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Anibamine, a Natural Product CCR5 Antagonist, as a Novel Lead for the Development of Anti Prostate Cancer Agents

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Abstract

Accumulating evidence indicates that the chemokine receptor CCR5 and the chemokine CCL5 may be involved in the proliferation and metastasis of prostate cancer. Consequently, chemokine receptor CCR5 antagonists could potentially act as anti prostate cancer agents. As the first natural product CCR5 antagonist, anibamine provides a novel chemical structural skeleton compared with other known antagonists identified through high-throughput screening. Our studies demonstrate that anibamine produces significant inhibition of prostate cancer cell proliferation at micromolar to submicromolar concentrations as well as suppressing adhesion and invasion of the highly metastatic M12 prostate cancer cell line. Preliminary in vivo studies indicate that anibamine also inhibits prostate tumor growth in mice. These findings indicate that anibamine may prove to be a novel lead compound for the development of prostate cancer therapeutic agents.

Keywords

chemokine receptor CCR5; antagonist; anibamine; prostate cancer

Prostate cancer is the most common non-cutaneous solid cancer occurring amongst men in the USA, and the second most common malignant cause of male death worldwide[1]. Current therapies remain limited to surgery, radiation, and/or androgen ablation[2]. Recent investigations indicate that there is a relationship between some inflammatory processes and cancer, specifically, prostate cancer development[3-13]. For example, the prostate cancer cell lines PC-3, DU145, and LNCaP express the chemokine CCL5 (RANTES) and the

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chemokine receptor CCR5. Furthermore, the chemokine receptor CCR5 antagonist, TAK-779 inhibited CCL5-induced proliferation of these prostate cancer cell lines[12]. Levels of CCL5 and CCR5 are also reported to be greater in prostate cancer specimens than in benign hyperplasia[13]. Collectively these findings in both patient-derived specimens and prostate cancer cell lines suggest that development of the appropriate chemokine receptor CCR5 antagonists could provide a novel prostate cancer therapy.

Anibamine (Figure 1), a novel pyridine quaternary alkaloid recently isolated from *Aniba sp*., was found to bind to CCR5 with an IC_{50} of 1 μM in competition with 125I-gp120, an HIV viral envelop protein[14]. Thus far, anibamine is the first known natural product acting as a CCR5 antagonist. While the chemokine receptor CCR5 has mainly been targeted in HIV therapies since it was first cloned more than a decade ago[15-21], CCR5 antagonists could provide a novel therapeutic approach for prostate cancer treatment through the inhibition of CCL5 induced cell proliferation.

Anibamine has a novel structural skeleton compared to other CCR5 antagonists identified through high-throughput screening. Considering the binding affinity to CCR5 of other original lead compounds[22-24], the inhibitory binding affinity of anibamine at 1 μM to CCR5 appears quite promising.

Recently, the total synthesis of anibamine has been reported by one of our laboratories[25]. The development of this synthetic pathway provides the opportunity for generating anibamine derivatives in order to explore their structure-activity relationships as CCR5 antagonists. The binding of anibamine to the chemokine receptor CCR5 has been characterized and compared with that of other CCR5 antagonists in different homology models of CCR5[26]. The binding pocket of anibamine shares some common features with other high affinity CCR5 antagonists, suggesting binding to similar binding sites. The current studies were designed to explore the utility of developing anibamine as a novel lead compound against prostate cancer.

As indicated previously, the expression of CCL5 and CCR5 has been observed in various prostate cancer cell lines, including PC-3, DU145, and LNCaP[12,13]. Expression of CCR5 and CCL5 mRNA was quantitated via qRT-PCR in the highly metastatic M12 prostate epithelial cell line, as well as in its non tumorigenic parental cell line P69[27]. The results, shown in Figure 2, indicate that while both genetically related sublines express CCR5, CCL5 expression was evident in the M12 tumorigenic subline but was barely detectable in the parental p69 line. From our results, the relatively elevated levels of CCL5 in the metastatic M12 cell line compared to the nontumorigenic parental p69 line suggest that CCL5 and its receptor CCR5 could be involved in prostate cancer metastatic progression, providing additional support for the potential value of targeting the chemokine receptor CCR5 in prostate cancer.

