The role of mTOR pathway as target for treatment in adrenocortical cancer

Maria Cristina De Martino1,2, Richard A. Feelders1, Claudia Pivonello2, Chiara Simeoli2, Fortuna Papa2, Annamaria Colao3, Rosario Pivonello2 & Leo J. Hofland1.

1Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.
2Dipartimento di Medicina Clinica e Chirurgia, Università Federico II, Naples, Italy.

Corresponding author:
Maria Cristina DE MARTINO, MD, PhD
Dipartimento di Medicina Clinica e Chirurgia
Università degli Studi di Napoli Federico II
Via Sergio Pansini 5
80131 Napoli, Italy
Tel. +39.0817462432; +393289622955
Abstract

Adrenocortical carcinomas (ACCs) are rare tumors with scant treatment options for which new treatments are required. The mTOR pathway mediates the intracellular signals of several growth factors, including the IGFs, and therefore represents a potential attractive pathway for the treatment of several malignancies including ACCs. Several mTOR inhibitors, including sirolimus, temsirolimus and everolimus have been clinically developed.

This review summarizes the results of the studies evaluating the expression of the mTOR pathway components in ACCs, the effects of the mTOR inhibitors alone or in combination with other drugs in preclinical models of ACCs and the early experience with the use of these compounds in the clinical setting.

The mTOR pathway seems a potential target for treatment of patients with ACC, but further investigation is still required to define the potential role of mTOR inhibitors alone or in combination with other drugs in the treatment of ACC patients.

Introduction

Adrenocortical carcinomas (ACCs) are rare tumors with scant treatment options for which new treatments are required.¹³ The limited efficacy of conventional antineoplastic treatment in ACCs increases the need for novel effective treatment options. During the past 15 years, progress in understanding the pathogenesis of tumors has encouraged the development of so-called “targeted drugs”, which are compounds that specifically interfere with molecular mechanisms involved in tumor cell growth and/or tumor vascular supply, leading to major advances in oncology.⁴,⁵ Targeted drugs include compounds interfering with growth factor receptors and their related signaling pathways. Mammalian target of rapamycin (mTOR) is a protein kinase of the phosphoinositide 3 kinase (PI3Ks)/protein kinase B (PKB or AKT) signaling pathway, which forms multimolecular intracellular complexes and functions as a gatekeeper of metabolism, as well as cell growth. mTOR receives signals from sensors of cell stress, intracellular nutrient levels and several growth factors, including vascular endothelial growth factor (VEGF), insulin-like growth factors (IGFs), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF).⁶⁻⁹ MTOR can be part of the two
functional complexes mTORC1 and mTORC2. Upon the binding of several growth factors to their cognate tyrosine kinase receptors, AKT is phosphorylated and activated, which in turn leads to the activation of mTOR as part of the mTORC1 complex. Activated mTORC1 complex regulates cell proliferation via the activation of mRNA translation and is mediated mainly via two downstream components, i.e. p70 ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E binding proteins (4EBP1). A more extensive description of this pathway has been previously reported and a schematic representation of the pathway is shown in figure 1. The mTORC2 complex regulates cytoskeleton function and seems to be involved in the activation of AKT function. Several drugs inhibiting the mTORC1 complex have been developed as anticancer treatment including sirolimus, temsirolimus and everolimus (traditional mTOR inhibitors) which have been approved for the treatment of different malignancy such as renal cell carcinoma and pancreatic neuroendocrine tumors. More recently some compounds which also target the mTORC2 complex have been proposed as anticancer treatment (i.e. OSI-027; AZD2014). Alterations of growth factors and their cognate receptors are considered to be involved in the pathogenesis of ACCs. Therefore, compounds interfering with tumor angiogenesis and growth factor signaling pathways represent a potential novel treatment option for the management of patients with ACCs. The mTOR pathway, being involved in both these processes, could represent a potential target for treatment of these malignancies. Moreover, the most common molecular alteration observed in ACCs is the increased expression of IGF2 mRNA which is reported in up 90% of cases. Therefore, IGF2 has been suggested to be involved in the pathogenesis of ACCs and represents a potential target for treatment in this malignancy. Since the mTOR pathway is one of the main mediators of the intracellular effects of IGFs, the study of the mTOR pathway in ACCs has been considered attractive as potential target for treatment and to better understand the pathogenesis of these tumors.

