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MDM2 antagonist nutlin-3a sensitizes tumors to V-ATPase inhibition

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ABSTRACT

Treating cancer is one of the big challenges of this century and it has become evident that single chemotherapeutic treatment is rarely effective. As tumors often carry multiple mutations using combination therapy which addresses different targets seems therefore more beneficial. One of the most frequently mutated genes in tumors is the tumor suppressor p53. Significant work has been put in the development of p53 activators, which are now in clinical studies against diverse cancers. Recently, we could show that inhibition of V-ATPase, a multi-subunit proton pump, by archazolid induces p53 protein levels in cancer cells. In this study, we provide evidence that the combination of archazolid with the p53 activator nutlin-3a is synergistically inducing cell death in different p53 wild type tumor cell lines. Mechanistically, this effect could presumably be attributed to reduction of glycolysis as TIGAR mRNA levels were increased and glucose uptake and Glut1 protein levels were reduced. In addition, combination treatment highly activated pro-apoptotic pathways including IGFBP3 and Bax inducing caspase-9 and PARP cleavage. Remarkably, combination of archazolid and nutlin-3a was more efficient in reducing tumor growth compared to single dose treatment in a U87MG mouse model *in vivo*. Hence, our findings suggest the combination of archazolid and nutlin-3a as a highly promising strategy for the treatment of p53 wild type tumors.

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1. Introduction

Cancer is a major health problem worldwide. It remains the second most common cause of death in the United States

(Siegel et al., 2015). The development of tumors is a multistep process involving four to seven independent mutations in most cancers. Targeting only one pathway in this complex disease network is, in many cases, insufficient to combat

Abbreviations: V-ATPase, vacuolar-type ATPase; OXPHOS, oxidative phosphorylation; TIGAR, TP53-induced glycolysis and apoptosis regulator; U87, U87MG; IGFBP3, insulin-like growth factor-binding protein 3.

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cancer. Hence, in these cases, combination drugs with multiple targets might be necessary to gain optimal therapeutic benefit (Zimmermann et al., 2007).

Recently, our group introduced archazolid, a myxobacteria-derived vacuolar-type ATPase (V-ATPase) inhibitor (Bockelmann et al., 2010; Huss et al., 2005; Menche et al., 2007; Sasse et al., 2003), as a promising new anti-cancer agent (Schneider et al., 2015; von Schwarzenberg et al., 2012; Wiedmann et al., 2012). V-ATPases are multisubunit proton pumps expressed on endolysosomal membranes of almost all eukaryotic cells. They are responsible for maintaining the cellular pH homeostasis and play an important role in the regulation of receptor-mediated recycling (Hinton et al., 2009). *In vitro* experiments with different cancer cells showed a high cytotoxic potential of archazolid (Schneider et al., 2015; von Schwarzenberg et al., 2012). Mechanistically, V-ATPase inhibition blocked the iron metabolism of cancer cells resulting in altered glucose metabolism and p53 stabilization. In mouse experiments, we could show that archazolid treatment reduced the tumor burden, however it was not successful in abrogating tumor growth completely. This matter asked for a rational and innovative combination strategy to achieve a better therapeutic efficacy for archazolid. Based on our previously published finding that archazolid stabilizes p53, we decided to combine in this study archazolid with the small molecule p53 activator nutlin-3a, which inhibits binding of p53 and MDM2 (Vassilev et al., 2004).

The transcription factor p53 is one of the most studied tumor suppressors, which plays a key role in maintaining genomic stability. In normal unstressed cells, p53 is expressed at a low level controlled by its negative regulators like MDM2 or MDMX. p53 gets activated in response to a variety of stress signals including DNA damage, hypoxia or activation of oncogenes (Brooks and Gu, 2006; Vousden and Prives, 2009). It is frequently mutated in human tumors and best known for regulating cell cycle arrest, senescence and apoptosis (Khoo et al., 2014).

Interestingly, in recent years, it has become obvious that p53 also plays a pivotal role in regulating tumor metabolism, which strongly contributes to its tumor suppressing abilities. It regulates glycolysis, pentose phosphate pathway, oxidative phosphorylation (OXPHOS) and lipid metabolism and can counteract many of the metabolic alterations associated with cancer development. Repression of glycolysis and activation of OXPHOS are the major metabolic functions of p53 which lead to tumor growth inhibition (Berkers et al., 2013). p53 activation not only directly inhibits glucose receptor transcription (Schwarzenberg-Bar-Yoseph et al., 2004) but also leads to an upregulation of TP53-induced glycolysis and apoptosis regulator (TIGAR), which inhibits glycolysis by decreasing fructose-6-bisphosphate concentration (Bensaad et al., 2006).

