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# LP2, a cyclic angiotensin-(1−7) analog extended with an N-terminal D-lysine, impairs growth of patient-derived xenografts of colorectal carcinoma in mice

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ABSTRACT

LP2 is a 4, 7 D, L lanthionine-stabilized analog of angiotensin-(1−7), with an N-terminal D-lysine, resistant to breakdown by peptidases. It is a specific agonist of the angiotensin II type 2 receptor. Consistent with its high specificity and stability, LP2 has shown excellent safety and pharmacokinetics in a first-in-human clinical phase Ia trial. Here, based on strong rationales, we studied the capacity of LP2 to inhibit the growth of patient-derived xenografts of colorectal cancer in mice. Prior to efficacy studies, immunohistochemistry on an untreated tissue array demonstrated that the $AT_2R$ expression is reduced in human colorectal cancer and in stroma when compared to tumor adjacent tissue. Subsequent studies demonstrated that LP2 at a subcutaneously injected dose as low as 0.2 μg/kg/day inhibited patient-derived xenografts of colorectal carcinoma in mice. Kinome analyses and validation of elected kinase inhibition indicated that LP2-mediated $AT_2R$ stimulation inhibited PI3K/AKT/mTOR which resulted in apoptosis via CDKs. LP2 acted synergistically with 5-FU and the EGFR inhibitor erlotinib. Taken together, the extremely low dose of LP2 at which antitumor activity is exerted, the synergism with selected drugs and, together with its excellent specificity, safety and stability, warrant further evaluation of LP2′s inhibitory potential of colorectal cancer.

## 1. Introduction

Lanthipeptides are lanthionine-constrained peptides. A lanthionine is composed of two alanines linked via a thioether bridge (Ala-s-Ala). The lanthipeptide LP2 is derived from angiotensin-(1−7), DRVYIHP. LP2 has the sequence dKDRV[dAIHA]s in which dK is a D-lysine, and in which [dA.A]s is a D, L lanthionine (dA-s-A). LP2 is thus protected against N-terminal degradation by aminopeptidases by the presence of the added N-terminal D-lysine. Furthermore, LP2 is stabilized by a D, L lanthionine which replaces the tyrosine4 and proline7 of natural angiotensin-(1−7). The conformational constraint imposed by a lanthionine, usually enhances the target selectivity of a peptide.

The octapeptide angiotensin II, DRVYIHPF, agonistically stimulates both the angiotensin II type 1 receptor ($AT_1R$) and the angiotensin II type 2 receptor ($AT_2R$) [10,24]. These two receptors generally exert opposite effects. Stimulation of the $AT_1R$ may lead to unwanted effects such as, for instance, a rise in blood pressure and fibrosis, whereas stimulation of the $AT_2R$ may reduce blood pressure and lead to anti-fibrotic effects.

In contrast to angiotensin II, which stimulates both $AT_1R$ and $AT_2R$

*Abbreviations:* ACEi, angiotensin converting enzyme inhibitor; AGTR1, angiotensin II type 1 receptor.; AKT, protein kinase B; ARB, angiotensin II type 1 receptor blocker; ATIP, angiotensin II type 2 receptor interacting protein; $AT_1R$, angiotensin II type 1 receptor; $AT_2R$, angiotensin II type 2 receptor; BW, body weight; CC3, cleaved caspase 3; CDK, cyclin dependent kinase; CMC, chemistry manufacturing and controls; CV, coefficient of variation; CRC, colorectal carcinoma; EGFR, epidermal growth factor receptor; 5-FU, 5-fluoruracil; GPCR, G-protein coupled receptor; i.p., intraperitoneally; JNK, C-JUN N-terminal kinases; MAPK, mitogen activated protein kinase; KRAS, kirsten rat sarcoma viral oncogene homolog; mTOR, mammalian target of rapamycin; MTUS, microtubule-associated scaffold protein; PDX, patient-derived xenograft; PI3K, phosphoinositide 3-kinase; PLZF, promyelocytic leukemia zinc finger, a direct adapter protein of $AT_2R$; p.o., orally; PTK, tyrosine kinase; Q7D, every second day; QD, daily; RFS, recurrence free survival; ROI, region of interest; s.c., subcutaneously; SHP2, protein-tyrosine phosphatase encoded by Ptpn11; STK, serine/threonine kinase; TMA, tissue microarray.

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[3], the lanthipeptide LP2 selectively stimulates $AT_2R$ [25]. Furthermore, in man, angiotensin II has a very short half-life of less than a minute [9], whereas LP2 has a half-life in man of 2.1–2.6 h. Twelve hours after administration by subcutaneous injection, 66 %− 85 % of the administered LP2 is found back intact in human urine. A clinical Phase Ia trial demonstrated excellent safety and pharmacokinetics of LP2 [17]. Taken together, the selectivity, safety and stability of LP2 might be compatible with significant therapeutic potential.