This review aims at summarizing the results of the studies evaluating the expression of the mTOR pathway components in ACCs, the effects of the mTOR inhibitors alone or in combination with other drugs in preclinical models of ACCs and the early experience with the use of these compounds in the clinical setting. Our research group largely contributed to the current knowledge on the subject.
The mTOR pathway in normal adrenals

In the normal adrenal gland a layer-specific protein expression pattern of the major components of the mTOR-pathway has been found, suggesting a uncharacterized role of the mTOR pathway in particular adrenal functions. For example, the stronger expression of several components (i.e total-mTOR, total-/phospho- 4EBP1 and total- /phospho- S6K1) of the mTOR pathway in the zona reticularis could suggest a role of this pathway in androgen production and the stronger expression of these components in the zona glomerulosa may be related to angiotensin II induced activation of the mTOR pathway. An anti-secretory effect (e.g. inhibition of cortisol production) of mTOR inhibitors in ACC cell lines has been reported, although up to date signs or symptoms of hypoadrenalism with the use of mTOR inhibitors in the clinical setting have not been clearly described. Further studies are required to clarify the specific role of the mTOR pathway in regulating steroid production.

Expression of the main components of the mTOR pathway in adrenocortical tumors

The expression of the main components of the mTOR pathway in adrenocortical tumors (ACTs) has been investigated in few studies. Only one study evaluated the mRNA expression of mTOR, S6K1 and 4EBP1 in a cohort of ACCs, demonstrating that the mRNA expression of S6K1 was significantly lower in ACCs than in benign ACTs (adrenocortical adenomas; ACAs). A highly variable protein expression of the main components of the mTOR pathway has been described in ACCs, and phospho-mTOR, phospho-S6K1 as well as phospho-4EBP1 were reported to be significantly expressed in 10-32%; 30-59% and 40-60% of cases respectively. This is summarized in TABLE 1. In the study from Nakamura, M. et al., the mean protein expression of several components of the mTOR pathway was lower in ACC samples than in ACA or normal adrenal samples, although the statistics were not reported. Similarly in a study from our group the protein expression of total and phospho-mTOR, total and phospho-S6K1 and total and phospho-4EBP1 was lower in ACCs compared to ACAs, although this difference was statistically significant only for total S6K1, and probably due to the small sample size. Even though these studies adopted different antibodies and methodologies, they all reported that the expression of the main phospho-proteins of
the mTOR pathway are not constantly found in these tumors, suggesting that this pathway is activated only in a subgroup ACCs. These data are partially in contrast with the study from Doghman, M. et al, who reported that mTOR signaling is active in childhood ACTs. These contrasting data further support the increasing body of evidence which suggests that adult ACCs and childhood ACTs are different entities. Based on these data, the mTOR pathway should not be expected to be widely involved in the pathogenesis of ACCs but might be involved in a subset of them.

Two studies investigated the potential prognostic value of the expression of some components of the mTOR pathway in ACTs (TABLE 1). We showed that S6K1 mRNA and protein expression are lower in ACCs than in ACAs, and ACC samples with a lower phospho- S6K1 and/or phospho- 4EBP1 protein expression had a significantly higher Weiss score than others. Additionally ACCs with a higher mitotic count (>5) presented a lower total S6K1 and phospho- 4EBP1 protein expression. Recently Germano et al. observed a negative phospho-mTOR staining in tumors with high Weiss score. In childhood ACTs, generally known to have a less aggressive phenotype than adult ACCs, Doghman, M. et al. reported a positive expression of some components of mTOR pathway. These data suggested that a subset of less differentiated ACCs could have an inactivation of the mTOR pathway. Therefore, the down-regulation of the mTOR pathway in ACCs warrants further investigation as a potential prognostic factor.

In the era of personalized medicine, the description of the main components of the mTOR pathway in ACCs is an important step to explore, as their presence can be considered as potential markers for treatment with mTOR inhibitors. Considering that molecular biomarkers capable to predict the clinical response to mTOR inhibitors have not been clearly identified yet, the currently available studies suggest that a subset of patients have potential molecular evidence of mTOR pathway activation. However, further studies are required to explore whether these molecular events could predict an increased sensitivity to mTOR inhibitors.