Our work unveils that combination of archazolid and nutlin-3a is highly efficient in inducing cell death *in vitro* and in reducing tumor growth *in vivo* in p53-positive tumors. This is mediated by counteracting the pro-glycolytic activities of archazolid leading to a decrease of glycolysis-related parameters which may contribute to the increased cell death induction. This work provides new insight in the role of V-ATPase in tumor metabolism and proposes targeting the

metabolic changes with nutlin-3a as a promising way for cancer therapy.

2. Material and methods

2.1. Cell lines and reagents

The mammary cancer cell line MCF7, the liver carcinoma cell line HepG2 and the cervical cancer cell line Hela were recently purchased from DSMZ (Braunschweig, Germany). The mammary cancer cell line MDA-MB-231 was obtained from Cell Line Service Eppelheim (Germany). The glioblastoma cell line U87MG (U87) was a kind gift from Prof. Adrian L. Harris (Department of Oncology, Weatherall Institute of Molecular Medicine, Oxford University). MCF7 cells were cultivated in RPMI 1620 complemented with 10% FCS, 1% nonessential amino acids, 1% pyruvate and 125 µg/L insulin. HepG2 and MDA-MB-231 cells were grown in DMEM High Glucose containing 10% FCS, Hela and U87 cells in RPMI 1620 supplemented with 10% FCS. Archazolid was isolated by Prof. Dirk Menche (Kekulé-Institute of Organic Chemistry and Biochemistry, University of Bonn, Bonn, Germany). Nutlin-3a was purchased from Sigma Aldrich (Taufkirchen, Germany).

2.2. Immunocytochemistry

2×10^4 MCF7 cells were seeded onto µ-slides 8-well ibidiTreat (IBIDI, Martinsried, Germany) and were treated as indicated. Then, cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100/PBS and blocked with 1% BSA/PBS containing 0.1% Triton X-100. Antibody against p53 (Cell Signaling Technology, Danvers, MA) and AlexaFluor 488-goat-antimouse (Invitrogen, Waltham, MA) were diluted in blocking solution. Nuclei were stained with Hoechst 33342® (Hoechst) (Sigma Aldrich).

2.3. Western blot

Western blot analysis was performed as described before (Schneider et al., 2015). Following antibodies were used: p53 (#9282), pAkt Ser⁴⁷³, Akt, Bid, caspase-9, PARP (Cell Signaling Technology), Glut1 (Novus, Littleton, CO), IGFBP3, HRP goat-antimouse (Santa Cruz Biotechnology, Heidelberg, Germany), Rb (BD Bioscience, Heidelberg, Germany), Bcl-2 (Calbiochem, Darmstadt, Germany), AIF (Chemicon, Billerica, MA), actin (Millipore, Darmstadt, Germany) and HRP-goat-antirabbit (Bio-Rad, Munich, Germany).

2.4. qPCR

RNA extraction, cDNA synthesis and qPCR were performed as described before (Schneider et al., 2015). All designed primers were purchased from Metabion (Planegg, Germany).

2.5. Cell death analysis

Cell death was assessed as described elsewhere (Schneider et al., 2015).

2.6. Measurement of metabolic activity

CellTiter-Blue Assay (Promega, Mannheim, Germany) was performed according to the manufacturer's protocol to quantify metabolic activity.

2.7. Measurement of glucose uptake, ATP levels, Bax activation

Quantifications were performed as reported previously (von Schwarzenberg et al., 2012).

2.8. In vivo mouse model

For *in vivo* studies, six-week old female BALB/cOlaHsd-Foxn1^{nu}/Foxn1⁺ mice (Harlan, Indianapolis, IN) were used. 5×10^6 U87MG cells were resuspended in PBS/matrigel (Corning, Wiesbaden, Germany) (1:1) and injected subcutaneously into the flank of each mouse. After formation of palpable tumors, mice were divided into groups and treated daily intraperitoneally with 0.2 mg/kg archazolid in 5% DMSO/10% solutol/PBS, 5 mg/kg nutlin-3a 5% DMSO/10% solutol/PBS, with both or solvent respectively. Measurement of tumors was done every 2–3 days with a caliper using the formula $a \times b^2/2$. Modeling was performed using the non-linear mixed effects modeling technique with the software NONMEM 7.3 (Simeoni et al., 2004). Nutlin-3a PK model was built based on literature

data (Zhang et al., 2010). Tumor growth curves were normalized to control. Animal experiments were approved by the District Government of Upper Bavaria in accordance with the German animal welfare and institutional guidelines.