Previously, M12 cells were shown to have a very high invasive ability[27]. It is also known that adhesion and invasion are important steps that further promote prostate tumorigenesis and metastasis. The growth inhibitory properties of anibamine were evaluated in the prostate cancer cell lines, PC-3, DU145, and M12. Results of these assays are summarized in Figure 3. Anibamine was observed to interfere with prostate cancer cell growth in a dose-dependent manner at micromolar to submicromolar concentrations in all three cell lines. The observations that anibamine can inhibit the invasion and adhesion of M12 cells support the possibility that anibamine may have anti metastatic properties against prostate cancer.

In invasion and adhesion assays, the addition of anibamine inhibited M12 invasive ability by 42 to 65% (Figure 4) depending on the dose and M12 adhesion up to 26% (Figure 5). No additional effects on adhesion were evident at higher concentrations (data not shown).

Further, M12 cells embedded in lrECM gels were studied to assess the effect of anibamine on tumor cell morphology. As shown in Figure 6, the M12 subline displayed a disorganized mass of cells when grown in 3D (which is in agreement with its metastatic character); the addition of anibamine reverted M12 cells to spheroid-like structures referred to as acini. These observations further support the premise that anibamine indeed could potentially inhibit prostate tumor metastasis.

After that, immunohistochemical staining for relevant cell proteins coupled with confocal microscopy was conducted to better examine the morphological differences displayed by these cells when grown embedded in lrECM gels. The M12 subline, which shows high expression of vimentin, spread throughout the lrECM as a disorganized mass, which reflects the highly tumorigenic/metastatic behavior of these cells when injected into male, athymic nude mice[28]. Interestingly, vimentin gene expression declined with the addition of anibamine (Figure 7). In addition, while the expression of α6- and β1- integrins was quite disorganized in M12 cells. The addition of anibamine reverted the disorganized mass of cells to an acinus with a distinct luman displaying basal polarization of α6- and β1- integrin as shown previously for the parental, benign P69 cells.

As with the development of any new class of drugs, it was critical to determine whether these compounds could be used at concentrations that are not toxic to normal cells. For our initial screening studies, we examined hemolysis of sheep red blood cells by anibamine, since this was thought to be a possible limitation on the use of this class of agents[29]. Our result indicates that no toxicity was observed in this assay below or at a concentration of 1 μM (Figure 8), which would support the potential selectivity of this agent.

In addition, preliminary data from an on-going in vivo analysis suggests that anibamine can reduce the subcutaneous growth of M12 tumor cells in athymic nude mice. In the three mice with subcutaneous M12 tumors, four days after four injections of anibamine, the size of the tumors was 321.4 mm^3, 80.0 mm^3, and 202.2 mm^3, respectively, averaging 201.2 ± 69.7 mm^3. In contrast, the size of the tumors injected with the solvent control was 421.6 mm^3, 182.6 mm^3, and 384.6 mm^3, respectively, averaging 329.6 ± 74.3 mm^3. Thus anibamine did appear to reduce the rate of growth of the M12 tumors by roughly 50% (Figure 9). Such observation that anibamine can reduce the subcutaneous growth of M12 tumor cells in athymic nude mice support the premise that anibamine and/or its derivatives could prove to have utility in the treatment of prostate cancer.

In summary, anibamine showed significant inhibition of prostate cancer cell proliferation at 1 μM and lower concentrations while direct hemolysis was not evident until an approximately 10-fold higher concentration. Anibamine also suppressed the invasive and metastatic properties of M12 cells and compromised the growth of these tumors in vivo. Overall these preclinical studies suggest that anibamine could have a reasonable therapeutic index, supporting the potential utility of this compound as the lead for future drug design and development.

One reservation is that the calculated log K_{ow} for anibamine is 9.1[29], which indicates that its lipophilicity is significantly higher than the value set forth by “Lipinski’s rule of 5” for drug-like compounds[30]. In comparing the chemical structure of anibamine with other known CCR5 antagonists (Figure 1), a major difference is that the anibamine side chains are simple, undecorated, aliphatic chains. Therefore, further drug development for CCR5 antagonists based on the chemical structure of anibamine, the first natural product with high binding affinity to the CCR5 chemokine receptor, may lead to a new type of therapeutic agent for metastatic prostate cancer therapy. Further studies of anibamine and its analogs