This study focused on the therapeutic potential of the $AT_2R$ agonist LP2 in colorectal cancer based on the following rationales from literature. In colorectal carcinoma, $AT_2R$ expression showed an inverse relation with tumor stage and metastasis [28]. Stimulation of the $AT_2R$ in colorectal liver metastases in mice inhibited tumor growth [1]. An $AT_2R$ agonist inhibited growth of pancreatic ductal adenocarcinoma grafts in mice [11]. $AT_2R$ has been reported to directly interact with three different tumor suppressor proteins [21]: SHP2 [5], PLZF [12] and MTUS/ATIP [4,29].

Colorectal carcinoma is the third most commonly diagnosed cancer and the second-leading cause of cancer deaths worldwide. Over 1.9 million new CRC cases and 930,000 deaths were estimated in the year 2020. The burden of CRC is projected to increase to 3.2 million new cases and 1.6 million deaths in the year 2040 [15]. Here we investigated whether LP2 could inhibit the growth of patient derived xenografts (PDX) of colorectal carcinoma in mice. As a first step we investigated the efficacy of LP2 in PDXs obtained from primary and metastatic colorectal carcinoma. Thereafter we performed mode of action studies on LP2 in colorectal carcinoma PDX.

## 2. Materials and methods

### 2.1. Synthesis of LP2

LP2 is a small peptide with the sequence dKDRV[dAIHA]$_s$ in which dK is a D-lysine, dA a D-alanine and [dA—A]$_s$ a D, L lanthionine composed of two alanines with a thioether bridge. Thanks to its charged residues, LP2 is perfectly hydrosoluble. It is easily synthesized via base-assisted sulfur extrusion of a D, L disulfide bridged precursor peptide, dKDRVdCIHC in which dK is a D-lysine and in which dC is a D-cysteine [17]. Base-assisted sulfur extrusion of a disulfide-bridged peptide usually yields a mixture of stereoisomers, but by chance in the case of this particular precursor peptide it is largely stereospecific yielding LP2 with a D, L lanthionine which can be easily purified to GMP quality. Hence, this process allows for low costs of goods of LP2. CMC aspects of this lanthipeptide LP2 are therefore most favorable.

### 2.2. $AT_2R$ expression in a human tissue array

Expression of $AT_2R$ was studied in a tissue array containing 50 independent samples of human colon adenocarcinoma and 50 matched adjacent normal colon tissues (BC05118d; US Biomax, Inc.). $AT_2R$ was stained using a highly specific $AT_2R$ antibody (MAB3659; R&D Systems, US). Following slide scanning an automated, unbiased quantitative image analysis was performed, at OracleBio, Glasgow, UK, using HALO Image Analysis Platform (Indica Labs, US) with HALO AI Deep Learning Classifier add-on for segmentation of Regions of Interest (ROIs) such as tumor and stroma. The stained cells have been classified into negative (0), weak (1 +), moderate (2 +) and strong (3 +) stained cells, based on the pixel intensities. To show overall IHC staining intensity in the ROI, the H-score has been applied. The percentage of cells at each staining intensity level is calculated per section and the following formula is used:

$$\text{H-Score} = [1 \times (\%\ \text{cells}\ 1+) + 2 \times (\%\ \text{cells}\ 2+) + 3 \times (\%\ \text{cells}\ 3+)]$$

This gives a value for the section in the 0–300 range, which allows a more effective comparison of the staining levels between samples. For statistical analysis of the H-score, non-parametric tests have been used: the Friedman test with Dunn's multiple comparisons test for three group comparison and Mann Whitney test for two group comparison.

### 2.3. Efficacy of LP2 against patient-derived xenografts of colorectal carcinoma tumors in mice

#### 2.3.1. Effect of LP2 on the tumor growth

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Research Council's Guide for the Care and Use of Laboratory Animals. All the PDX studies were done in accordance with the United Kingdom Coordinating Committee on Cancer Research regulations for the Welfare of Animals [27] and of the German Animal Protection Law and approved by the local responsible authorities, Berlin, Germany (Approval No. A 0010/19, LaGeSo Berlin, Germany). From arrival onwards, the animals were group-housed in individually ventilated cages (type GM 500) and placed in a TouchSLIMPlustm air handling unit (Techniplast S.p.A., Italy) providing HEPA filtered air. The animals were kept with a piece of gnaw wood and paper rolls (Enviro-dri) as environmental enrichment. Drinking water and autoclaved cereal-based commercial rodent VRF1 diet were provided ad libitum from the arrival of the mice until the end of the study. The NMRI nu/nu female mice at age 6–8 weeks were inoculated with PDX tumor subcutaneously (for a list of PDXs see Table S1) and the tumor was grown to reach palpable tumor size before any treatment was initiated. Allocation of animals to the treatment arms allowed uniform distribution of initial tumor volume in both placebo and verum groups. LP2 was dissolved in sterile PBS while PBS alone served as placebo (vehicle). Animals were treated once daily (0.2 μg/kg BW) with s.c. injections for maximal 28 days. During each study tumor volume and body weight were measured three times a week. At the study-end mice were sacrificed and the tumor samples were snap frozen (for kinome analysis) or formalin fixed (for immunohistology). For statistical analysis two-way ANOVA with Šídák's multiple comparisons test was used.