2.9. Statistical analysis

For all statistics, GraphPad Prism was used. Error bars display SEM, except *in vivo* tumor growth curves which show SD. Synergism was calculated with the Bliss formula $v = x_{combination}/((x_A - x_B) - (x_A * x_B))$ with $v > 1$ indicating synergism.

3. Results

3.1. Combination of archazolid and nutlin-3a strongly induces p53

We could recently show that the V-ATPase inhibitor archazolid induces p53 protein expression in breast cancer cells (Schneider et al., 2015). Figure 1A shows that while low dose archazolid had no effect on p53 protein level, a combination with nutlin-3a even raises p53 protein levels over the level of nutlin-3a alone. To more deeply investigate this phenomenon, we analyzed the translocation of p53 to the nucleus – a prerequisite for p53 to be activated. We found that p53 is mostly located in the cytoplasm of untreated cells, whereas

Figure 1 – p53 translocates to the nucleus in archazolid- and nutlin-3a-treated MCF7 cells. (A) p53 protein levels were analyzed by western blot in MCF7 cells after 24 h of treatment. (B) p53 and nuclei were stained in permeabilized MCF7 cells after 24 h of indicated treatment. Pictures of cells were taken with confocal microscopy. Scale bars, 10 μ m. (A, B) Representative experiments out of three independent experiments are shown. (C) p53 mRNA levels were quantified by qPCR in MCF7 cells after 24 h of treatment. Bars are the SEM of three independent experiments performed in duplicate.

it partially translocates to the nucleus after high dose archazolid treatment as well as after nutlin-3a treatment. Interestingly, a combination of the two compounds led to an almost complete reduction of cytosolic p53 and showed only activated p53 in the nucleus (Figure 1B). This increased activation is due to stabilization of the protein and not enhanced transcription as there is no elevated mRNA level after combined treatment with archazolid and nutlin-3a (Figure 1C). Interestingly, treatment with nutlin-3a even decreases p53 transcription which might be a feedback mechanism due to an increased amount of active p53. Taken together, these findings indicate that combination of archazolid and nutlin-3a might have an improved anti-tumor activity in wild type p53 cells compared to the two compounds alone.

3.2. Combination of archazolid and nutlin-3a induces synergistic cell death in cancer cells

To analyze whether the increased p53 activation by archazolid and nutlin-3a has an effect on cell death induction, we used

different p53 wild type tumor cell lines with different MDM2 and MDMX expression profiles and one breast cancer cell line with mutated p53 and treated them with single drugs and combination. Subsequent flow cytometry analysis showed for MCF7, Hela and HepG2 cells synergistic cell death induction after combination treatment and for the glioblastoma cell line U87 reduced metabolic activity (Figure 2A). As expected, combination treatment in MDA-MB-231 cells, which have mutated p53, did not show any effects (Figure 2B). These results suggest that the synergistic cell death induction depends on p53 activation.

3.3. Treatment with combination of archazolid and nutlin-3a reduces glycolysis

To find a molecular explanation for the synergistic effects of archazolid and nutlin-3a, we investigated the cellular glucose metabolism since p53 has effects on tumor metabolism (Madan et al., 2012; Matoba et al., 2006; Schwartzberg-Bar-Yoseph et al., 2004). Firstly, mRNA expression levels of TIGAR

Figure 2 – Archazolid and nutlin-3a synergistically induce cell death in p53 wild type tumor cells. (A, B) Cell death induction after archazolid and nutlin-3a treatment (48 h in MCF7, Hela and MDA-MB-231 cells; 24 h in HepG2 cells) was assessed by flow cytometry (quantification of PI-permeable cells for MCF7, Hela and HepG2 cells; quantification of SubG1 for MDA-MB-231 cells). Metabolic activity of U87 cells was analyzed by Cell Titer Blue assay. Bars are the SEM of three independent experiments performed in triplicate. Synergism was calculated with the Bliss formula (values: 1,59 for MCF7; 2,45 for Hela; 2,43 for HepG2 and 1,25 for U87).