should also serve to clarify the mechanisms by which targeting the chemokine receptor CCR5 may suppress metastatic processes of prostate cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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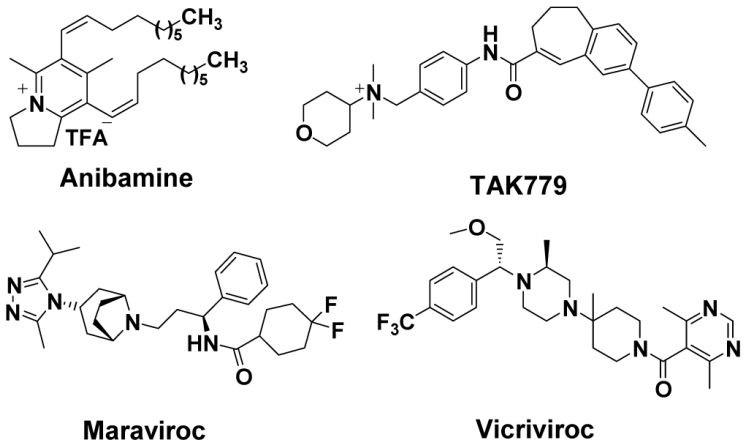

Figure 1.
Anibamine and some known CCR5 antagonists

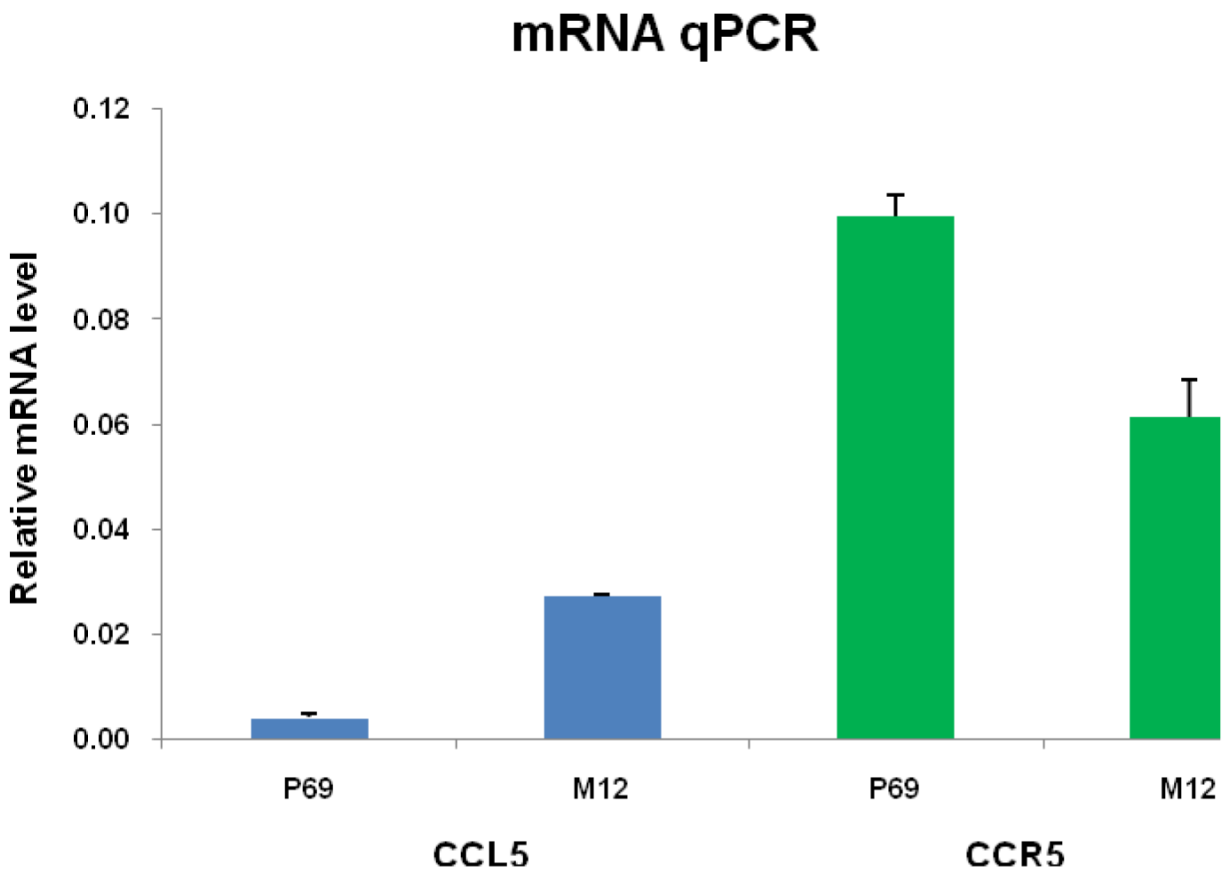

Figure 2.
Differential expression of CCL5 and CCR5 in isogenic P69 and M12 prostate cancer sublines. SYBR-based qRT-PCR was performed with total RNA extracted from P69 and M12 sublines as described in Materials