For drug combination studies with LP2, 5-FU and erlotinib were selected. 5-FU was administered Q7D at a dose of 80 mg/kg BW i.p., while erlotinib was given QD at a dose 25 mg/kg BW p.o. Those drugs were given alone or in combination with LP2 (0.2 μg/kg/d). For statistical evaluation Two-Way ANOVA with Tukey's multiple comparisons post-test was used. To determine influences of drug combinations (e.g., synergism) the following approximation has been applied:

Antagonistic(AB)/C > (A/C) x (B/C).
Additive (A/B)/C = (A/C) x (B/C).
Synergistic(A/B)/C < (A/C) x (B/C).

Where A is response to treatment 1; B is response to treatment 2; C is response to vehicle (placebo) treatment and AB is combination treatment of drugs 1 and 2 [8].

#### 2.3.2. Immunohistological assessment of apoptosis and proliferation

Tumor tissues obtained from PDXs studies have been processed for immunohistological assessment of apoptosis and proliferation. For immunostaining the following antibodies have been selected: for apoptosis cleaved caspase 3 antibody (9661S; Cell Signaling Technology; US) and for proliferation Ki37 antibody (ab15580; Abcam; UK) in combination with hematoxylin counterstain. Like in the tissue array study, HALO AI Deep Learning classifier was applied for segmentation of tumor and stroma ROI. For Cleaved Caspase 3 the positive stained area while for Ki67 cell counts were computed and calculated as percentage of target ROI. For statistical analysis unpaired T-test was applied.

#### 2.3.3. Kinase activity profiling

*2.3.3.1. Kinase activity.* At PamGene, Den Bosch, NL, to elucidate

molecular mechanisms underlying LP2-mediated tumor inhibition, tumor tissue extracted from mice harboring colorectal carcinoma PDXs and treated either with LP2 or vehicle, was subjected to kinase activity profiling and this was subsequently validated with specific kinase inhibitors. For the analysis, a PDX was selected that harbors a KRAS mutation, one of the key mutations in the colorectal carcinoma transition. In the first part, tumor tissue lysates extracted from mice (either vehicle- or LP2-treated; 10 animals per group) were subjected to tyrosine and serine/threonine kinase activity profiling using PTK and STK chips (PamGene, NL) with spotted multiple peptides serving as targets for kinases [7]. Following image analysis, a list of differential phosphosites was generated and a corresponding list of putative kinases was calculated (BioNavigator, PamGene, NL and R, The R Foundation for Statistical Computing, AT). Pathway/network generation and interpretation was conducted with three independent computing tools: GeneGo/MetaCore (Clarivate Analytics, US), Enrichr (Icahn School of Medicine at Mount Sinai, US) and Proteome Map (Greifswald University, DE).

*2.3.3.2. Validation of the kinase activity profiling by kinase inhibitors.* Inhibitors were dissolved in DMSO and diluted in DMSO to 50x the final concentration. Kinase inhibitors were selected based on their differential effect on vehicle- and LP2-pretreated lysates from CRC PDX. For this screening 3 PTK targets (FGFRs, HER2 and MET) and 6 STK targets (CDKs, MAPKs, JNK, p38, AKT and PI3K/mTOR) were selected from the kinome profiling and 21 specific kinase inhibitors of these targets. Then, on single vehicle and LP2-treated lysates, 6 concentrations of the selected kinase inhibitors were tested. The concentration of the inhibitor was selected that yielded the most differential effect when comparing vehicle and LP2 treated lysates. Thereafter, at this selected inhibitor concentration, lysates, at N = 9–10, were subjected to the kinase inhibitor. Relative inhibition of specific peptides was calculated using the after wash integrated relative signal intensities of each compound in comparison to DMSO control. A nonlinear regression curve fitting model was applied to relative signal intensity to get the inhibitor-response graph for each specific peptide set for respective kinase. In vehicle and MOR107-treated tumor lysates, relative inhibition was calculated for biological replicates (n = 10) on-chip treated with Erdafitinib, BEZ-235, MK-2206 and PHA-793887 in comparison to DMSO control. Relative inhibition differences between conditions were evaluated using the two-tailed t-tests (significance limit $p < 0.05$).