were analyzed in MCF7 cells. TIGAR is a known target of p53 and reduces glycolysis by lowering levels of the glycolytic intermediate fructose 2,6-bisphosphate (Bensaad et al., 2006). Archazolid alone slightly downregulated TIGAR mRNA levels and nutlin-3a showed as expected a strong induction. Interestingly, combination of the two drugs led to a 14-fold induction of TIGAR which is even higher than nutlin-3a alone (Figure 3A). Along this line, combination of archazolid and nutlin-3a showed reduced glucose uptake (Figure 3B) and Glut1 protein levels in MCF7 cells (Figure 3C), which is not the case for each drug alone. Furthermore, archazolid increases the mRNA level of aldolase c, a key enzyme in glycolysis, which was diminished by combination with nutlin-3a (Figure 3D). Consistently, ATP levels were strongly reduced in MCF7 cells treated with combination of archazolid and nutlin-3a (Figure 3E). These observations indicate that nutlin-3a counters the pro-glycolytic activities of archazolid.

3.4. Combination treatment targets apoptotic pathways

Next, we wanted to connect the inhibition of glycolysis, mediated by combination of archazolid and nutlin-3a, with the apoptosis-inducing properties. First, we analyzed the expression of the p53 target proteins insulin-like growth factor-binding protein 3 (IGFBP3) and Rb. Both can inhibit glycolysis and Akt (Clem and Chesney, 2012; Elzi et al., 2012; Lee et al.,

2004) and can lead to apoptosis induction or growth arrest (Elzi et al., 2012; Fan and Steer, 1999; Lee et al., 2004). We could show that the combination of archazolid and nutlin-3a leads to a strong induction of IGFBP3 and dephosphorylation of Rb (Figure 4A,B). This effect is accompanied by an inhibition of Akt phosphorylation (Figure 4C). Interestingly, both drugs alone did not show an inhibitory effect on Akt. In addition, combination strongly increases active Bax levels (Figure 4D), but had no effect on Bcl-2, Bid and AIF (Figure 4E). Finally, cleavage of caspase-9 (Figure 4F) and PARP (Figure 4G) is induced, altogether suggesting activation of pro-apoptotic pathways.

3.5. Combination of archazolid and nutlin-3a reduces tumor growth in vivo

Finally, to prove that combination of archazolid and nutlin-3a is efficient in reducing tumor growth *in vivo* we performed a xenograft mouse model using U87MG cells. 28 Female SCID mice were treated with archazolid 0.2 mg/kg, nutlin-3a 5 mg/kg or combination over 17 days and tumor burden was measured and normalized to control (Figure 5A). Growth rate of tumors treated with combination was significantly reduced compared to control. Moreover, combination was most effective in reducing tumor volume (50.4%). Along this line, tumors treated with combination showed highest doubling times

Figure 3 – Reduced glycolysis in cells treated with archazolid and nutlin-3a. (A, D) qPCR analysis was performed in MCF7 cells to quantify TIGAR and aldolase c mRNA levels after 24 h of treatment. Bars are the SEM of three independent experiments performed in duplicate. (B) To assess glucose uptake, flow cytometry analysis was performed after 8 h of treatment in MCF7 cells. Therefore, cells were incubated with 100 μM 2-NBDG (2-[N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino]-2-Deoxy-D-Glucose) in HANKS buffer for 30 min at 37 °C. Synergism was calculated with the Bliss formula (value: 1,05). (C) Western blot analysis was performed in MCF7 cells after 48 h of treatment to detect Glut1 protein levels. One representative experiment out of three independent experiments is shown. (E) ATP levels were determined by Cell Titer Glow assay. Therefore, MCF7 cells were treated with archazolid and nutlin-3a for 24 h, subsequently incubated with CTG and analyzed with a luminometer. Synergism was calculated with the Bliss formula (value: 1,32). (B, E) Bars are the SEM of three independent experiments performed in triplicate.

Figure 4 – Combination treatment targets apoptotic pathways including IGFBP3 and Bax. (A–C, E–G) MCF7 cells were treated for 24 h and western blot analysis was performed to detect IGFBP3, Rb (48 h), pAkt, Akt, Bcl-2, Bid, AIF, caspase-9, PARP (48 h), tubulin and actin protein levels. Representative experiments out of three independent experiments are shown. (D) Flow cytometry analysis was used to quantify active Bax levels after 24 h of archazolid and nutlin-3a treatment in MCF7 cells. Bars are the SEM of three independent experiments performed in triplicate. Synergism was calculated with the Bliss formula (value: 3,13).

(Figure 5B). These data render combination of archazolid and nutlin-3a promising and attractive strategy for the treatment of p53 wild type tumors.

