1 alleviates NMDA-induced excitotoxicity in cultured hippocampal neurons against autophagy via the NR2B/PI3K-AKT-mTOR pathway. *J. Cell. Physiol.* **229,** 1618–1629 (2014).

119. Shehata, M. & Inokuchi, K. Does autophagy work in synaptic plasticity and memory? *Rev. Neurosci.* **25,** 543–557 (2014).

120. American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders(5th ed.). Arlington, VA: American Psychiatric Publishing.

9 Danksagung

Ohne meine Tätigkeit als studentische Hilfskraft in der Abteilung Translationale Forschung des Max-Planck-Instituts für Psychiatrie unter Leitung von Dr. Elisabeth Binder wäre diese Arbeit nicht möglich geworden. Für seine freundliche Aufnahme in die Projektgruppe „Molekulare Signalwege der Depression“, seine Ermunterung und Geduld danke ich PD Dr. Theo Rein sehr herzlich. Er und Nils Gassen waren es, die mich auf das interessante Thema gebracht haben.

Mein besonderer Dank gilt meinem Betreuer Dr. Nils Gassen, der mir bei meinen Experimenten und bei der Anfertigung der Studie stets mit Rat und Tat beiseite stand. Seine unermüdliche Unterstützung und vielen Anregungen, vor allem aber die konstruktive und kreative Diskussion meiner Ergebnisse waren eine überaus wertvolle und von mir hoch geschätzte Hilfe.

Kathrin Hafner habe ich für ihre exzellente technische Assistenz und wunderbare Hilfe beim Erlernen von Western blotting zu danken.

Für meine Arbeit durfte ich auch Daten aus den Tierexperimenten von Georgia Balsevich nutzen. Das hat sie deutlich abrunden und eine wichtige Verbindung zur Klinik schaffen können, wofür ich sehr dankbar bin.

Last, but not least, danke ich Professor Dr. Günter Stalla, dass er mich und diese Arbeit als Doktorvater unter seine Fittiche genommen hat.

Eidesstattliche Versicherung

Frevert, Marie Louise

Name, Vorname

Ich erkläre hiermit an Eides statt,
dass ich die vorliegende Dissertation mit dem Thema

„24 drugs as potential SKP2 Inhibitors: a novel approach to finding new antidepressants“

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Berlin, den 13.07.2016

Ort, Datum Unterschrift Doktorandin/Doktorand