Effects of mTOR inhibitors in ACCs

The testing of mTOR inhibitors in preclinical models of ACCs is a mandatory step to explore whether these compounds could represent a novel treatment opportunity for the management of ACCs. Few
studies have evaluated the effects of different mTOR inhibitors, sirolimus, everolimus and/or temsirolimus on human ACC cancer cell lines (NCI-H295R, their clone HAC15 and SW13) and primary ACC cell cultures. Using different methodologies (TABLE 2), it was demonstrated that mTOR inhibitors inhibit the proliferation in ACC cell lines (including NCI-H295R) and showed anti-proliferative effects in some but not all ACC primary cell cultures. However, it should be considered that while NCI-H295R cells are well accepted as a good model of ACCs, a debate is still open about the appropriateness of SW13 cells as a model for this type of cancer. Taking into account this and the other potential limitations of ACC cell lines as preclinical model of ACCs, the results of the current studies might suggest that among ACC patients it could be possible to find subgroups of patients with a higher sensitivity to mTOR inhibitors.’’ The anti-proliferative effects of mTOR inhibitors in ACC cells seem to be associated with cell cycle inhibition and/or apoptosis induction although these effects have been observed only at high of the concentrations tested. Based on current data the antiproliferative effects of mTOR inhibitors at concentrations that are potentially reachable in vivo seem to be predominantly cytostatic. An anti-secretory effect of sirolimus in ACC cells has also been reported. In mice, the inhibition of NCI-H295R xenograft growth has been reported using high everolimus dose. Additionally, sirolimus was found to significantly reduce cell survival and cortisol secretion only in selected ACC primary cultures. These data suggest that a subset of patients with ACCs might be more sensitive than others to this treatment. Therefore, further studies are warranted to find potential biomarkers predictive of response to treatment with mTOR inhibitors in ACCs. In this respect, the protein expression of the main components of the mTOR pathway was investigated in relation to the in vitro effects of mTOR inhibitors in ACC primary cultures. However, the expression of none of the evaluated proteins correlated with the in vitro response to these drugs. This absence of a correlation could be due to the low number of primary cultures used in this study. However, specifically designed clinical trials can appropriately evaluate for biomarkers predictive of response to treatments. Unfortunately, this type of clinical trials is extremely difficult to perform in such a rare cancer as ACCs. Therefore, progress in this direction can only be awaited from the results of clinical trials in other more common
types of cancer. Once a clear predictive biomarker is identified in other cancers, its value in ACCs should be explored.

To the best of our knowledge, the effects of compounds targeting the mTORC1 and 2 complex in ACC cell lines have not been explored yet. A recent study reported that n-3 polyunsaturated fatty acids prevent ACC growth by inhibiting mTORC1/2 in preclinical models of ACCs, which suggests that both mTORC complexes might play a role in ACC cell proliferation\textsuperscript{33}. Another compound that was reported to inhibit ACC growth in preclinical models of ACCs is the dual PI3-kinase/mTOR inhibitor NVP-BEZ235\textsuperscript{34}. These new class of compounds require future investigations.

**Relationship between the mTOR and the IGF pathways in ACCs**

The relationship between the mTOR and the IGF pathways in ACCs has been scantily investigated\textsuperscript{10}. As the mTOR pathway mediates some of the IGFs effects\textsuperscript{10, 35, 36}, it could be involved in mediating the pathogenic effects of IGFs in ACCs. Therefore it might be important to understand whether a differential expression of the main components of the IGF pathway could influence the *in vitro* sensitivity to mTOR inhibitors and whether there is a rational to combine drugs targeting the IGF and the mTOR pathways.

The relationship between the mTOR- and the IGF pathways in the NCI-H295R and SW13 ACC cell lines is addressed in a few studies\textsuperscript{24, 28}. These studies demonstrate that both ACC cell lines have a similar protein expression of IGF1R and the main components of the mTOR pathway, but both mRNA and protein expression of IGF2 were considerably higher in NCI-H295R compared with SW13. IGF1 significantly stimulated AKT and S6K1 phosphorylation in both NCI-H295R and SW13, demonstrating that the mTOR pathway acts as an intracellular mediator of IGFs in both human ACC cell lines\textsuperscript{24}. A schematic representation of the pathway is shown in figure 1. Therefore, the mTOR pathway could also be involved in mediating the proliferative effects of IGFs in ACC cell lines.