4. Discussion

Our study suggests combination of the V-ATPase inhibitor archazolid with the p53 activator nutlin-3a as a promising and viable strategy for the treatment of p53 wild type tumors and reveals novel insights into archazolid's mode of action.

Cancer is a multigenic disease. The process of oncogenesis requires in most tumors four to seven mutations. As only a few single-target drugs are able to efficiently combat this disease, a change to multi-target therapeutics has occurred in recent years. Combination drugs can be more efficient in controlling complex diseases and can overcome drug resistance. For example, patients suffering from breast cancer benefit from a combination of trastuzumab targeting ErbB2 with the anti-VEGF antibody Avastin®. Along this line, multi-target

therapies with traditional chemotherapeutics are well established in the clinical treatment of cancers (Zimmermann et al., 2007).

One very promising target for combination therapy is p53. A lot of work has been put in the development of p53 activators, which are now in clinical studies against diverse cancers. One very promising approach is to activate p53 through small molecule MDM2 antagonists, and recently also by MDMX inhibitors. Several groups also developed dual MDM2/MDMX inhibitors to increase efficiency, especially in cells overexpressing MDM2 and/or MDMX (Chang et al., 2013; Zaytsev et al., 2015). In order to restore p53 function, in cases where it is mutated, efforts have been made to identify drugs which stabilize p53 conformation by acting as p53 chaperones through binding to it. Moreover, gene therapy has been used to manipulate the p53 network (Khoo et al., 2014).

It was shown that the MDM2 antagonist nutlin-3 alone was efficient in reducing tumor growth of SJSA-1 xenografts (Vassilev et al., 2004), and others revealed its antitumor activity in MHM, LnCap and 22Rv1 xenografts (Tovar et al., 2006).

Figure 5 – Combination of archazolid and nutlin-3a efficiently reduces tumor growth of U87 cancer cells *in vivo*. (A) U87 tumor cells were subcutaneously injected into the flanks of 28 SCID mice (co: n = 6; arch: n = 7; nutlin-3a: n = 7; combination: n = 8). Mice were treated daily intraperitoneally with 0.2 mg/kg archazolid, 5 mg/kg nutlin-3a, both or equal amounts of solvent. Tumor growth curves are normalized to control. Bars display SD. (B) Growth rate, reduction and doubling time were calculated from data in Figure 5A. Growth rate was calculated based on control growth rate. Reduction is in relation to control.

Moreover, efficient reduction of tumor growth was shown in combination studies. For example, targeting MDM2 with nutlin-3 showed superior *in vivo* efficacy in leukemia when combined with valproic acid, which inhibits histone deacetylases (McCormack et al., 2011).

Although p53 is mostly known for its DNA repair, apoptosis and senescence inducing effects, in recent years, it became obvious that the ability of p53 to suppress glycolysis in tumors strongly contributes to its anti-cancer activity (Berkers et al., 2013).

Interestingly, our recently published study showed an upregulation of p53 by the V-ATPase inhibitor archazolid, which is involved in apoptosis induction (Schneider et al., 2015). In the last decade, the anti-tumor activity of V-ATPase inhibitors was intensively investigated. Their effectiveness *in vitro* was mostly based on a disturbance of the pH homeostasis and endocytotic recycling (Koul et al., 2013; Nakashima, 2003; Ohta et al., 1998; Schneider et al., 2015; von Schwarzenberg et al., 2012; Wu et al., 2009). But in recent years, it emerged that the V-ATPase is also strongly involved in regulating metabolism and nutrient sensing. Kozik et al. could show that V-ATPase knockdown interferes with cholesterol metabolism, Zoncu et al. reveals that V-ATPase is important for amino acid sensing and our group could show that archazolid leads to a shift towards glycolysis, which might be an escape mechanism (Kozik et al., 2013; Schneider et al., 2015; Zoncu et al., 2011).

Nevertheless, especially effectiveness of V-ATPase inhibitors in impeding solid tumor growth remains disappointing, which might be partly due to high toxicity preventing the use of higher doses (Drose and Altendorf, 1997; Lim et al., 2006; Ohta et al., 1998). This fact predestines V-ATPase inhibitors for a combination therapy which we did in this study with nutlin-3a.

As both drugs influence glycolysis – archazolid by activating it and nutlin-3a by inhibiting it – our aim was to use this combination to decrease glycolysis induced by archazolid and, as a consequence, increase cell death induction.

And indeed, p53 wild type tumor cells showed synergistic cell death induction *in vitro*, while p53 mutant cells showed no effect, suggesting a p53 dependent mechanism. To reach an increased apoptosis induction in cell lines with certain p53 mutations, a combination with a small molecule stabilizer would be an interesting option to increase cytotoxic effects.

