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The Renin Angiotensin System (RAS) mediates bifunctional growth regulation in melanoma and is a novel target for therapeutic intervention

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Abbreviations: AngII: Angiotensin II; AZA: Azacytidine; AT1R: angiotensin receptor type 1; AT2R: angiotensin receptor type 2; BT: Breslow Thickness; GBM: Glioblastoma multiforme; MVP:
Methylation Variable Position; RAS: renin angiotensin system; SSM: superficial spreading melanoma; TSA: Trichostatin A; VGP: vertical growth phase; RGP: radial growth phase.

Abstract

Despite emergence of new systemic therapies, metastatic melanoma remains a challenging and often fatal form of skin cancer. The renin-angiotensin system (RAS) is a major physiological regulatory pathway controlling salt-water equilibrium, intravascular volume and blood pressure. Biological effects of the RAS are mediated by the vasoactive hormone angiotensin II (AngII) via two receptor subtypes, AT1R (encoded by AGTR1) and AT2R (encoded by AGTR2). We report decreasing expression and increasing CpG island methylation of AGTR1 in metastatic versus primary melanoma and detection in serum of methylated genomic DNA from the AGTR1 CpG island in metastatic melanoma implying that AGTR1 encodes a tumour suppressor function in melanoma. Consistent with this hypothesis, antagonism of AT1R using losartan or shRNA-mediated knock-down in melanoma cell lines expressing AGTR1 resulted in acquisition of the ability to proliferate in serum-free conditions. Conversely, ectopic expression of AGTR1 in cell lines lacking endogenous expression inhibits proliferation irrespective of the presence of AngII implying a ligand-independent suppressor function for AT1R. Treatment of melanoma cell lines expressing endogenous AT2R with either AngII or the AT2R-selective agonist Y6AII induces proliferation in serum-free conditions whereas the AT2R-specific antagonists PD123319 and EMA401 inhibit melanoma growth and angiogenesis and potentiate inhibitors of BRAF and MEK in cells with BRAF V600 mutations. Our results demonstrate that the RAS has both oncogenic and tumour suppressor functions in melanoma. Pharmacological inhibition of AT2R may provide therapeutic opportunities in melanomas expressing this receptor and AGTR1 CpG island methylation in serum may serve as a novel biomarker of metastatic melanoma.
Introduction

Stage IV (metastatic) melanoma frequently presents as large volume, multi-focal disease often with central nervous system (CNS) involvement, with at least 40% of melanoma patients developing brain metastases. Recent therapeutic advances have improved outcomes for some patients with advanced melanoma. These include the use of targeted agents such as the BRAF and MEK kinase inhibitors in patients whose melanomas contain mutations in BRAF and NRAS and immunotherapy with the anti-CTLA4 antibody ipilimumab (1, 2, 3, 4, 5) and anti-PD-1 agents such as nivolumab and pembrolizumab (6, 7). Despite the introduction of these new approaches to clinical practice, many patients continue to have poor outcomes and there remains a clear need for both novel therapeutic strategies as well as serum biomarkers to inform adjuvant or earlier treatment with immunotherapy.

The renin-angiotensin system (RAS) is a key physiological pathway involved in intravascular volume regulation. Angiotensin II (AngII) an octapeptide hormone is the biological effector and key player with important functions in maintenance of salt-water balance, blood pressure control and other fundamental biological pathways. The effects of AngII are mediated through two G protein coupled receptors, the AT1R (encoded by AGTR1) and AT2R (encoded by AGTR2). Activation of the receptors is thought in some systems to have opposing effects (8). Relatively little is known of the involvement of the RAS in cancer. However, a small subset of oestrogen receptor (ER) positive and HER2 negative breast cancers over-express AT1R and their growth can be partially inhibited by the selective AT1R antagonist losartan commonly used as an anti-hypertensive agent (9, 10, 11). Consistent with these observations, down-regulation of AGTR1 by the miRNA miR-410 suppresses growth, invasiveness, angiogenesis and migration of pancreatic cancer cells, suggesting that AT1R may also have oncogenic properties in pancreatic cancer (12). In melanoma, inhibition of angiogenesis by losartan was observed in a murine model (13) but no studies have been carried out in human melanoma and the associated function of AT2R signalling remains unexplored.

Previous studies have identified an array of genes subjected to methylation-dependent transcriptional silencing in melanoma. Furthermore, detection of methylated genomic DNA in peripheral blood is an attractive potential source of cancer biomarkers because of the stability of DNA and the relative specificity of methylation for malignant disease (14). The requirement for sensitive and specific biomarkers of recurrent and particularly unresectable/metastatic melanoma has been emphasised by
evidence from recent clinical trials that efficacy of anti-melanoma therapies is superior, with improved clinical outcomes, when such drugs are deployed in low volume disease (15). We previously showed that serum detection of methylated genomic DNA from TFPI2 is associated with metastatic melanoma (16) and others have also shown the utility of this approach (17, 18). Herein, we explored the involvement of the two principal angiotensin receptors in melanoma and demonstrate the potential to exploit RAS alteration both for therapeutic and diagnostic purposes.

Results

Expression of AGTR1 and AGTR2 in melanoma cell lines

We analysed the expression of AGTR1 and AGTR2 in a panel of melanoma cell lines arising respectively from radial growth phase (RGP; PMWK, WM35, SBCL2), vertical growth phase (VGP; WM902.B (in figure 1A it is WM902.6), SKMEL224, MEL505 and metastatic melanoma (WM266.4, SKMEL2, SKMEL23, SKMEL30, SKMEL147, SKMEL173, SKMEL501, COLO829, A375). AGTR1 mRNA was detectable in normal human melanocytes and in each of the RGP cell lines, with the highest expression in PMWK but was greatly down regulated in the WM902B VGP cell line (Figure 1A) and undetectable in SKMEL224 and MEL505. In metastatic melanoma cell lines, expression was undetectable in SKMEL23, WM266.4, SKMEL2 and A375M and greatly down-regulated in SKMEL30 and COLO829 (Fig 1A). Although expressed at lower levels than AGTR1, AGTR2 mRNA was nonetheless detectable in most cell lines with the exception of SKMEL2 and SKMEL224 where there was no detectable expression. Highest expression was observed in PMWK, WM35, MEL505, COLO829 and SKMEL23 (Figure 1A). We also determined whether expression of AGTR1 and AGTR2 was affected by serum-free conditions. PMWK, SKMEL23 and SKMEL224 cells were grown in serum-free conditions and mRNA was harvested at various time points and analysed for AGTR1 and AGTR2 expression by qPCR. No change in expression of either gene was detected up to 48 hours (data not shown).