and Methods. The Y-axis represents the relative mRNA level of CCL5 or CCR5 normalized to RNU48 as an internal control. The standard error of the mean is shown as error bars. Student's t-test indicates a significant difference with a *P*-value <0.001 for both CCL5 and CCR5.

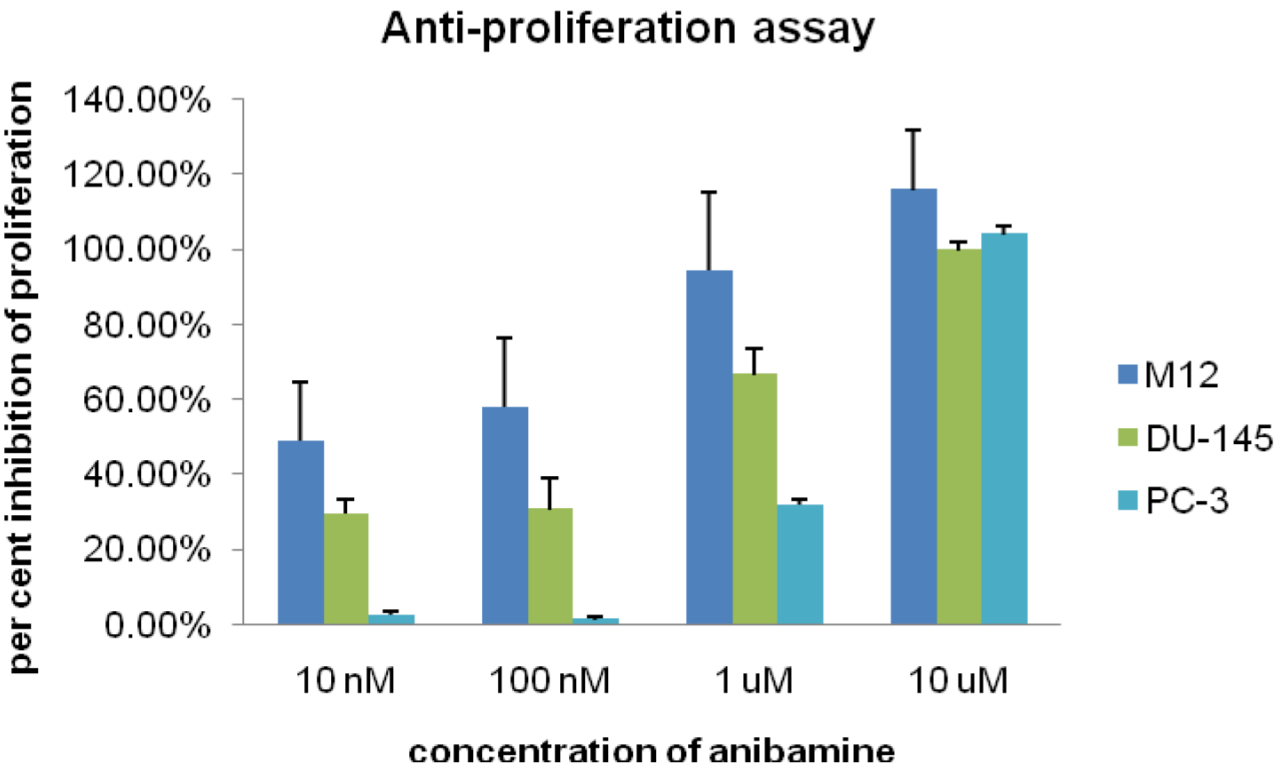

Figure 3.
Inhibition of prostate cancer cell proliferation by anibamine. Three prostate cancer cell lines, M12, PC-3, and DU-145 were exposed to a series of concentrations of anibamine. Proliferation was assessed using the WST-1 Cell Proliferation Reagent (Roche).