To connect cell death induction with glycolysis, we analyzed some key mechanisms of the glycolytic pathway like ATP level, glycolytic intermediates and glucose uptake or receptor expression and found in all cases a strong reduction and partly a reversal of archazolid-induced glycolysis. Moreover, other proteins indirectly supporting glycolysis were affected by the combination. The tumor suppressor IGFBP3, which is a p53 target gene and involved in glycolytic regulation, was shown to attenuate tumor growth in several cancers (Alami et al., 2008; Butt et al., 2000) and was strongly induced by the combination. In addition, combination treatment influenced a further player in the p53 network as it strongly dephosphorylates and activates Rb. Rb is best known to lead to cell cycle arrest, but interestingly, it was recently shown that deregulation of the protein promotes glycolysis (Clem and Chesney, 2012).

The often constitutive activated serine/threonine kinase Akt, which strongly promotes aerobic glycolysis, was also significantly decreased (Elstrom et al., 2004). Interestingly, Akt directed glucose metabolism can inhibit Bax activation and therefore apoptosis induction (Rathmell et al., 2003). We could importantly show a strong induction of Bax activation after the combination treatment strengthening the hypothesis that synergistic cell death induction is based on inhibition of glycolysis. Most importantly, the combination of nutlin-3a and archazolid not only showed an effect *in vitro* but also strongly reduced growth of a wild type p53 glioblastoma xenograft mouse model compared to each drug alone.

Taken together, targeting the p53 pathway at two distinct points with archazolid and nutlin-3a unveiled strong tumor-inhibiting capabilities *in vitro* and *in vivo* in p53 wild type tumor cells. Mechanistically, combination treatment affects several parts in the p53 network and manages to counteract archazolid-induced glycolysis. These data therefore give a first insight into the molecular mechanism of the combination therapy using p53 activators and V-ATPase inhibitors.

Conflict of interest

There is no conflict of interest.