AGTR1 silencing occurs in melanoma cell lines via CpG island methylation

Expression analysis revealed down-regulation of AGTR1 in multiple VGP and metastatic melanoma cell lines (Figure 1A). We wished to explore the mechanistic basis for this observation. A CpG island
is located in the 5´ regulatory sequences of AGTR1 and we performed pyrosequencing to measure methylation in the cell line panel. Representative pyrograms are shown in Figure 1B and methylation density profiles are presented in Figure 1C. In normal melanocytes, each of the 7 analysed methylation variable positions (MVP) was entirely unmethylated (Figure 1C). In contrast, we observed dense methylation in several of the melanoma cell lines (Figure 1C; Table 1). Of note, the majority of cell lines with dense AGTR1 CpG island methylation were VGP and metastatic melanomas whereas the RGP cell lines PMWK, SBCL2 and WM35 all showed only low level methylation. In general, there was a good correlation between methylation and transcriptional silencing of AGTR1. To further confirm that CpG island methylation causes silencing of AGTR1, PMWK, SKMEL23 and SKMEL224 cells were grown in the presence or absence of the demethylating agent 5'-azacytidine. 5'-Azacytidine had no effect on AGTR1mRNA levels in PMWK cells (CpG island unmethylated) but resulted in increased levels in SKMEL23 and SKMEL224 (CpG island methylated) (Figure 1D). Addition of the HDAC inhibitor Trichostatin A (TSA) caused a further increase in AGTR1 mRNA levels in SKMEL23 and SKMEL224 (Figure 1D).

**AGTR1 is down regulated by CpG island methylation in metastatic melanoma**

Together, the above data imply that AT1R may have tumour suppressor function in melanoma and suggests that inactivation of AGTR1 via CpG island methylation occurs as a late event in melanoma. To validate this hypothesis in clinical tumour samples, we tested a series of paired primary and metastatic melanomas together with control benign nevi for AGTR1 expression and CpG island methylation using qPCR. AGTR1 was readily detectable using qPCR in control benign nevi.

Expression was typically greatly down regulated in metastatic melanomas relative to matched primary and frequently undetectable in the metastasis (Figure 2A). We then tested AGTR1 CpG island methylation in primary and metastatic melanomas, again using histologically confirmed benign pigmented nevi as controls. Representative pyrograms are shown in Figure 2B. In each of the benign nevi the AGTR1 CpG island was unmethylated or methylated at a very low level showing that methylation is a feature of malignant melanocytes (N1-N8, Figure 2C). Similarly, in a series of primary melanomas with a Breslow thickness (BT) of 1mm or less which had not metastasised, the CpG island was invariably unmethylated (P1-P10, Figure 2C). In contrast, in primary melanomas from which metastatic disease subsequently developed, there was a clear increase in methylation compared to low-risk melanomas (P11-P16, Figure 2C). Moreover, in 5/6 cases where matched
primary and metastasis was available, methylation increased from the primary to the metastatic lesion (P11-P16 and M11-M16, Figure 2C).

**AGTR1 CpG island methylation is a serum biomarker of metastatic melanoma**

The association of AGTR1 CpG island methylation with metastatic melanoma prompted us to explore whether methylated genomic DNA from the AGTR1 CpG island is detectable in peripheral blood samples of patients with melanoma. We analysed a cohort of sera from 63 patients with (13) or without (50) distant metastatic disease and as controls we used sera from healthy individuals. Serum was isolated using a standardised protocol and methylation in the AGTR1 CpG island analysed using pyrosequencing. Representative methylation profiles indicating the % methylation at each of the 7 MVPs in the analysed fragment are shown in Figure 2D wherein the increased methylation levels present in sera from patients with metastatic disease are clearly evident. Methylated genomic DNA from AGTR1 was not detected in the serum of healthy controls. However, positivity for detection of methylated AGTR1 (defined as % methylation of ≥ 5 at 3 or more MVPs) was significantly higher in patients with metastatic disease (bone, lung liver, spleen) than in those without metastases: 7/13 (54%) vs 6/50 (12%), p=0.0058.

**Down-regulation of AGTR1 with over-expression of AGTR2 in melanoma CNS metastases**

Central nervous system (CNS) metastasis is a common event in melanoma patients. Given the relationship implied between loss of AGTR1 and development of metastatic melanoma, we were interested therefore to examine the potential involvement of the RAS in CNS metastasis and to determine the expression patterns of AGTR1 and AGTR2. To address this, we generated four novel CNS metastatic melanoma cell lines de novo from tissue obtained at surgical resection of intracranial metastases confirmed by histopathology to be metastatic melanoma and expression of AGTR1 and AGTR2 was determined by qPCR in early passage cells. AGTR1 was expressed in one cell line but was undetectable in the other 3 (Figure 2E). In contrast, AGTR2 was expressed in 3/4 melanoma brain metastasis cell lines but was not expressed in control brain tissue (Figure 2E).

**Antagonism of AT1R confers serum independence to melanoma cell lines**
Transcriptional silencing of AGTR1 implies a tumour suppressor rather than oncogenic function for AGTR1 in melanoma. To seek mechanistic evidence to support this hypothesis, we investigated the effect of antagonism of AT1R. Since expression of AGTR1 was highest in PMWK and SKMEL147 (Figure 1A), we selected these cell lines to initially assess the effect of antagonism using the selective AT1R blocker losartan. In serum replete conditions, losartan had no effect as both cell lines readily proliferated. However, in serum free conditions an increase in proliferation was observed in both cell lines on day 3 and day 5 in the presence of losartan (Figure 3A and 3B). Promotion of PMWK and SKMEL147 proliferation by losartan was reproducibly observed in multiple independent experiments (PMWK: p=0.0066, SKMEL147: P=0.005). To confirm the specificity of the effect of losartan on proliferation we tested SKMEL224 cells that do not express AGTR1. These cells grew readily in serum-free conditions and growth was unaffected by the addition of Losartan in contrast to the clear growth promoting effect of losartan on PMWK cells (Figure 3C). Similarly, losartan had no effect on the proliferation of MEL505 and SKMEL30 that also lack expression of AGTR1 but promoted serum-free growth of C8161 and SKMEL173 both of which express AGTR1 (data not shown). We conclude, therefore, that the ability of losartan to promote melanoma growth requires expression of AGTR1.