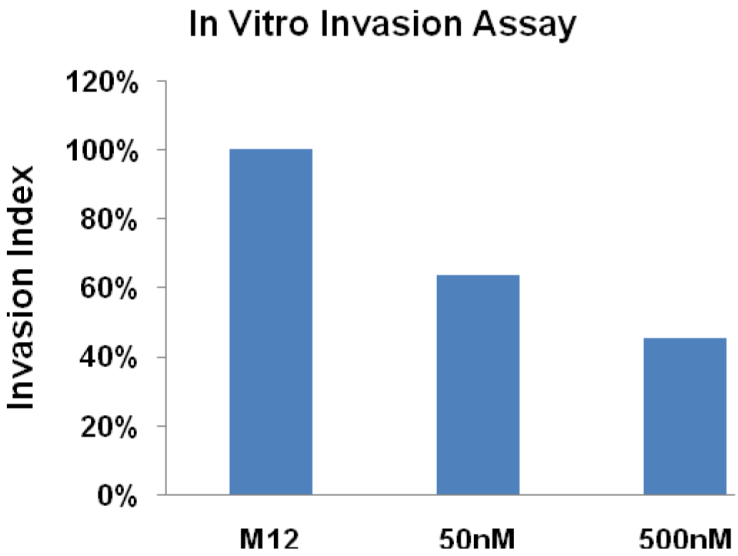

Figure 4.

Effect of anibamine on invasive capability of M12 cells. Equal numbers of M12 cells were plated in Transwell chambers −/+ anibamine at the indicated concentrations as described in Materials and Methods. Filters were coated with 1:10 diluted lrECM prior to cell plating. Medium containing 20% FBS, EGF (20 ng/ml) and 5ng/ml CCL5 was added as a chemo-attractant to the lower chamber. Bars indicate standard error. ANOVA testing indicates a significant difference with a *P*-value <0.001.

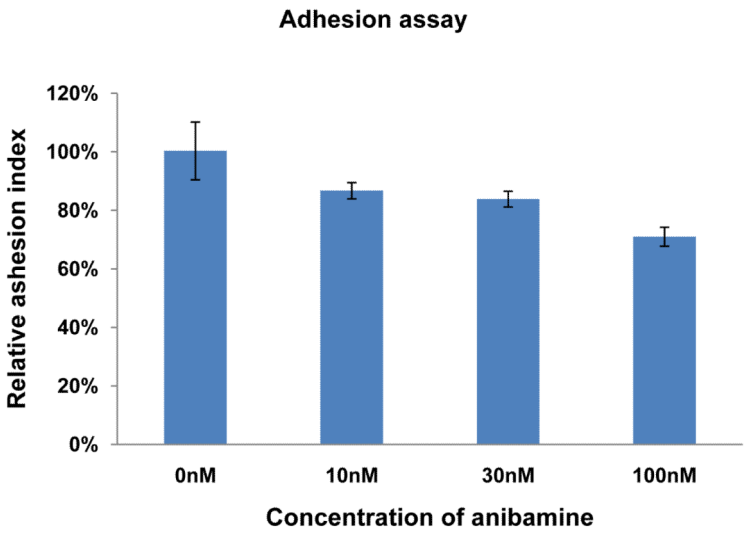

Figure 5.

Effect of anibamine on M12 cell adhesion. Equal numbers of M12 cells were pre-treated +/− anibamine at the indicated concentrations for 24 hours and were then plated in 96-well cells coated with diluted lrECM as described in Materials and Methods. The x-axis represents the concentration of anibamine, while the Y-axis represents the relative adhesion index normalized to the M12 control without drug. Bars indicate standard error. ANOVA test indicates a significant difference with a P-value of <0.001 (F=26.8).