However, the effects of the mTOR inhibitor sirolimus on the IGF-activated intracellular pathways were different between NCI-H295R and SW13 cells. At the experimental condition tested, IGF1 induced the activation of the AKT/mTOR pathway in both cell lines, but ERK activation was observed only in NCI-H295R. Sirolimus efficiently suppressed the mTORC1 activity in both cell lines. However, only in NCI-H295R cells, the inhibition of mTORC1 activity was associated with the
activation of AKT, likely representing an escape pathway. This activation was further enhanced by IGF1 administration which also induced ERK stimulation in the sirolimus treated NCI-H295R cells. Therefore, the NCI-H295R cell line seems to have two potential pathways of escape to treatment with traditional mTOR inhibitors: the AKT and ERK pathways (see figure 1 for the potential escape pathways) 35, 37. The activation of these escape pathways could be related, at least partially, to the IGF2 overexpression in NCI-H295R, which is not found in the SW13 cell model. Therefore, it could be speculated that high IGF2 expression could negatively influence the in vitro sensitivity of ACC cell lines to mTOR inhibitors, which supports the rationale to combine mTOR inhibitors and drugs specifically targeting the IGF pathway in ACCs 31. In another study everolimus has been reported to inhibit S6K1 phosphorylation in both NCI-H295R and SW13, to only slightly reduce AKT phosphorylation at the highest drug concentration used and to have no effect on ERK phosphorylation 30. High everolimus doses might reduce AKT phosphorylation sequestering of the mTOR as part of the mTORC1 complex and subsequently inhibiting the mTORC2 activity 10.

IGF2 overexpression is very common in ACC (about 80%) 18, whereas only a subset of ACC samples strongly expressed the components of the mTOR pathway, particularly the phospho-proteins 28. In the studies from our research group a subgroup of 16 ACC samples was characterized for protein expression of the main components of both mTOR and IGF pathway, including IGF2 28, 31. Within this subgroup of ACC samples, we were not able to find correlations between these proteins (TABLE 3; personal unpublished data). Therefore, the expression of the main components of the mTOR and the IGF pathways seem not to be strongly related, which raises the questions whether in ACCs there is a dissociation between the expression of IGF2 and the activation of the classical IGF stimulated intracellular pathways, and whether the role of IGF2 in the pathogenesis of adult ACCs may have been overestimated, in agreement with some other recent speculations 38. However, it should also be considered that the complexity of the IGF system may have been underestimated since ACC express other components of the IGF pathway as well, such as the insulin receptor subtype A and the IGF2R 31, 36. These components have been scantily considered up today. As such, before to finally declare a “game over” 38 for the role of IGF2 in
adrenocortical tumorigenesis and as a potential target for novel treatment in ACC patients, it could be probably useful to return to the bench and try to better explore the IGF pathway in ACCs in its whole complexity.

**Effects of mTOR inhibitors in combination with other drugs in ACTs**

The data derived from the use of the mTOR inhibitors alone in preclinical studies\(^24, 25, 28-30\), together with the expected heterogeneity of ACCs\(^25, 28, 39\), suggest that caution is required before using this class of drugs in unselected ACC patients. Such caution was also suggested by preliminary clinical experience with the use of everolimus in some ACC patients with a late stage of disease \(^40\).

Unfortunately, due to the current lack of molecular biomarkers capable to predict the response to mTOR inhibitors in ACCs\(^25, 28\), it is difficult to define selection criteria for ACC patients that are candidate for treatment with this class of drugs. Therefore, combination of mTOR inhibitors with other drugs, potentially active in ACCs, could be a more prudent clinical approach than the use of these inhibitors as monotherapy in unselected ACC patients.

Until recently, the IGF pathway was considered as the most attractive target for new treatment in ACCs\(^10, 41, 42\) with a potential rationale to combine mTOR inhibitors with drugs targeting the IGF pathway\(^11, 24\). Linsitinib (OSI-906) is an IGF1-R/Insulin receptor blocker that has recently been tested in a phase III trial in ACC patients\(^43\). It was shown that only a very small subgroup of patients seems to benefit from treatment with this drug, but the anticipated improvement in overall or progression-free survival was not observed. This observation again illustrates that ACCs is a very heterogenous disease. However, whether combining drugs that target the IGF-system with other compounds, such as mTOR inhibitors, could be more effective requires further investigation. A recent study explored the \textit{in vitro} effects of mTOR inhibitors in combination with linsitinib and showed that, particularly when cells were cultured in medium with low serum, combined treatment of mTOR inhibitors with linsitinib has additive growth inhibitory effects on ACC cells at pharmacological concentrations\(^31\). This supports a potential role for treatment strategies combining mTOR inhibitors and drugs targeting the IGF pathway in ACCs. These results are in line with a recently published phase I study demonstrating
that a subgroup (about 40%) of ACC patients treated with cixutumumab (IGF1R inhibitor) and temsirolimus (mTOR inhibitor) experienced long term disease stabilization (longer than 6 months) 44.