**Loss of AT1R confers growth factor independence in melanoma**

Rather than acting as an oncogenic receptor (as reported in a subset of breast and other cancers), our results imply a negative growth regulatory function for AT1R in melanoma. To further examine the association between the absence of AT1R and serum-free growth in melanoma we used inhibitory RNA to modulate expression of AGTR1. We generated stable knockdowns using the TRIPZ Inducible Lentiviral shRNA vector system (Dharmacon) that allows reversible, controlled gene silencing. The vector is designed to be Tet-On® such that shRNA expression is induced in the presence of doxycycline. PMWK cells stably expressing the AGTR1 shRNA vector or the non-silencing control vector were cultured in 10% FBS, 2% FBS and 1% FBS conditions in the presence or absence of doxycycline. Doxycycline was added at 2μg/ml daily for the course of the experiment to maintain AGTR1 knockdown. Knockdown of AGTR1 was confirmed by qPCR and western blotting (Figure 4A, upper panels). Proliferation of cells was assessed on day 5. All cells maintained in 10% serum were able to proliferate in the presence or absence of doxycycline (Figure 4A, lower panels). However, in 1% serum conditions, PMWK cells expressing AGTR1 shRNA grew more
efficiently in the presence of doxycycline (where AGTR1 is knocked down) than in its absence and more efficiently than the non-silencing cells in both conditions (p<0.05; Figure 4B). These data show that even modest decreases in levels of AGTR1 result in enhanced proliferation in conditions of growth factor limitation.

**AGTR1 is a ligand independent suppressor of melanoma growth**

Next, we examined the effect of ectopic expression of AGTR1 in cells that lack endogenous expression of the gene. We transfected the AGTR1 null cell line SKMEL224 with escalating concentrations of AGTR1 expression plasmid or control plasmid (0.1µg -1µg) and selected transfectants in G418. Surviving colonies transfected with the control plasmid were obtained at comparable frequency in all control plasmid concentrations but no transfectants were obtained at any of the AGTR1 expression plasmid input concentrations (Figure 4C). Importantly, the colony suppressing effect of ectopically expressed AGTR1 did not require exogenous AngII. Further attempts to generate stable AGTR1 expressing clones in serum and serum-free conditions and using another null line (SKMEL23) were also unsuccessful (Figure 4C). We also performed clonogenic assays by transiently transfecting SKMEL224 and SKMEL23 cells in serum and serum free conditions as described in methods. However, as with our previous experiments, in both serum conditions colonies were only obtained with the control plasmid. To rule out a potential non-specific toxic effect of the AGTR1 expression plasmid, we transfected the AGTR1 expressing line, PMWK with this plasmid and successfully generated stable clones (Figure 4C). Taken together, these results imply that ectopic expression of AGTR1 is detrimental to cell lines lacking expression of the endogenous gene and this effect is independent of AngII.

**AngII drives proliferation of melanoma cell lines expressing AT2R**

AngII has been shown to promote proliferation in a subset of breast cancers which overexpress AT1R and given the evidence of transcriptional silencing of AGTR1 in melanoma we wished to determine the effect of AngII on melanoma proliferation. Accordingly, we initially tested the effect of varying concentrations of AngII (1pM-100pM) on the proliferation of melanoma cell lines with differing expression of AT1R and AT2R (as determined in Figure 1A), namely PMWK (AGTR1 +/ AGTR2 +), SKMEL 224 (AGTR1 -/ AGTR2 -) and SKMEL23 (AGTR1 -/ AGTR2 +). Under normal culture conditions in media supplemented with 10% serum (non-starved) AngII had no effect on the
proliferation of GBM cell lines irrespective of their expression of angiotensin receptors (Figure 4D). Foetal bovine serum (FBS) contains an abundance of growth promoting as well as inhibitory factors that could mask the growth enhancing effects of angiotensin II (19). We therefore repeated this experiment in serum free conditions and under these conditions significant differences in growth were observed (Figure 4D). In serum-free medium and in the absence of AngII, PMWK and SKMEL23 cells proliferated minimally. However, in the presence of AngII both cell lines exhibited a significant dose-dependent increase in growth (PMWK: 1µM, p=0.0896; 10nM, p=0.01; 100pM, p=0.0026; 1pM, p=0.041. SKMEL23: 1µM, p=0.0288; 10nM, p=0.0372; 100pM, p=0.0185; 1pM, p=0.8004). The maximum growth-promoting effect occurred in both cell lines at 100pM AngII (Figure 4D). Essentially similar effects of AngII were also observed in SKMEL505 (AGTR1 - / AGTR2 +). In contrast, AngII had no effect on proliferation of SKMEL224 in serum-free medium (Figure 4D). These results are consistent with the growth-promoting effects of AngII being mediated via AT2R. To test this hypothesis, we determined the effect on melanoma cells of Y6AII which we recently showed to be a highly selective agonist of AT2R (20). PMWK cells were cultured in serum-free medium in the presence or absence of varying concentrations of Y6AII and proliferation assessed on day 5. As shown in Figure 4E, there was a clear dose-dependent increase in proliferation in cells treated with Y6AII, with 100 µM Y6AII causing an increase in proliferation at levels comparable to stimulation with 100pMAngII (Figure 5B). Y6AII caused an increase in proliferation of PMWK across a range of concentrations and this was maximal at10µM (p=0.048) and100µM (p=0.0003). In contrast, Y6AII had no effect on proliferation of the AGTR2 negative cell lines SKMEL2 and SKMEL224 (Data not shown).

Selective blockade of AT2R inhibits melanoma growth in vitro and in vivo

These results imply a role for AT2R rather than AT1R in promoting melanoma growth and prompted us to determine whether pharmacological inhibition of AT2R inhibits melanoma growth. We first evaluated the effect of PD123319 a selective, non-peptide AT2R inhibitor. In the absence of exogenous AngII, PD123319 had no effect on proliferation of any of the tested cell lines in either 10% serum or serum-free conditions (Data not shown). However, PD123319 efficiently blocked the growth promoting effects of AngII in serum free conditions in both PMWK (p=0.05) and SKMEL23 (p=0.0096) (Figure 5A). In SKMEL224, neither AngII nor PD123319 had any effect on proliferation (Figure 5A). Having observed that PD123319 inhibits melanoma cells expressing AT2R, we tested
the more potent EMA401, a highly selective tetrahydroisoquinoline AT₂R antagonist (21). EMA401 inhibited proliferation of PMWK cells, with almost complete inhibition at 30μM in the absence of exogenous AngII (Figure 5B). We then wished to determine whether the ability of AT2R-blocking agents to inhibit melanoma was also seen in vivo. For this, we used a zebra fish model which is recognised as an excellent system in which to study melanoma (23,24). SKMEL23 cells were injected into zebrafish yolksac and the fish grown with or without 30μM EMA401 in their medium. The presence of EMA401 greatly reduced the proliferation of the melanoma cells (Figure 5C). Similar inhibition of proliferation was seen in PMWK cells.