## 3. Results

### 3.1. $AT_2R$ expression in human CRC samples – Tissue array study

Prior to studying antitumor action of LP2, we studied the presence of its receptor, $AT_2R$, in a tissue array containing 50 independent samples of human colon adenocarcinoma and 50 matched adjacent normal colon tissues. Neither these tumor tissues nor the adjacent tissues had been treated with LP2. HALO software precisely segmented regions of interest and well detected $AT_2R$-positive cells in stroma (Fig. S1E) and tumor (Fig S1F). In contrast to a previously published study [2], $AT_2R$-positive cells were assessed using an automated quantitative image analysis approach that ensures high accuracy and unbiased results, and were determined not only in the tumor area but also in the stroma. $AT_2R$ expression was significantly lower in tumor tissue and stroma compared to adjacent tissue (Fig. 1). This is in accordance with previous findings which demonstrated significant lower expression of $AT_2R$ in CRCs with high local invasion, high stage, high nodal and vascular invasion [2]. Furthermore, the expression of $AT_2R$ is significantly higher in female than in male tumor (Fig. S2A) and in female stroma than in male stroma (Fig. S2B) and similar in female and male adjacent area of CRC tissue (Fig. S2C). Loss of $AT_2R$ expression in advanced CRC cells might lead to the avoidance of $AT_2R$-mediated anti-tumor signals and thus to CRC progression.

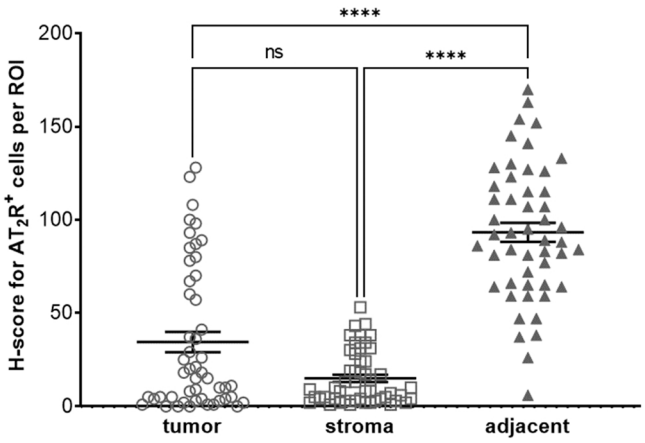


**Fig. 1.** Reduced $AT_2R$ expression in tumor and stroma. Expression of $AT_2R$ is reduced in CRC tumor tissue and tumor stroma as compared to the adjacent area. N = 50. ****$p < 0.0001$ by non-parametric Friedman test with Dunn's multiple comparison test.

### 3.2. In vivo screening and efficacy of LP2 in patient-derived xenografts

In subsequent experiments, antitumor efficacy of LP2 was studied. In a screening study the in vivo effect of LP2 was studied on the growth of different human PDXs of colorectal carcinoma, and of peritoneal carcinomatosis, a metastatic form of colorectal carcinoma. Table S1 indicates susceptibility of diverse PDXs, including KRAS-mutated PDX, to LP2 treatment. Subsequent studies on LP2 with selected PDX using bigger cohorts of animals (N = 9–11 per treatment arm) demonstrated significantly decreased tumor growth of colorectal carcinoma (Fig. 2) and peritoneal carcinomatosis (Fig. S3). In none of the studies any toxicity of LP2 was detected, which is consistent with the excellent safety observed in preclinical and clinical development of LP2 [17].

### 3.3. In CRC PDXs LP2 promotes tumor cell apoptosis and inhibits proliferation in stroma

Tumor tissue was extracted following in vivo PDX studies and further analyzed with immunohistochemistry for apoptosis and proliferation markers. The HALO AI Deep Learning software correctly segmented tumor and stroma ROI (Figs. S4, S5). In KRAS-mutated CRC tumors, LP2 significantly induces apoptosis within tumor ROI, but has no effect on apoptosis within stroma ROI, as compared to the vehicle (Fig. 3). Furthermore, LP2 has no impact on cell proliferation within tumor but