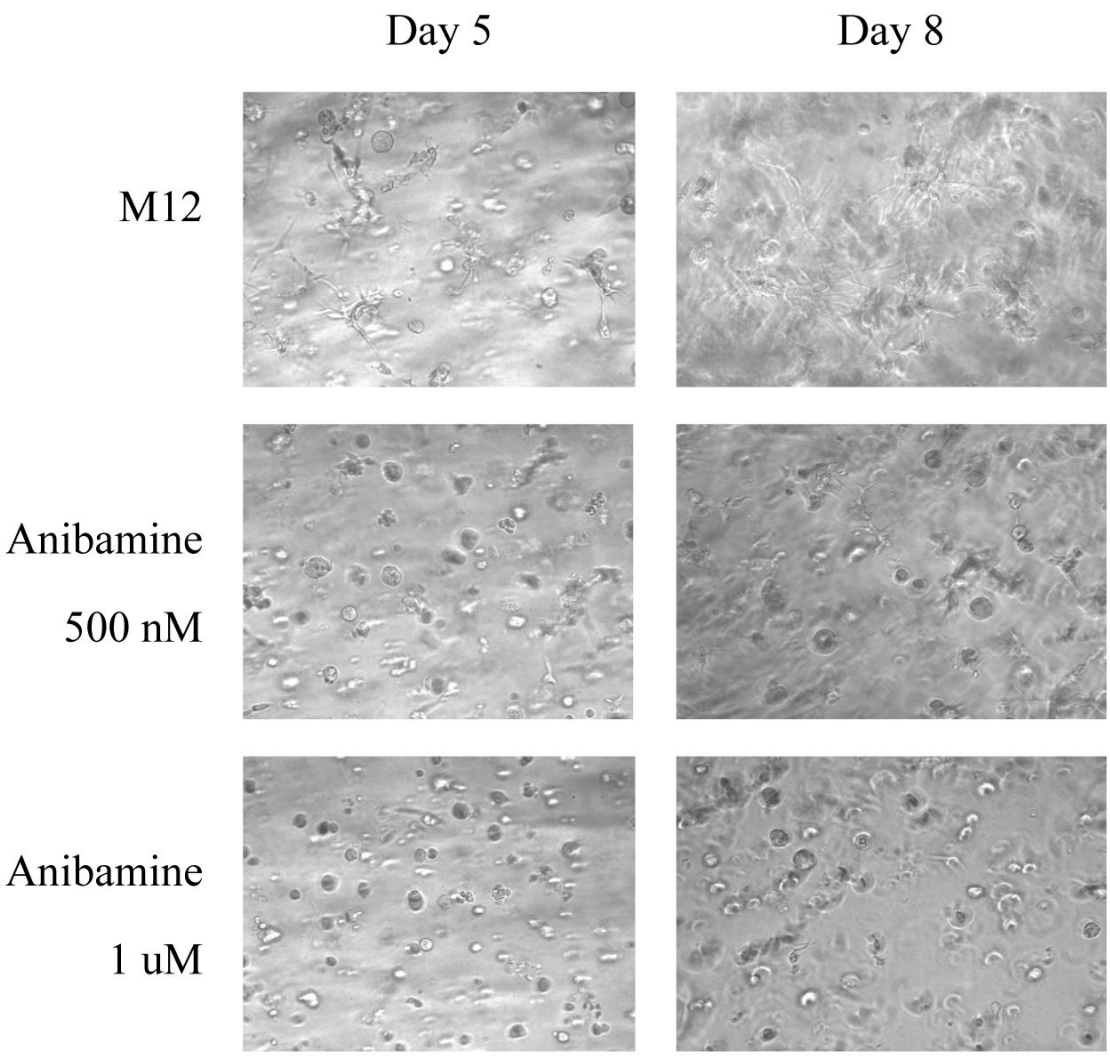

Figure 6.
Growth properties of M12 cells +/− anibamine in 3D lrECM gels. M12 cells (1×10^5) were mixed with 100μl of undiluted lrECM gel +/− 500nM or 1μM anibamine and then plated in 96-well plate as described in Materials and Methods. Light microscopy images were taken from cultures at day 5 and 8 as indicated. Magnification is at 10X.