**Selective blockade of AT₂R inhibits melanoma angiogenesis**

AngII signalling is known to be an important determinant of angiogenesis. We therefore sought to determine whether the ability of melanoma cells to promote angiogenesis is affected by selective blockade of AT₁R and AT₂R. To assess the role of angiotensin receptor signalling in the induction of tumour associated angiogenesis, we utilised the assay described by Carpentier et al (22). Human microvascular endothelial cells (hCMEC/D3) were exposed to conditioned media (CM) collected from PMWK or SKMEL23 cells treated with angiotensin II alone or in combination with the angiotensin receptor antagonists Losartan, PD123319 and EMA401. The effects on endothelial tube formation (master segments length) and on network integrity (number of meshes) was determined after 24 hours. Exposure of hCMEC/D3 cells to medium conditioned by PMWK or SKMEL23 resulted in an increase in master segments length and tube formation. This was not potentiated by exposure of cells to exogenous AngII during the conditioning phase. However, both losartan and PD123399 blocked the increase brought about by the CM with EMA401 demonstrating superior efficacy (Figure 6A and 6B).

**Selective blockade of AT₂R potentiates anti-BRAF therapy**

We noted that some of the cell lines with low expression of AGTR1 contained the BRAF V600 mutation which confers sensitivity to BRAF and MEK inhibitors (A375, WM239, WM35, COLO829). This led us to evaluate whether inhibition of AT₂R affects response to these agents. We tested this question in A375 and WM329 cell lines which express low to undetectable AGTR1 and readily detectable AGTR2 and in two further cell lines (WM164 and WM793) which express
AGTR2 and positive for BRAF V600 mutation. Cells were grown in medium containing 1% serum and challenged with various concentrations of dabrafenib with or without EMA401. As observed previously, PD123319 alone had no effect on proliferation of cell lines under these growth conditions (data not shown). However, EMA increased the sensitivity of all the tested cell lines to dabrafenib, implying that inhibition of AT2R increases cellular sensitivity to targeted agents in melanoma (Figure 6C). This is most clearly illustrated in A375 cells in which dabrafenib and EMA401 individually only incompletely inhibit proliferation but together produce complete inhibition.
Discussion

The importance of the RAS in regulating intravascular volume is well-recognized and therapeutic manipulation of the RAS is a mainstay of antihypertensive and heart failure management. There is evidence in breast cancer that over-expressed AT1R has oncogenic function(s) (9, 10, 11), but this is restricted to a subset of oestrogen receptor positive HER2 negative cases in which AngII promotes and losartan inhibits proliferation. In the current work, we report that AngII drives proliferation of melanoma and to the best of our knowledge this is the first demonstration that AngII is mitogenic in human melanoma cells. We also provide several lines of evidence that (in contrast to the situation in breast cancer) AT1R acts as a tumor suppressor rather than as an oncogenic receptor and, in addition, AT2R acts as an oncogene in melanoma. Together, the opposing functions of AT1R and AT2R reveal that the RAS is a bifunctional growth regulator in melanoma. Fundamental to our investigation of the function(s) of AT1R and AT2R in melanoma is the use of highly selective agonists and antagonists to manipulate the endogenous activity of each receptor, rather than cell lines that have been engineered to ectopically over-express either gene. Additional work to investigate the precise mechanism by which AT1R acts as a tumour suppressor in melanoma is being undertaken using inducible expression in appropriate systems.

We are not aware of previous experimental evidence implying a tumor suppressor function for AT1R in human malignancy but we now provide multiple, complementary lines of evidence to support the candidacy of AGTR1 (encoding AT1R) as a tumor suppressor in melanoma. First, antagonism of AT1R by losartan promotes the growth in serum-free medium of melanoma cell lines that express AGTR1. We first observed this effect in PMWK (RGP) melanoma cells, but also subsequently in SKMEL147 (metastatic), C8161 (metastatic) and other melanoma cell lines that express AGTR1. The effect of AT1R blockade in promoting proliferation is thus common to melanoma cell lines expressing ATGRI, losartan having no effect in cell lines lacking AGTR1 due to transcriptional silencing, affirming the specificity of the effect. The ability of losartan to promote melanoma proliferation is in clear contrast to its anti-proliferative effect in breast cancer cells over-expressing AT1R (9, 10, 11) and to a study in murine melanoma cells (13). To the best of our knowledge, there are no previous reports that pharmacological blockade of AT1R promotes cancer cell growth.

Second, targeted down-regulation of AGTR1 using inducible shRNA conferred increased ability to
proliferate in serum-free and low serum medium in cell lines expressing AGTR1 and thereby mimicked the effect of losartan. Again, we are not aware of previous evidence demonstrating this effect. Third, ectopic expression of AGTR1 in cell lines with transcriptional silencing of the endogenous gene resulted in cell death making it impossible to derive clones stably expressing AGTR1 from parental cells with no or low endogenous expression. However, no inhibitory effect on proliferation was seen in cell lines that express endogenous AGTR1. This effect was reproducibly observed in the absence of exogenous AngII and in both serum replete and serum-free conditions. These observations are consistent with a tumour suppressor function for AGTR1 that can occur in a ligand-independent manner. We note that ligand-independent activation of AT1R (and AT2R) is well-recognised (25, 26). Fourth, AGTR1 expression is silenced by CpG island methylation in melanoma but not in control benign nevi. Transcriptional silencing in neoplastic but not equivalent normal tissue is a recognized feature of tumour suppressor genes and we are not aware of any previous reports of methylation-dependent transcriptional silencing of AGTR1 in cancer. The frequency with which AGTR1 is silenced implies strong selective pressure to abrogate the function of AT1R in advanced melanoma and is consistent with our functional analyses demonstrating that inactivation (either pharmacologically with losartan or by shRNA) confers a growth advantage to melanoma cells in allowing serum-free proliferation and with the proposed function of AGTR1 as a ligand-independent tumour suppressor. The specificity of AGTR1 methylation for malignant melanocytes and its association with advanced / metastatic melanoma is also consistent with a tumour suppressor function for AT1R.

Despite the negative growth regulatory effect of AGTR1, we show in this study that AngII nonetheless promotes serum-free proliferation in melanoma cell lines and we demonstrate several lines of evidence to support the hypothesis that this effect occurs via AT2R. First, AngII had no mitogenic effect on the serum-free proliferation of cell lines lacking expression of AT2R, as exemplified by comparison of SKMEL 23 and SKMEL224. In both cell lines expression of AGTR1 is silenced by CpG island methylation whilst AT2R is expressed in SKMEL23 but not in SKMEL224 and AngII promoted serum-free growth in SKMEL23 but not in SKMEL224. Furthermore, A375 cells which, like SKMEL23, have a silenced AGTR1 but express AGTR2 also demonstrate growth promotion in serum-free conditions by AngII. Second, growth of melanoma cells expressing AGTR2 was blocked by the AT2R selective antagonists PD123319 and EMA401. Third, Y6AII, a highly selective AT2R agonist (20) phenocopied the mitogenic effect of AngII but only in melanoma
expressing AT$_2$R. Promotion of proliferation by Y6AII strongly supports our hypothesis that AngII acts as a mitogen in melanoma via AT$_2$R and not AT$_1$R. Fourth, AGTR2 is over-expressed in several novel early passage cell lines established de novo in our laboratory from histologically confirmed melanoma CNS metastases. Given the low expression of AGTR2 in normal melanocytes, the frequent over-expression of AGTR2 implies that this confers a growth advantage in advanced melanoma. Of note, AGTR1 was undetectable in the majority of these cell lines, consistent with a model in which silencing of AGTR1 abrogates the ligand-independent growth suppressor effect of AGTR1 and over-expression of AGTR2 promotes proliferation via ligand-dependent mechanisms.