Another attractive candidate for new combination treatment strategies in ACCs is mitotane, since this drug is currently considered as a key drug in the treatment of patients with advanced ACCs. Unfortunately the majority of studies suggest that about two-thirds of patients do not respond and/or do not tolerate this drug 1, 45-47. In ACC cell lines, two studies reported the effects of mTOR inhibitors in combination with mitotane22, 25. One study demonstrated that sirolimus had some additive effects with mitotane, but only when mitotane was used at low concentration22; whereas another study reported that mitotane blocked the anti-proliferative effects of everolimus25. Although these studies are contrasting in their main final conclusions, both studies show that the effects of mitotane can, at least in some conditions, overcome the effects of the mTOR inhibitors thus limiting the usefulness of combining full doses of these two therapeutic agents. As above-mentioned, the preclinical results show that the addition of sirolimus to low concentrations of mitotane has stronger antiproliferative effects than mitotane alone22. If these results could be translated to humans, they suggest that the addition of sirolimus might add to the antitumor action of mitotane, thereby reducing the mitotane dose required to obtain a desired clinical effect with potentially fewer side effects. In a clinical setting, mTOR inhibitors can be metabolised by the microsomal liver enzyme cytochrome P450 (CYP3A4/5). Drugs as mitotane are capable to induce these enzymes, and might increase the liver metabolization of mTOR inhibitors, potentially reducing the plasma concentration of these compounds to sub-therapeutic levels44, 48. The combination of the mTOR inhibitor everolimus and the tyrosine kinase inhibitor sorafenib has been evaluated in preclinical models of ACCs in which it is shown that combined treatment was more effective than single drug treatment both in ACC cell lines and in xenograft models. These data support the rationale for combined treatment in this type of malignancy30.

**New potential targets for ACCs in addition to the IGF and mTOR pathways**

Up today, most of the early clinical experience with targeted drugs, including drugs targeting the IGF pathway, failed to demonstrate the desired effects in patients with ACCs 2, 38, 43. This raises the
question whether molecular events, potentially targetable with currently developed drugs, are present in at least a subset of ACC patients. Using hotspot gene sequencing and comparative genomic hybridization, the presence of a large number of mutations and copy number abnormalities of potential interest for therapeutic aims, were evaluated in a large group of adult ENSAT stage III-IV ACC samples. No relevant alteration in the evaluated components of the mTOR and IGF pathways were found with these techniques and no simple targetable molecular event emerged\(^\text{21, 39}\). Therefore, based on genomic alterations, the cell cycle appeared to be the most relevant new potential therapeutic target for patients with advanced ACC (figure 1). Recent data from exome sequencing confirm that the cell cycle or WNT pathways might be future target for treatment in ACCs\(^\text{20, 49}\). Further studies to explore the effects of these compounds in preclinical models of ACCs are warranted.

Overall current data underline that, despite the fact that during the last 10 years much progress has been made in describing the molecular alteration in ACCs, the translation of these progress from bench to the bedside with the aim to improve the treatment of patients with ACCs has not been easy, so far.

**Conclusion and future directions**

In conclusion, the mTOR pathway seems a potential target for treatment of a subset of patients with ACCs, but treatment strategies combining mTOR inhibitors with other drugs are expected be more effective than the use of mTOR inhibitors alone. Additionally, considering the potential heterogeneity of this malignancy, treatment strategies based on the selection of patients with a potentially higher chance to respond to mTOR inhibitors according to their tumor characteristics, might be more effective than the use of mTOR inhibitors in unselected patients. Unfortunately, molecular biomarkers capable to predict a clinical response to mTOR inhibitors have not been clearly identified yet. Therefore, further preclinical and clinical investigations are required to find new molecular biomarkers useful to predict tumor response to both conventional and novel treatments for patients with ACCs and to address the role of mTOR inhibitors, alone or in combination with other drugs, in selected subgroups of patients with these tumors. All these data could help to move into the direction of a more
personalized approach to the treatment of ACCs, and hopefully this approach could lead to progress in the clinical management of this rare but aggressive disease.