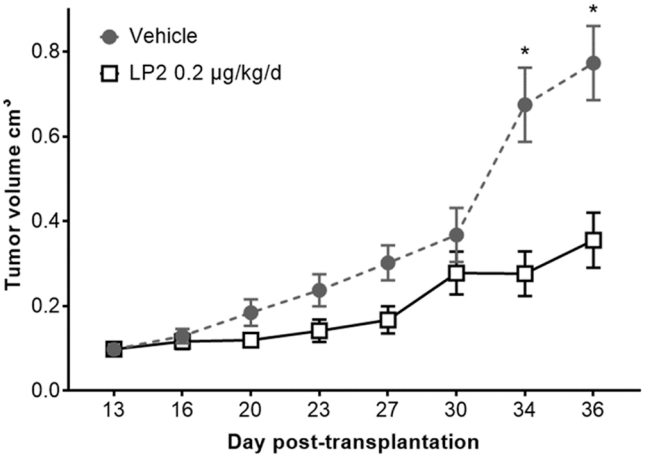


**Fig. 2.** LP2 inhibits the growth of colon cancer PDX in mice. N = 8–9, * $p < 0.05$ by Two-way ANOVA with Šídák's multiple comparisons test.

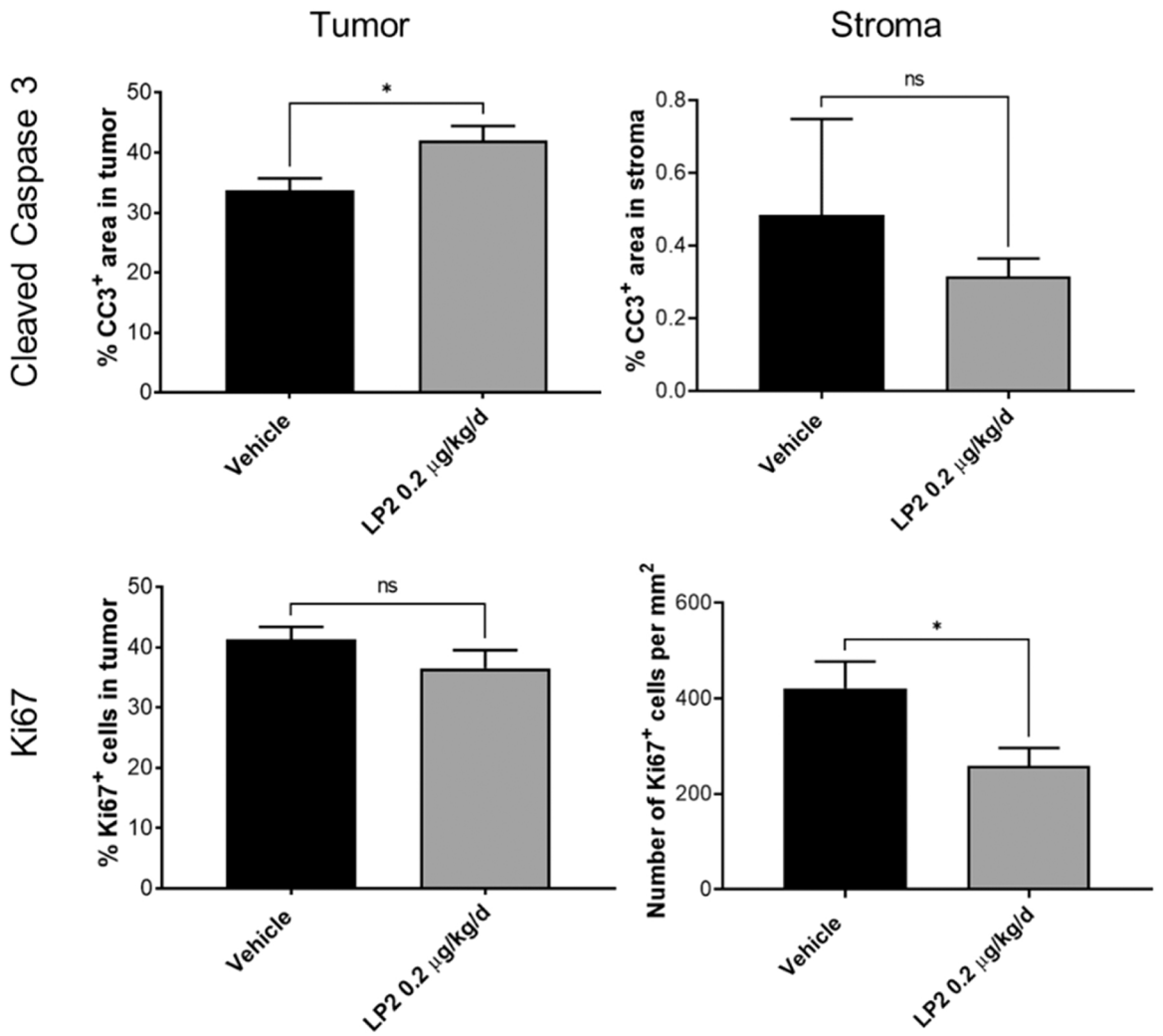


**Fig. 3.** LP2 induces tumor apoptosis and inhibits proliferation in stroma. LP2 at 0.2 µg/kg/d promotes apoptosis within tumor (Cleaved Caspase 3) and inhibits proliferation (Ki67) within stroma of CRC PDX. N = 9 * $p < 0.05$ by unpaired T-test; ns non-significant.

significantly inhibits proliferation within stroma of CRC PDXs (Fig. 3).