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REFERENCES

- Alami, N., Page, V., Yu, Q., Jerome, L., Paterson, J., Shiry, L., Leyland-Jones, B., 2008. Recombinant human insulin-like growth factor-binding protein 3 inhibits tumor growth and targets the Akt pathway in lung and colon cancer models. *Growth Horm. IGF Res.* 18, 487–496.
- Bensaad, K., Tsuruta, A., Selak, M.A., Vidal, M.N.C., Nakano, K., Bartrons, R., Gottlieb, E., Vousden, K.H., 2006. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* 126, 107–120.
- Berkers, C.R., Maddocks, O.D., Cheung, E.C., Mor, I., Vousden, K.H., 2013. Metabolic regulation by p53 family members. *Cell Metab.* 18, 617–633.
- Bockelmann, S., Menche, D., Rudolph, S., Bender, T., Grond, S., von Zezschwitz, P., Muench, S.P., Wiczorek, H., Huss, M., 2010. Archazolid binds to the equatorial region of the c-ring of the vacuolar H⁺-ATPase. *J. Biol. Chem.* 285, 38304–38314.
- Brooks, C.L., Gu, W., 2006. p53 ubiquitination: Mdm2 and beyond. *Mol. Cell* 21, 307–315.
- Butt, A.J., Firth, S.M., King, M.A., Baxter, R.C., 2000. Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. *J. Biol. Chem.* 275, 39174–39181.
- Chang, Y.S., Graves, B., Guerlavais, V., Tovar, C., Packman, K., To, K.H., Olson, K.A., Kesavan, K., Gangurde, P., Mukherjee, A., Baker, T., Darlak, K., Elkin, C., Filipovic, Z., Qureshi, F.Z., Cai, H., Berry, P., Feyfant, E., Shi, X.E., Horstick, J., Annis, D.A., Manning, A.M., Fotouhi, N., Nash, H., Vassilev, L.T., Sawyer, T.K., 2013. Stapled alpha-helical peptide drug development: a potent dual inhibitor of MDM2 and MDMX for p53-dependent cancer therapy. *Proc. Natl. Acad. Sci. U. S. A.* 110, E3445–E3454.
- Clem, B.F., Chesney, J., 2012. Molecular pathways: regulation of metabolism by RB. *Clin. Cancer Res.* 18, 6096–6100.
- Drose, S., Altendorf, K., 1997. Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *J. Exp. Biol.* 200, 1–8.
- Elstrom, R.L., Bauer, D.E., Buzzai, M., Karnauskas, R., Harris, M.H., Plas, D.R., Zhuang, H., Cinalli, R.M., Alavi, A., Rudin, C.M., Thompson, C.B., 2004. Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res.* 64, 3892–3899.
- Elzi, D.J., Lai, Y., Song, M., Hakala, K., Weintraub, S.T., Shio, Y., 2012. Plasminogen activator inhibitor 1-insulin-like growth factor binding protein 3 cascade regulates stress-induced senescence. *Proc. Natl. Acad. Sci. U. S. A.* 109, 12052–12057.
- Fan, G., Steer, C.J., 1999. The role of retinoblastoma protein in apoptosis. *Apoptosis* 4, 21–29.
- Hinton, A., Bond, S., Forgacs, M., 2009. V-ATPase functions in normal and disease processes. *Pflugers Arch.* 457, 589–598.
- Huss, M., Sasse, F., Kunze, B., Jansen, R., Steinmetz, H., Ingenhorst, G., Zeeck, A., Wiczorek, H., 2005. Archazolid and apiculan: novel specific V-ATPase inhibitors. *BMC Biochem.* 6, 13.
- Khoo, K.H., Verma, C.S., Lane, D.P., 2014. Drugging the p53 pathway: understanding the route to clinical efficacy. *Nat. Rev. Drug Discov.* 13, 217–236.
- Koul, H.K., Kiyoshima, T., Yoshida, H., Wada, H., Nagata, K., Fujiwara, H., Kihara, M., Hasegawa, K., Someya, H., Sakai, H., 2013. Chemoresistance to concanamycin A1 in human oral squamous cell carcinoma is attenuated by an HDAC inhibitor partly via suppression of Bcl-2 expression. *PLoS One* 8, e80998.
- Kozik, P., Hodson, N.A., Sahlender, D.A., Simecek, N., Soromani, C., Wu, J., Collinson, L.M., Robinson, M.S., 2013. A human genome-wide screen for regulators of clathrin-coated vesicle formation reveals an unexpected role for the V-ATPase. *Nat. Cell Biol.* 15, 50–60.
- Lee, H.Y., Moon, H., Chun, K.H., Chang, Y.S., Hassan, K., Ji, L., Lotan, R., Khuri, F.R., Hong, W.K., 2004. Effects of insulin-like growth factor binding protein-3 and farnesyltransferase inhibitor SCH66336 on Akt expression and apoptosis in non-small-cell lung cancer cells. *J. Natl. Cancer Inst.* 96, 1536–1548.
- Lim, J.H., Park, J.W., Kim, M.S., Park, S.K., Johnson, R.S., Chun, Y.S., 2006. Bafilomycin induces the p21-mediated growth inhibition of cancer cells under hypoxic conditions by expressing hypoxia-inducible factor-1alpha. *Mol. Pharmacol.* 70, 1856–1865.
- Madan, E., Gogna, R., Kuppusamy, P., Bhatt, M., Pati, U., Mahdi, A.A., 2012. TIGAR induces p53-mediated cell-cycle arrest by regulation of RB-E2F1 complex. *Br. J. Cancer* 107, 516–526.
- Matoba, S., Kang, J.G., Patino, W.D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P.J., Bunz, F., Hwang, P.M., 2006. p53 regulates mitochondrial respiration. *Science* 312, 1650–1653.
- McCormack, E., Haaland, I., Venås, G., Forthun, R.B., Huseby, S., Gausdal, G., Knappskog, S., Micklem, D.R., Lorens, J.B., Bruserud, Ø., Gjertsen, B.T., 2011. Synergistic induction of p53 mediated apoptosis by valproic acid and nutlin-3 in acute myeloid leukemia. *Leukemia* 26, 910–917.
- Menche, D., Hassfeld, J., Li, J., Rudolph, S., 2007. Total synthesis of archazolid A. *J. Am. Chem. Soc.* 129, 6100–6101.
- Nakashima, S., 2003. Vacuolar H⁺-ATPase inhibitor induces apoptosis via lysosomal dysfunction in the human gastric cancer cell line MKN-1. *J. Biochem.* 134, 359–364.
- Ohta, T., Arakawa, H., Futagami, F., Fushida, S., Kitagawa, H., Kayahara, M., Nagakawa, T., Miwa, K., Kurashima, K., Numata, M., Kitamura, Y., Terada, T., Ohkuma, S., 1998. Bafilomycin A1 induces apoptosis in the human pancreatic cancer cell line Capan-1. *J. Pathol.* 185, 324–330.
- Rathmell, J.C., Fox, C.J., Plas, D.R., Hammerman, P.S., Cinalli, R.M., Thompson, C.B., 2003. Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. *Mol. Cell Biol.* 23, 7315–7328.
- Sasse, F., Steinmetz, H., Höfle, G., Reichenbach, H., 2003. Archazolid, new cytotoxic macrolactones from archangium gephyra (Myxobacteria) production, isolation, physico-chemical and biological properties. *J. Antibiot. (Tokyo)* 56, 520–525.
- Schneider, L.S., von Schwarzenberg, K., Lehr, T., Ulrich, M., Kubisch-Dohmen, R., Liebl, J., Trauner, D., Menche, D., Vollmar, A.M., 2015. Vacuolar-ATPase inhibition blocks iron metabolism to mediate therapeutic effects in breast cancer. *Cancer Res.* 75, 2863–2874.
- Schwartzberg-Bar-Yoseph, F., Armoni, M., Karnieli, E., 2004. The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. *Cancer Res.* 64, 2627–2633.
- Siegel, R.L., Miller, K.D., Jemal, A., 2015. Cancer statistics, 2015. *CA Cancer J. Clin.* 65, 5–29.
- Simeoni, M., Magni, P., Cammia, C., De Nicolao, G., Croci, V., Pesenti, E., Germani, M., Poggesi, I., Rocchetti, M., 2004. Predictive pharmacokinetic-pharmacodynamic modeling of