**Declaration of interest:** there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding:** this research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

**References**

11. Robbins HL & Hague A. The PI3K/Akt Pathway in Tumors of Endocrine Tissues. *Front Endocrinol (Lausanne)* 2015 **6** 188.


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**Figure legend**

**Figure 1.** Schematic representation of the potential molecular pathways representing potential targets for treatment in patients with ACC, based on the results presented in the current thesis. GFs: growth factors; GFR: growth factor receptor. Brown lines shows two potential escape pathways to the treatment with mTOR inhibitors: AKT and ERK activation.
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<th>Number of ACA</th>
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<th>Comparison with ACC</th>
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<td>mTOR monoclonal; Phospho-mTOR (Ser2448) monoclonal; p70 S6 Kinase monoclonal; Phospho-p70 S6 Kinase (Thr389) monoclonal; 4E-BP1 monoclonal; Phospho-4E-BP1 (Thr37/46) polyclonal</td>
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<td>Tissue microarrays. Staining was assessed for all but one antibody, using a binary scoring system based on the evaluation of cytoplasmic/membrane staining: score 0 = no staining; score 1 = positive staining.</td>
<td>phospho-Akt (Ser473) polyclonal; phospho-mTOR (Ser2448) monoclonal; Phospho-p70 S6 Kinase (Thr389) monoclonal; Phospho-4E-BP1 (Ser65) polyclonal</td>
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<td>Doghman M et al</td>
<td>H295R; SW13; Primary pediatric ACT cells</td>
<td>Everolimus</td>
<td>Cells were counted after 6 days of culture in the presence of the drug.</td>
<td>Inhibition of cell proliferation</td>
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<td>H295R xenografts in NOD/SCID/γc null mice treated with placebo or with RAD001 (10/mg/kg/d). Tumor growth was significantly different in animals treated with the drug (P &lt; 0.01).</td>
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<td>De Martino MC et al</td>
<td>H295R; HAC15; SW13</td>
<td>Sirolimus or everolimus</td>
<td>After 24 hours, 3, 6, and 9 days of treatment, the cells were harvested for DNA measurement.</td>
<td>Sirolimus and everolimus significantly suppressed the cell growth in a dose- and time-dependent manner.</td>
<td>Anti-IGF2-neutralizing antibody</td>
<td>72-hour treatment with sirolimus combined with anti-IGF2 Abs almost totally blocked H295R cell proliferation (90% inhibition). Sirolimus or anti-IGF2 antibodies significantly reduced SW13 cell proliferation of 64 and 62% respectively.</td>
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<td>Marinello B et al</td>
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<td>Everolimus</td>
<td>After 2 days of culture, cells were maintained overnight in low serum medium and drug incubation was started. Cell viability studies employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were performed 24 and 72 hours after treatment.</td>
<td>Dose-dependent cytotoxicity on SW13 and H295R.</td>
<td>Cell Viability Assay (WST-1)</td>
<td>Sorafenib MTT assay. The combination of everolimus with sorafenib produced a further reduction of SW13 cell viability but not in H295R. Sorafenib monotherapy showed no significant activity on either SW13 and H295R xenografts, whereas administration of everolimus alone delayed SW13 tumor growth but was ineffective against H295R xenografts. Combination therapy produced remarkable tumor growth inhibitory effects on both SW13 and H295R xenografts; pharmacological treatments affected median survival of SW13 and H295R treated xenografts.</td>
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<td>After 72 hours treatment a Cell Viability Assay (WST-1) was performed</td>
<td>Everolimus induced a dose-dependent decrease of cell viability in the two adrenal cancer cell lines.</td>
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<td>Sirolimus and everolimus inhibited cell proliferation in H295R and HAC15 cells in a dose-dependent manner.</td>
<td>Linsitinib DNA measurement.</td>
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**ACT:** Adrenocortical Tumors
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NA: not available; *Considerable expression defined as an intermediate to high expression as reported in De Martino et al. Endocrine Relatede cancer 2014