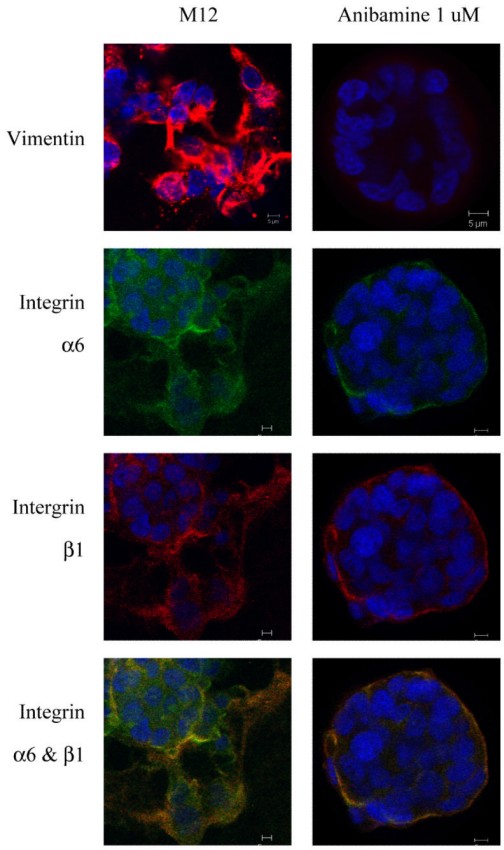

Figure 7.
Comparison of content and localization of vimentin and integrin within the morphological structures formed by the M12 prostate sublines +/− anibamine grown embedded in lrECM gels. Confocal immunofluorescence microscopy of structures formed at day 8 stained with antibodies to vimentin (red, top panel), α6-integrin (green) and β1-integrin (red) as indicated. The overlay of α6β1-integrin is shown on the bottom panel. All pictures are taken at a magnification of 63X and nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI; blue) as discussed in Materials and Methods.

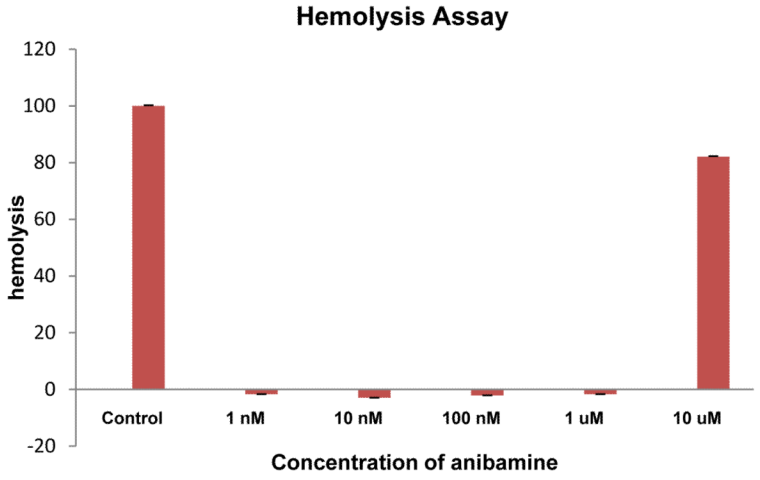

Figure 8.

Assessment of red blood cell hemolysis by anibamine. Sheep red blood cells were exposed to anibamine in PBS for 60 min. Samples were centrifuged and the absorbance of the supernatant determined at 540nm. Lysis in distilled water was used as a positive control.

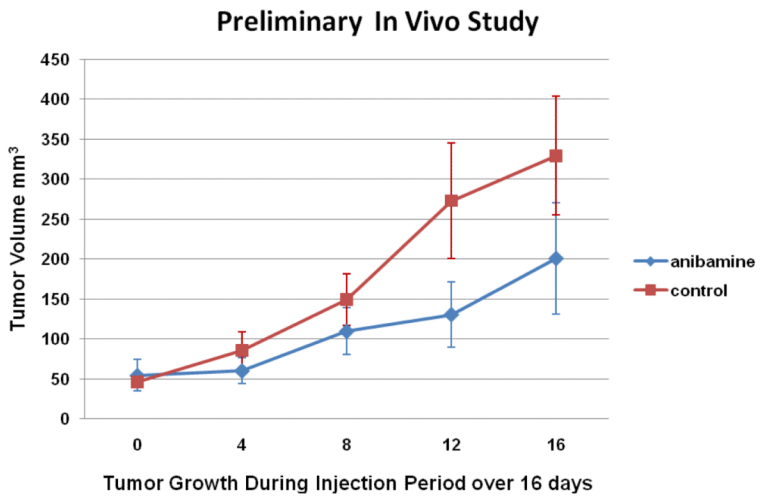

Figure 9.

Influence of anibamine on prostate tumor growth in vivo. Six athymic nude mice were injected subcutaneously with 2000 M12 cells. After the tumors became visible, the mice were injected intravenously via a lateral tail vein with 0.3mg/kg of anibamine or with 0.3mg/kg of saline over a 16 day period for four injections (once every fourth day). The tumor size was recorded accordingly as the number of day right after the first injection.