Together these mechanistic studies suggest that loss of AT$_1$R confers a more aggressive phenotype to a subset of melanomas. Accordingly, the expression of AGTR1 was greatly decreased in metastatic relative to matched primary clinical melanomas and, conversely, AGTR1 methylation quantitatively increased in metastatic derivatives of primary clinical melanomas. We propose, therefore, a model in which the status of the RAS influences the biology of melanoma. In early disease, exemplified by the RGP cell line PMWK, AGTR1 is expressed and the clinical phenotype is typically not aggressive. With malignant progression, AGTR1 is silenced by CpG island methylation conferring a more aggressive phenotype (for example by allowing a proliferative advantage in low growth factor conditions), whereas AGTR2 is expressed and acts as the transducing receptor for the mitogenic effects of AngII.

Our data have clear potential clinical implications. First, we have shown that blocking AT$_2$R with the antagonists PD123319 and EMA401 inhibits proliferation of melanoma cells expressing AGTR2 in vitro and in vivo in a zebrafish model. The most robust inhibition was seen with EMA401. This agent is a first in class potent and highly selective antagonist of AT$_2$R and is under clinical development to treat peripheral neuropathic pain (27). Whereas the effect of PD123319 was evident in the presence of exogenous AngII, EMA401 exhibited complete inhibition of melanoma proliferation in the absence of exogenous AngII. Of note, the anti-proliferative effects of AT$_2$R blockade occurred in both BRAF V600 mutation positive, NRAS mutation positive and in double negative (BRAF & NRAS wild-type) cell lines implying that inhibition of AT$_2$R may be a viable therapeutic strategy in melanomas lacking common driver mutations. We also show that antagonism of AT$_2$R potentiates the anti-proliferative effect of small molecule BRAF inhibitors. We demonstrate potentiation of dabrafenib in 4 independent BRAF V600 mutation positive, AT$_2$R positive cell lines. Therapeutic synergy without additional toxicity implies that this combination may improve the
clinical utility of currently used anti-melanoma drugs. Studies to determine the optimal combinations of AT_2R blockers with BRAF and MEK inhibitors using in vivo models are in progress. Third, the ability of AngII to drive proliferation of melanoma implies that agents which reduce serum levels of AngII may have growth inhibitory activity. The Angiotensin Converting Enzyme inhibitors (ACEI) such as lisinopril and ramipril, although producing only incomplete inhibition of ACE (28), are widely used in cardiovascular medicine. Later generation agents that inhibit renin mediate substantial reductions in serum AngII levels (29). One such agent, Aliskiren, produces highly efficient reduction in serum AngII and has potent anti-hypertensive properties. It would be of interest to determine the effect of Aliskiren on melanoma growth in vivo. We show in our study that AGTR2 is over-expressed in early passage cell lines developed in our laboratory from CNS melanoma metastases. Importantly, the use of Aliskiren to deplete serum AngII as a potential therapeutic strategy for CNS melanoma metastasis would abrogate the requirement for passage of the drug across the blood : brain barrier.

Indirect support for the hypothesis that AngII may promote cancer growth and that expression of AGTR1 may correlate with a better prognosis is afforded by the results of a number of epidemiological studies. Yoon et al (29) demonstrated a generalized decreased risk of cancer associated with use of AT_1R blockers in cohort and nested case-control studies and in studies with long-term follow-up of more than five years. Critically however, in subgroup meta-analysis an increased risk specifically of melanoma and renal cancer was reported in patients using ATR blockers. Of note, AGTR1 is also subject to transcriptional silencing in metastatic clear cell carcinoma of the kidney (unpublished observations). In a study in lung cancer, low ACE levels were predictive of OS benefit from cediranib (30) and higher activity of the ACE-AngII-AGTR1 axis associated with a better response to bevacizumab in cancer (31).

We have shown in the present study that methylation of AGTR1 is associated with metastatic melanoma. Furthermore, analysis of a series of patient-matched primary: metastatic melanomas demonstrated that methylation levels in the AGTR1 CpG island frequently increase in the progression of a primary cutaneous melanoma to a metastatic lesion. This association of AGTR1 CpG island methylation with metastatic disease was reflected in the presence of methylated genomic DNA in the peripheral blood of a subset of patients with metastatic disease, implying that detection may have utility as a biomarker, particularly of visceral and CNS metastatic disease. We are investigating this
possibility in large, prospectively collected series of sera with known clinical outcome analyzing AGTR1 both alone and in combination with TFPI2 (16).

In conclusion, we show here that the status of the RAS is an important determinant of the biological properties of melanoma and may be exploitable both diagnostically and therapeutically.

Materials and Methods

Cell lines and drugs

Melanoma cell lines were routinely grown as described previously (16). Primary cultures of brain metastatic melanomas M5, M78, M80 and M88 were established from fresh tumour tissue obtained from neurosurgical resection of intracranial lesions confirmed to be metastatic melanoma by histopathology. Tumours were washed in DMEM: F12 (1:1) (Gibco), minced through a cell strainer (BD Falcon) to obtain a single cell suspension and cells pelleted by centrifugation for 10mins at 12K rpm. The pellets were briefly re-suspended in 1ml of sterile dH20 to lyse contaminating red blood cells followed by the addition of 10ml of DMEM: F12 + 10% FBS to neutralise the effects of dH20. Cells were pelleted as before and cultured in fresh DMEM: F12 +10% FBS at 37°C. The selective AT1R inhibitor losartan and the highly selective AT2R agonist Y6AII recently developed by us were synthesised as described previously (20). AngII and PD123319 were purchased from Sigma. EMA401, a highly selective tetrahydroisoquinoline AT2R antagonist (21), was kindly provided by Novartis pharmaceuticals. Demethylation experiments using azacytidine and trichostatin were done as described previously (33, 34). Dabrafenib was purchased from Sigma.