### *3.4. LP2 acts synergistically with clinically relevant chemotherapeutic agents in CRC PDXs*

In clinical use there are no approved monotherapies with sufficient efficacy for colorectal carcinoma. Therefore, efficacy of LP2 was tested in combination with a most frequently used drug, 5-FU. Moreover, it was investigated whether the combination of LP2 with an EGFR inhibitor in KRAS mutated PDXs might sensitize those tumors to EGFR inhibition. The combination of LP2 with 5-FU significantly reduced tumor volume by 56 % as compared to the vehicle and (Fig. S9). Testing for additivity/synergism indicates synergism at Day 38. The combination of LP2 with the EGFR inhibitor erlotinib significantly reduced tumor volume by 57 % as compared to the vehicle (Fig. 4) exhibiting synergism at day 31, day 35 and day 38. Since normally EGFR inhibitors only act on KRAS wild-type tumors, these data suggest that LP2 acts as sensitizer allowing application of EGFR inhibitors for treatment of KRAS mutated tumors. Patient-Derived Xenografts are the best in vivo models of human malignancies, well reflecting the tumor cell composition and molecular features. However, they are also characterized by high response variability to tumor inhibiting agents, similarly to the response variability observed in clinical trials. To assess the variability, we have compared the coefficients of variation within each treatment groups for each day of tumor volume assessment (Table S3). In the LP2/erlotinib combination

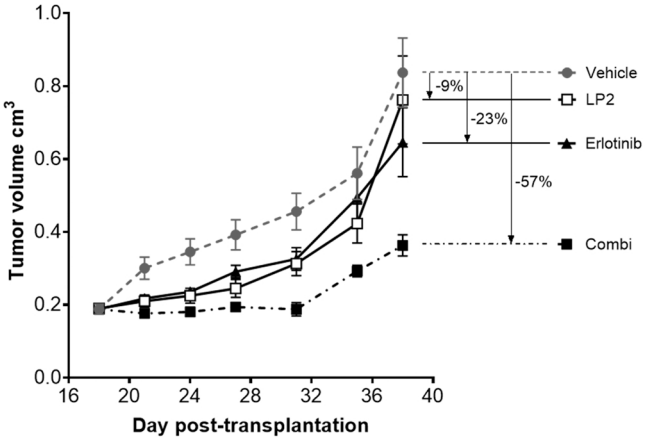


**Fig. 4.** Synergism of LP2 with erlotinib. Efficacy of LP2 combined with EGFR inhibitor erlotinib in colorectal carcinoma PDX. Arrows indicate percentage of tumor volume reduction by each of the compounds or their combination, as compared to the vehicle. N = 10–11 2-Way ANOVA showed significant differences for the Time Factor ($p < 0.0001$) and for the Treatment Factor ($p = 0.0007$). Tukey's multiple comparisons post-test detected significant differences for Combination vs. Vehicle ($p = 0.0023$) and Combination vs. LP2 ($p = 0.0353$) at the study end.

study the highest coefficient of variation values were observed within the vehicle group, while the lowest within the LP2 / erlotinib group. This low coefficient of variation would favor the eventual clinical evaluation of the combination of LP2/erlotinib.

### 3.5. In CRC PDXs LP2 acts via inhibition of PI3K/Akt/mTOR pathway and RTKs

To elucidate molecular mechanisms underlying LP2-mediated tumor inhibition, tumor tissue extracted from mice harboring colorectal carcinoma PDXs and treated either with LP2 or vehicle, was subjected to kinase activity profiling and validated with specific kinase inhibitors. For the analysis, a PDX was selected that harbors a KRAS mutation. In the first part, tumor tissue lysates extracted from mice, either vehicle- or LP2-treated and 10 animals per group, were subjected to tyrosine and serine/threonine kinase activity profiling using PTK and STK chips with spotted multiple peptides serving as targets for kinases. Following image analysis, a list of differential phosphosites was generated and a corresponding list of putative kinases was calculated. Fig. S6 shows the ranking of differentially regulated kinases based on their significance and specificity. While on a PTK chip only LMR1 and HER2 are highly significant and specific, the STK chip shows much more kinases, including multiple CDKs, ERKs and JNKs. With GeneGo Clarivate Analytics a set of relevant canonical pathways was found (Fig. S7). Here, the pathways including PDGF signaling via MAPK cascade and Angiotensin II signaling via p38, ERK and PI3K appear to be relevant for the observed effects. The Enrichment Analysis was used for the identification of classes of proteins that are over-represented (Table S2). Here, several pathways that are down-regulated in LP2-treated tumors have been identified, including MAPK, PI3K, Rap1 and Ras signaling pathways. Finally, a Proteome Map was generated, demonstrating significantly affected pathways with corresponding proteins (Fig. S8). Here, the MAPK has been identified as a main signaling pathway that includes MAP kinases, EGFR, AKT. The second major pathway identified includes FGFRs. The common pathways, based on the three pathway analysis methods, that are altered in LP2-treated colon tumors, are: MAPK (MAPK1, MAPK3, MAPK7, MAPK12 and AKT1) and Ras (FGFRs, FLT1/VEGFR1, CSF1R) signaling pathways.