- tumor growth kinetics in xenograft models after administration of anticancer agents. *Cancer Res.* 64, 1094–1101.
- Tovar, C., Rosinski, J., Filipovic, Z., Higgins, B., Kolinsky, K., Hilton, H., Zhao, X., Vu, B.T., Qing, W., Packman, K., Myklebost, O., Heimbrook, D.C., Vassilev, L.T., 2006. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc. Natl. Acad. Sci. U. S. A.* 103, 1888–1893.
- Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., Liu, E.A., 2004. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303, 844–848.
- von Schwarzenberg, K., Wiedmann, R.M., Oak, P., Schulz, S., Zischka, H., Wanner, G., Efferth, T., Trauner, D., Vollmar, A.M., 2012. Mode of cell death induction by pharmacological vacuolar H⁺-ATPase (V-ATPase) inhibition. *J. Biol. Chem.* 288, 1385–1396.
- Vousden, K.H., Prives, C., 2009. Blinded by the light: the growing complexity of p53. *Cell* 137, 413–431.
- Wiedmann, R.M., von Schwarzenberg, K., Palamidessi, A., Schreiner, L., Kubisch, R., Liebl, J., Schempp, C., Trauner, D., Vereb, G., Zahler, S., Wagner, E., Muller, R., Scita, G., Vollmar, A.M., 2012. The V-ATPase-Inhibitor archazolid abrogates tumor metastasis via inhibition of endocytic activation of the Rho-GTPase Rac1. *Cancer Res.* 72, 5976–5987.
- Wu, Y.C., Wu, W.K.K., Li, Y., Yu, L., Li, Z.J., Wong, C.C.M., Li, H.T., Sung, J.J.Y., Cho, C.H., 2009. Inhibition of macroautophagy by bafilomycin A1 lowers proliferation and induces apoptosis in colon cancer cells. *Biochem. Biophys. Res. Commun.* 382, 451–456.
- Zaytsev, A., Dodd, B., Magnani, M., Ghiron, C., Golding, B.T., Griffin, R.J., Liu, J., Lu, X., Micco, I., Newell, D.R., Padova, A., Robertson, G., Lunec, J., Hardcastle, I.R., 2015. Searching for dual inhibitors of the MDM2-p53 and MDMX-p53 protein-protein interaction by a Scaffold-Hopping approach. *Chem. Biol. Drug Des.* 86, 180–189.
- Zhang, F., Tagen, M., Throm, S., Mallari, J., Miller, L., Guy, R.K., Dyer, M.A., Williams, R.T., Roussel, M.F., Nemeth, K., Zhu, F., Zhang, J., Lu, M., Panetta, J.C., Boulos, N., Stewart, C.F., 2010. Whole-body physiologically based pharmacokinetic model for nutlin-3a in mice after intravenous and oral administration. *Drug Metab. Dispos.* 39, 15–21.
- Zimmermann, G.R., Lehár, J., Keith, C.T., 2007. Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov. Today* 12, 34–42.
- Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., Sabatini, D.M., 2011. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H⁺-ATPase. *Science* 334, 678–683.