Plasmid vectors and generation of stable clones

To generate an AGTR1 expression vector, the AGTR1 ORF was purchased from Origene and sub-cloned into pcDNA3-DEST40 using Invitrogen’s Gateway Technology to generate pcDNA3.2/V5/GW/AGTR1. pcDNA3.2/V5/GW/CAT served as the control plasmid. The sequence of both plasmids was verified by sequencing. The TRIPZ AGTR1 shRNA plasmids (set of 5) for inducible knockdown of AGTR1 and the non-silencing control plasmid were purchased from DE Dharmacon. Cells were transfected with 1ug of each of the plasmids using metafectine (Biontex) as
per manufacturer’s instructions and transfectants selected in 0.5\(\mu\)g/mL puromycin (Sigma).

Expression of the \textit{AGTR1} or non-silencing shRNA was induced by adding 2 \(\mu\)g/mL doxycycline (Sigma) every 48 hours. Transfectants were analysed for knockdown of \textit{AGTR1} by qPCR and by Western blotting using the Novus Biologicals antibody (NBP-77078) at a dilution of 1:250.

\textbf{Proliferation analysis}

Cell proliferation was measured using the sulforhodamine b (SRB) colorimetric assay. Cells were seeded into 96 well plates (Costar) at a density of 4\(\times\)10\(^3\) cells per well in media containing 10\% FCS. 24hrs after plating culture media was replaced either with media containing 10\% FBS (non-starved) or 0\% FBS (starved). After a further 24hrs hours cells were treated with drugs as appropriate and incubated for various time points (D1, D3, D5) and harvested by fixing in 10\% cold trichloroacetic acid (TCA) for at least 1 hour at 4\(^\circ\)C. Fixed plates were washed 4 times with dH\(_2\)O and air dried at room temperature prior to staining with 60 \(\mu\)L 0.4\% SRB for 1h at RT. Unbound SRB was removed by washing plates 4 times using 0.1\% acetic acid and allowed to air dry at room temperature. Bound SRB was re-suspended in 150\(\mu\)L 10 mmol/L Tris pH 10.5 and the absorbance read at 490nm using an ELx800 microplate reader (BioTek).

\textbf{Clonogenic Assays}

Clonogenic assays using transiently transfected cells were performed by plating 2\(\times\)10\(^5\) cells / 6cm dish and transfected after 24h with 1\(\mu\)g of pcDNA3.2/V5/GW/AGTR1 or control vector pcDNA3.2/V5/GW/CAT using Metafectine (Biontex) according to the manufacturer’s instructions. Transfection efficiency was 25–30\% under these conditions as measured by immunofluorescence staining using V5 antibody. Essentially transfected cells were fixed with 4\% paraformaldehyde for 15 minutes, permeabilized with 0.25\% Triton™ X-100 for 10 minutes, and blocked with 5\% BSA for 1 hour at RT. The cells were labelled with V5 Tag Mouse Monoclonal Antibody (Invitrogen) at a dilution of 1:400 in 1\% BSA and incubated for 3 hours at RT and then labelled with Rabbit Anti-mouse FITC Secondary Antibody (Sigma) at a dilution of 1:100 for 30 minutes at RT. Percentage of fluorescent cells was detected using a Nikon microscope at 20X magnification. 48 hours after transfection puromycin (0.5\(\mu\)g/ml) was added and medium containing drug was replaced on day 3 and 5. On day 8, cells were washed with PBS and fed with fresh medium without drug. Medium was replaced every
72–96 h for the remainder of the experiment. Cells were monitored daily by light microscopy for small colonies of proliferating cells. Colonies were counted 3–4 weeks after the removal of drug by staining with Coomassie Brilliant Blue. Cell clusters of 50 cells were defined as colonies.

**Angiogenesis assays**

To assess the role of angiotensin receptor signalling in the induction of tumour associated angiogenesis by melanoma cells, human microvascular endothelial cells (hCMEC/D3) were exposed to conditioned media (CM) collected from PMWK cells treated with angiotensin II alone or in combination with losartan and PD123319. To prepare conditioned medium (CM) PMWK cells were seeded onto 6 well plates (Corning, USA) at 1.5x10^5 cells per well and allowed to adhere for 24h. Cells were washed twice with warm PBS and media replaced with 2mL per well of EBM-2 basal medium without any supplements. The EBM-2 basal medium was conditioned for 24h, then carefully collected and centrifuged at 300 RCF for 5 minutes at RT to pellet cells and debris. The CM was moved to a fresh tube and used immediately in the endothelial cell tube formation assay of angiogenesis. To perform this assay, µ-slide angiogenesis slides (Ibidi, Germany) were coated with 10µL growth factor reduced matrigel (10 mg/mL, Corning, USA) per well using cooled pipette tips to prevent early polymerisation of the matrigel. Coated slides were then placed in 10cm dishes to prevent scratching and incubated at 37°C for 30 min. During the incubation, hCMEC/D3 cells were washed twice with PBS, trypsinised and counted. hCMEC/D3 cells were aliquoted into sterile 1.5 mL micro-centrifuge tubes (Eppendorf, Germany) at 4x10^4 cells and pelleted by 5 min centrifugation at 300 RCF. Cell pellets were resuspended in 200µL CM and plated onto matrigel coated angiogenesis slides in triplicate (10000 cells in 50µL per well). This was done to ensure maximum exposure time of endothelial cells to the CM. Tube formation was monitored throughout the experiment and imaged at 6 hours post-plating using a TMS inverted phase contrast microscope (Nikon, Japan) with a DinoEye AM7023 eyepiece camera (Dino-Lite, Taiwan) at 2X magnification. Tube formation was analysed using the ImageJ angiogenesis analyser plugin (22).

**Zebrafish assays**

SKMEL23 cells were transplanted into 2-day-old casper zebrafish, Cells were stained prior to injection with cell tracker (CMTPX-Red, Molecular Probes) for 30min at 37°C and washed twice with PBS.
Later the cells were trypsinised and re-suspended in 500µl RPMI medium. The cells were incubated for 20 minutes on ice and 100 cells were injected into the yolk sac of 20-30 casper zebrafish/treatment (35). Following recovery for one hour at 28°C, zebrafish were maintained at 35°C, and screened for Red fluorescence at the injection site. At 48h post-injection the zebrafish were treated for 10 days with 30µM PD or EMA401 in E3 medium (1mM NaCl, 0.17mM KCl, 0.33 mM CaCl₂, 0.33mM MgSO₄, 10⁻⁵ % Methylene Blue). Tumour growth was assessed by fluorescent imaging every 48 hours.

Clinical material

The study was approved by the Local Research Ethical Committee, East London and City Health Authority and the Tayside Tissue Bank, under delegated authority from the Tayside Local Research Ethics Committee. Tissue for culture of CNS melanoma metastasis was obtained from Imperial College Tissue bank. Tissues and sera were used after informed consent had been obtained. In all cases, micro-dissection of tissue sections was performed to enrich for melanoma cells prior to isolation of nucleic acids. Benign pigmented nevi (n=8) from sun-exposed skin were used as control tissues. We used proteinase K digestion to isolate genomic DNA from all tissue sections. Sera were collected from patients (n=63) at the time of diagnosis of metastatic disease or during routine follow up. Sera were harvested using a standard operating procedure in which peripheral blood was subject to centrifugation immediately after venesection, then snap frozen in aliquots that were stored at -80°C. 200 µl units of serum were thawed immediately prior to use and genomic DNA isolated using the QIAamp DNA Blood Mini kit (Qiagen, Germany) and DNA modified with sodium bisulphite as described below.