Subsequently, validation of the observed kinase inhibition was performed. Following kinase activity profiling and validation, significant differences were observed for PI3K/mTOR (using inhibitor: BEZ-235), AKT (using inhibitor: MK-2206), CDKs (using inhibitor: PHA-793887) and FGFR (using inhibitor: Erdafitinib). These data were used for hypothesizing a $AT_2R$-mediated pathway in colorectal carcinoma (Fig. 5). Selective stimulation of $AT_2R$ with lanthipeptide LP2 inhibits receptor tyrosine kinases (RTK; e.g., FGFRs) probably via SHP2. Moreover, $AT_2R$ stimulation inhibits PI3K/AKT/mTOR which results in apoptosis and anti-proliferative effect via CDKs. Here, as intermediator, autophagy has been postulated (Fig. 5).

## 4. Discussion

Colorectal carcinoma (CRC) is an increasingly important disease and in particular metastatic CRC and KRAS-mutated tumors of CRC are difficult to treat. Existing treatments have severe side effects. Patient-derived xenografts in mice are a well-established in vivo model to study treatments of tumors, with significant capability of translational prediction. Some general in silico methods exist on the discovery of antitumor peptides [28]. In contrast, based on strong rationales, we here studied a highly specific agonist of the $AT_2$ receptor. In contrast to angiotensin II which stimulates both the $AT_1R$ and the $AT_2R$, LP2 specifically stimulates $AT_2R$. As LP2 differs from natural linear angiotensins, by having a D, L lanthionine and an N-terminal D-Lysine, it might be of interest to study its interaction with $AT_2R$ in silico docking studies. The D, L lanthionine-imposed constraint increases the target specificity, whereas both the D-lysine and the D, L lanthionine enhance the resistance to breakdown by peptidases. Indeed, most of the s.c. administered LP2 was recovered intact from human urine [17]. LP2′s specificity and stability are consistent with its efficacy and safety. LP2 is in vivo active at extremely low dose. It might be of interest to perform head-to-head comparisons of LP2 with other peptide $AT_2R$ agonizts, like NP-6A4, in the here used PDX model.

The first step in this study clearly showed that $AT_2R$ expression is reduced in untreated human tumor and stroma tissue compared to adjacent tissue. Previous work showed that $AT_2R$ expression inversely correlates with the pathological stage and liver metastases in CRC [30]. These data might suggest that tumor development may be facilitated by lower expression of $AT_2R$ or, in other words, that $AT_2R$ (over)expression may counter tumor development. Indeed, $AT_2R$-knock-out CRC cell line CMT93 shows in vitro enhanced cell growth, invasion, increased VEGF expression, and decreased apoptosis [20]. And indeed, adenovirus-mediated overexpression of $AT_2R$ inhibited tumor growth of prostate cancer in vivo [13]. Viral vector mediated overexpression of $AT_2R$ promoted apoptosis and reduced VGEF in bladder cancer [20]. Interestingly, $AT_2R$ stimulation by $AT_2R$ agonist NP-6A4 caused enhanced expression of $AT_2R$ [22]. Therefore, it is tempting to speculate that a combination of LP2-mediated $AT_2R$-linked pathway stimulation and an autofeedback loop leading to enhanced expression of $AT_2R$ may