Methylation analysis

The 5’ end of AGTR1 contains a CpG island (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). Genomic DNA (1µg) was extracted and modified by sodium bisulphite as described previously (27, 28). Pyrosequencing was performed using the PyroMark ID System (Biotage) and primers were designed to cover a region including 7 CpG dinucleotides of the CpG island. The primers were as follows: 5’- biotin-GTTAGGATT'TAGGTAGTAG -3’ (forward primer) and 5’- CTCAACCCTCCCAT -3’ (reverse primer), amplifying a 100bp region. PCR was performed in a final volume of 30µl,
containing 0.5µM of each primer, 200µmol/L of each dNTP, 0.05 units of AmpliTaq Gold 360 DNA polymerase in buffer containing 1.5mmol/L MgCl2, 3µl of 360GC enhancer (Applied biosystems) and 2µl of bisulphite-treated DNA as template. The initial denaturation step (95°C, 10 min) was followed by 35 cycles of 30 s at 95°C, 30 s at 53°C, 40 s at 72°C, and a final extension step at 72°C for 10 min. Three µl of the PCR products were visualised by gel electrophoresis and 25µl were subjected to pyrosequencing using the reverse as sequencing primer at a final concentration of 0.5µM. Purification and subsequent processing of the biotinylated single-strand DNA was done according to the manufacturer’s instructions using the Pyro Gold reagents kit (Biotage). Resulting data were analyzed and quantified with the PyroMark CpG Software (Biotage). Positive (commercial methylated DNA) and negative (placenta DNA) controls were included and treated as well as samples. Pyrograms of the control DNA were analysed to confirm complete bisulfite conversion. Sera were deemed positive if the % methylation was ≥ 5 at 3 or more Methylation Variable Positions (MVP) in the amplified fragment of the CpG island.

**Gene expression analysis**

Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions including the optional on-column DNase step. Total RNA was reverse transcribed using random primers (Invitrogen) following the M-MLV Reverse Transcriptase protocol (Promega) and cDNA was stored at -20°C until used. TaqMan® probe-based gene expression analysis (Applied Biosystems) was used to detect *AGTR1* (assay ID: Hs00258938_m1) and *AGTR2* (assay ID: Hs02621316_s1). qPCR reactions were carried out in triplicate following the manufacturers protocol on a C1000 Thermal Cycler combined with a CFX96 detection module (Bio-Rad). 50ng of template cDNA was used per reaction. No-template controls and no-RT controls were included in each run to exclude false positives due to impurities. Expression data was normalised to the mean Ct value of the reference genes TATA-binding protein (*TBP*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and displayed as $2^{-\Delta \text{Ct}}$.

**Statistics**

Statistical analyses were performed using Prism 5 (GraphPad software, Inc., La Jolla, CA, USA). The unpaired Mann-Whitney *t*-test was used to compare methylation levels between different groups for
both cell lines and tissue samples. Significant differences in proliferation were also established by unpaired t-test.

**Conflict of interest**

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Expression of AGTR1 is silenced by CpG island methylation in melanoma.

A: qPCR analysis of AGTR1 and AGTR2 in melanoma cell lines. Data shown are mean expression +/- 1SD from 2 experiments. B: Representative pyrograms of AGTR1 CpG island analysis in melanoma cell lines. Cell lines were subjected to pyrosequencing analysis as described in Methods. C: Summary of quantitative methylation analysis of AGTR1 CpG island. The number denotes each individual CpG dinucleotide in the amplified fragment analysed by pyrosequencing. Five levels of methylation are represented, the level of methylation increasing with increasing intensity of shading in the circles as indicated. D: Demethylation reactivates expression of AGTR1 in melanoma cell lines with transcriptional silencing. The indicated melanoma cell lines were grown in the presence of Trichostatin (T), Azacytidine (A) or both drugs (A & T). Expression of AGTR1 was determined by qPCR. Data shown are mean expression relative to untreated controls (C) +/- 1SD.
**Figure 2. Methylation associated silencing of AGTR1 is associated with metastasis in melanoma.**

A: qPCR analysis of AGTR1 in paired primary : metastatic melanomas. The figure shows relative expression in 9 paired cases. Expression is down regulated in 7 of the 9 cases. In 3 cases expression was undetectable in the metastasis. For clarity of the figure these cases are shown as 0.01 relative expression.  

B: Representative pyrograms of AGTR1 CpG island analysis in clinical cases of melanoma showing unmethylated and methylated cases as indicated.  

C: AGTR1 CpG island methylation is increased in primary melanomas which metastasize and in metastatic lesions. Genomic DNA was isolated from benign nevi (N), primary melanomas (P1-P10) which were all of BT < 1mm and did not subsequently metastasise and from high-risk primary melanomas P11-16 and their matched, subsequent metastatic lesions (M11-M16). In all cases, pyrosequencing was performed as described in Methods. The degree of methylation at each analysed MVP is represented by the intensity of shading in the circles as shown in the figure.  

D: Methylated genomic DNA from the AGTR1 CpG island is detectable in the serum of patients with metastatic melanoma. The figure shows representative methylation profiles from 6 patients: 2 without metastatic disease (no metastases) and 4 with metastatic disease. The sites of metastases are: bone (Bo), lung (Lu), lymph node (LN) and sub-cutaneous (SC). The profiles show % CG methylation at each of 7 MVPs in the AGTR1 CpG island determined by pyrosequencing.  

E: Expression of AGTR1 and AGTR2 in early passage CNS metastatic melanoma cell lines is consistent with a tumour suppressor function for AGTR1 and oncogenic function for AGTR2. Novel CNS metastatic melanoma cell lines were derived as described in Methods and expression of AGTR1 and AGTR2 was assessed by qPCR.

**Figure 3. Antagonism of AT1R promotes melanoma proliferation**

The selective AT1R antagonist losartan promotes serum-free proliferation of the AGTR1-expressing melanoma cell line PMWK. PMWK cells were grown in serum-free medium without (control) or in the presence of 100μM losartan and photographed after 96 hours.  

B: Growth promotion of PMWK and SKMEL147 cells by losartan occurs in serum-free medium. Cells were grown in medium containing 10% FBS or in serum-free conditions in the presence (black squares) or absence (empty circles) of 100μM losartan as indicated. Cell numbers were estimated at various time points as described in Methods. Data shown are A490 values +/- 1SD.  

C: Losartan-dependent proliferation in serum-free conditions requires expression of AGTR1. PMWK and SKMEL224 cells were grown in...
serum-free medium in the presence (black squares) or absence (clear circles) of 100μM losartan as indicated and cell numbers estimated as above. Data shown are A490 values +/- 1SD.

**Figure 4. AGTR1 is a growth suppressor in melanoma but AngiotensinII promotes melanoma proliferation.**

A: Stable knock down of AGTR1 promotes low serum growth of PMWK cells. PMWK cells stably expressing the AGTR1 shRNA vector or non-silencing control vector were cultured in 10% FBS in the absence (-) or presence (+) of doxycycline (2μg/ml) and knockdown of AGTR1 was assessed by qPCR after 48 hours (left hand figure) and western blotting (middle figure). Image analysis (right hand figure) confirms knock down. Cells stably expressing either the control plasmid (control) or AGTR1 knockdown plasmid (AGTR1) were then grown in 10% FBS, 2% FBS or 1% FBS in the absence (-) or presence (+) of doxycycline as shown and A490 determined after 72 hours. Data shown are mean A490 values +/- 1SD. C: Ectopic expression of AGTR1 inhibits proliferation in melanoma cells lacking endogenous expression. SKMEL224, SKMEL23 and PMWK cells were transfected with 0, 100 or 1000 ng of pcDNA3AGTR1 then grown in medium with 10% serum together with G418. Data shown are colony number +/- 1SD relative to cells receiving vector only from at least two experiments. D: Dose : response effect of AngII on proliferation of melanoma cell lines. The upper panel shows growth in 10% foetal bovine serum in which exogenous AngII has no effect on proliferation. The lower panel shows growth in 0% serum in which the proliferation of PMWK and SKMEL23, but not that of SKMEL224, is promoted in a dose-dependent manner by AngII. Cell lines were grown in the presence of the indicated concentrations of AngII and proliferation determined as described in Methods. Data shown are mean A490 values +/- 1SD. E: Y6AII, a potent and highly specific agonist of AT2R, promotes serum-free proliferation of cells expressing AGTR2. PMWK cells were grown in serum-free medium in the presence of increasing concentrations of Y6AII or 100 pM AngII as indicated and proliferation assessed. UC1: at start of experiment; UC5: untreated control after 5 days. Data shown are A490 values +/- 1SD.

**Figure 5. Inhibition of AT2R suppresses proliferation of melanoma cell lines**

A: Effect of PD123319 on proliferation of melanoma cell lines. PMWK (AGTR1 + / AGTR2 +), SKMEL23 (AGTR1 - / AGTR2 +) and SKMEL 224 (AGTR1 - / AGTR2 -) cells were grown in
either 10% serum (left hand panels) or 0% serum (right hand panels). Cells grown in 0% serum were either untreated control cells (clear circles), treated with AngII (A, black squares) or AngII + PD123319 (A + PD123319, black diamonds). B: Suppression of proliferation of AGTR2 expressing melanoma cells by inhibition of AT2R. SKMEL23 cells were grown in medium containing 1% serum in the absence of exogenous AngII but in the presence of varying concentrations of either PD123319 or EMA401 as shown and cell numbers estimated by measurement of A490. C: AT2R blockade inhibits melanoma growth in vivo. SKMEL23 cells were injected into Zebrafish yolksac as described in Methods. -: control fish not injected; +U injected but untreated control; +EMA injected and grown in the presence of 30μM EMA.

Figure 6. Antagonism of AT2R inhibits proliferation and angiogenesis and potentiates targeted therapy in melanoma.

A: Blocking AT2R inhibits angiogenesis. Angiogenesis assays in PMWK and SKMEL23 cells were performed as described in Methods. The upper panels show representative appearances of SKMEL23 untreated controls (UC) and cells exposed to EMA401 and the lower panels PMWK untreated controls (UC) and cells exposed to PD123319. B: Quantitative analysis of total segment length (upper panels) and number of meshes (lower panels). PMWK and SKMEL23 cells were treated in the indicated way (CM: conditioned medium; AngII: 100 pM angiotensinII; LOS: 100μM losartan; PD30: 30μM PD123319; Y6: 100μM Y6AII; EMA30: 30μM EMA401). Data are mean +/- 1SD. C: Effect of AT2R antagonism on sensitivity to BRAF inhibition in melanoma. SKMEL23 (BRAF wild-type), A375, WM164 and WM793 (all BRAF V600 mutation positive) cells were grown in 1% serum in the presence of EMA401 (30μM), dabrafenib (5nM) or both agents as shown and cell numbers directly counted. Data shown are mean relative cell number +/- 1SD from 2 independent experiments.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>BRAF / NRAS</th>
<th>AGTR1</th>
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Abbreviations: RGP: Radial Growth Phase; SSM: Superficial Spreading Melanoma; VGP: Vertical Growth Phase Melanoma; Wt: Wild Type. M: methylated; U: unmethylated.
FIGURE 1

A

AGTR1

AGTR2

B

PMWK (unmethylated)  MEL505 (methylated)

C

HUMA  HUMN1  SBCL2  PMWK  WM35  WM902B  SKMEL224  MEL505

WM266.4  SKMEL2  SKMEL23  SKMEL30  SKMEL147  SKMEL173  SKMEL501  COLO-829

D

Relative AGTR1

0  50  100

C  T  A  A&T  C  T  A  A&T  C  T  A  A&T
FIGURE 4

A

Control    AGTR1

REL AGTR1

-    +    -    +    -    +

B

Control

AGTR1

A<sub>490</sub>

-    +    -    +    -    +

C

SKMEL224    SKMEL23    PMWK

REL COLONIES

-    +    -    +    -    +

D

PMWK    SKMEL23    SKMEL224

A<sub>490</sub>

-    +    -    +    -    +

E

A<sub>490</sub>

-    +    -    +    -    +

C1    C5    1    10    100    AngII
Figure 5

A

PMWK

SKMEL23

SKMEL224

10% FBS

0% FBS

B

A

A + PD123319

Control

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

1 2 3 4 5

DAYS

A490

Control

PD10

PD30

PD50

PD100

EMA10

EMA30

EMA50

EMA100

C

-  + U  + EMA

Head

Trunk

Tail

Yolk
FIGURE 3

A

PMWK Control

PMWK + losartan

B

10% FBS

0% FBS

PMWK

0% FBS

SKMEL147

C

PMWK

0% FBS

SKMEL224
Angiogenesis assays

Tumour associated vasculature

PMWK 24h CM Master segments length

Experiment #2

Results tube formation: PMWK CM + treatments for 24h
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Abbreviations Wt: wild-type; U: unmethylated; M: methylated.