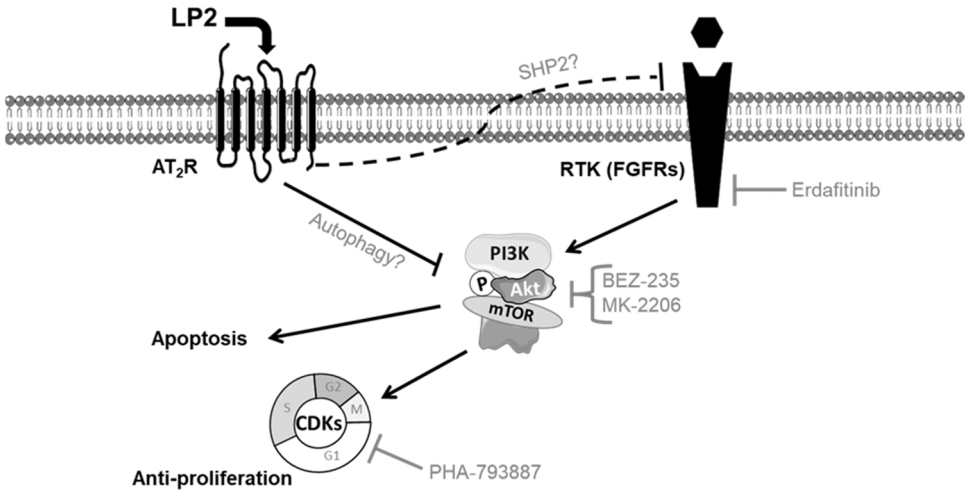


**Fig. 5.** Signaling pathway model. Model based on kinome analyses and validation using specific kinase inhibitor profiling on LP2 and vehicle-treated CRC PDX tumor lysates.

contribute to the efficacy of LP2 at a low dose. LP2 acted synergistically with erlotinib (Fig. 4) which makes sense in the light of the proposed mechanism of action (Fig. 5). LP2 inhibits not only RTKs, which are target to EGFR inhibitors like erlotinib, but also the PI3K/AKT/mTOR pathway downstream of RTKs (Fig. 5). This is particularly relevant for treating KRAS mutated tumors, where EGFR inhibitors are thus far excluded from therapeutic schedules due to lack of efficacy.

Unbalanced stimulation of $AT_1R$ can be tumor-promoting, proliferative, pro-angiogenic, fibrotic and inflammatory. Angiotensin Receptor Blockers (ABRs) block $AT_1R$ and thereby access of Ang II to $AT_1R$. Resulting unbound Ang II may stimulate $AT_2R$. Thus, the therapeutic effect of ARBs results from both $AT_1R$ blockage and $AT_2R$ stimulation. Whereas the $AT_1R$ blockage mainly reduces blood pressure, prevents renal salt and water retention, the $AT_2R$ stimulation has tissue protective effects and may cause apoptosis and anti-proliferation. These additional effects of ARBs have been observed already in 2000 [23], whereas the tumor-suppressive effects mediated by ARBs have been observed retrospectively in humans for several tumor types [14].

Clinical evidence shows the relevance of the renin angiotensin system in cancer. Clinical data show that high AGTR1 ($AT_1R$) expression level is associated with poor recurrence-free survival (RFS) and RFS was significantly better in the ACE inhibitor/ARB group [18]. ARBs alter the KRAS-mutated CRC oncogenic signaling resulting in improvement in patient outcome or, through a reversion to a KRAS wild-type phenotype, in improved response to anti-EGFR treatment [26]. The use of ARBs decreases colorectal cancer risk and mortality [6]. Furthermore, among patients taking ACE inhibitors / ARBs, a significant 3-fold increase in the rate of pathologic complete response to radiation therapy was observed [16].

The effects of ARBs on $AT_2R$ simulation are relatively weak due to the low concentration, pico- to femtomolar, of the labile Ang II which insufficiently stimulate the $AT_2R$. In contrast, the direct $AT_2R$ stimulation with a selective and stable agonist, fully activates $AT_2R$ providing relevant anti-tumor effects in vivo. Direct stimulation of $AT_2R$ is tumor-suppressing. Combined $AT_1R$ blocking and $AT_2R$ stimulation acted synergistically in ovarian cancer [19].

## 5. Conclusion

In summary, LP2, a selective $AT_2R$ agonist, impairs the growth of patient-derived xenografts of colorectal carcinoma. Synergism has been demonstrated for the combination of LP2 with 5-FU and the EGFR inhibitor erlotinib. These effects are obtained at an extremely low dose of 0.2 μg/kg/d subcutaneously injected LP2. While the observed efficacy in KRAS mutated PDX and peritoneal metastatic PDX are relevant, future evaluation using larger number of PDXs may elucidate any association between gene mutation pattern and response to LP2. Together with the previously reported excellent safety in man, excellent pharmacokinetics and very favorable CMC these studies argue for continued evaluation of the hypothesis of therapeutic use of LP2 in colorectal cancer.

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## CRediT authorship contribution statement

All authors have contributed to the conceptualization and design of the study and contributed to discussion and evaluation of the obtained data. PN selected the methodologies and performed analyses. GNM and PN wrote the manuscript.

## Conflict of interest

GNM is owner of Lanthio Holding B.V.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.peptides.2022.170920.

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