



Published in final edited form as:

Int J Cancer. 2015 August 1; 137(3): 744–749. doi:10.1002/ijc.29414.

Dopamine is a safe anti-angiogenic drug which can also prevent 5-fluorouracil induced neutropenia

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Abstract

The role of vascular endothelial growth factor - A (VEGFA) in tumor angiogenesis is well established and accordingly, molecules targeting VEGFA or its receptors are being presently used in the clinics for treatment of several types of cancer. However, these anti-angiogenic agents are expensive and have serious side effects. Thus identification of newer drugs with manageable systemic side effects or toxicities is of immense clinical importance. Since we have reported earlier that dopamine (DA) inhibits VEGFA induced angiogenesis in experimental tumor models, we therefore sought to investigate whether DA treatment results in similar toxicities like other anti-angiogenic agents. Our results indicated that unlike sunitinib, another commonly used anti-angiogenic agent in the clinics which targets VEGF receptors, DA (50mg/kg/d × 7days i.p.) not only could inhibit tumor angiogenesis and growth of HT29 human colon cancer and LLC (Lewis lung carcinoma) in mice, it also did not cause hypertension, hematological, renal and hepatic toxicities in normal, HT29 and LLC tumor bearing animals. Furthermore and interestingly, in contrast to the currently used anti-angiogenic agents, DA also prevented 5-fluorouracil (5FU) induced neutropenia in HT29 colon cancer bearing mice. This action of DA was through inhibition of 5FU mediated suppression of CFU-GM colony forming units in the bone marrow. Thus our results indicate that DA may be safely used as an anti-angiogenic drug for the treatment of malignant tumors.

Keywords

Dopamine; anti-angiogenic drugs; 5-FU; toxicity; Lung Cancer; Colon Cancer

Introduction

Neovascularization or formation of new blood vessels from adult endothelial cells (angiogenesis) and bone marrow (BM) -derived endothelial progenitor cells

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(vasculogenesis) is essential for the growth, progression and metastasis of malignant tumors (1–3). This process is tightly regulated by both pro- and anti-angiogenic factors, and a shift of the balance towards the pro-angiogenic factors results in the generation of neovessels in tumor microenvironment (2). Among the pro-angiogenic factors, the role of VEGFA in mediating tumor angiogenesis is well established (2). VEGFA mainly binds to VEGF receptor-2 (VEGFR-2) to induce neovascularization in tumors (2). Accordingly, VEGFA- and VEGF receptor targeting monoclonal antibodies and tyrosine kinase inhibitors have been developed to treat several types of solid tumors (4, 5). Since these agents unlike conventional cytotoxic drugs usually target the functions of this growth factor in proliferating endothelial cells and because endothelial cells are normally quiescent in absence of stimuli, it is thus expected that these drugs will be generally well tolerated mainly due to their specific cellular effects (6, 7). However, these anti-VEGF agents are not only expensive, but they also have serious toxicities, which precludes their uses in many patients (8, 9). We and others have reported that the neurotransmitter DA can inhibit VEGFA induced angiogenesis and vasculogenesis in experimental tumors (10–14). DA acting through its D₂ receptors present in the adult endothelial and BM-derived endothelial precursor cells suppresses pro-angiogenic signaling pathways of VEGFA (10, 13). Because DA inhibits the actions of VEGFA, it will therefore be important to investigate if the anti-angiogenic dose of DA has similar side-effects that are observed with the currently used anti-VEGF drugs (8, 9). The results of this study will further help to translate the anti-angiogenic action of DA for the treatment of cancer.

Materials and Methods

Cells and Reagents

HT29 cells (ATCC) were cultured in McCoy's 5A (ATCC) medium, supplemented with 10% FBS (12). Highly angiogenic and VEGFA producing type LLC cells (clone D122-96) was provided by Dr. L. Eisenbach (Weizman Institute of Science, Rehovot, Israel) and were cultured in DMEM (ATCC) containing 10% FBS (15).

Mice, Tumors

Animal experiments were undertaken after approval by the Ohio State University Animal Care and Use Committee. Male athymic nude (Charles River Laboratory) and C57BL/6 (Jackson Laboratory) (6–8 wk old) mice were used. The HT29 orthotopic colon cancer model was developed as before (12). Briefly, 1×10^6 HT29 cells in 50 μ L medium were injected into the cecal wall from the serosal side with a 30-gauge needle. After tumor cell transplantation, the cecum was returned to the abdominal cavity, and the wound was closed. The abdomen of the animals were surgically reopened approximately 60 days after tumor implantation to detect tumors in the cecum and then closed. (12). Tumor bearing animals were divided randomly into vehicle and DA treatment groups. DA (American Reagent Laboratories) was administered once daily intraperitoneally (i.p.) at a dose of 50 mg/kg for 7 consecutive days (12). This dose of DA corresponds to 5% of the LD₅₀ in mice (11, 12) and raises the plasma DA level in mice to 1.2 ± 0.01 nmol/mL in 1 min from 1.5 ± 0.01 pmol/mL (normal plasma level in mice) (11). Furthermore, this raised level of DA is comparable to the plasma level of DA reached in human subjects treated with an intravenous

infusion of the dopaminergic dose of DA (16). Sunitinib malate (Selleckchem) was administered at a dose 40mg/kg once daily by oral gavage for 7 consecutive days as this dose (40mg/kg orally once daily) was reported to inhibit angiogenesis in mice bearing HT29 and LLC tumors (17). 5-FU (APP Pharmaceuticals) was administered at a dose of 20 mg/kg once daily for 5 consecutive days (18) either alone or after DA treatment (50mg/kg) of HT 29 tumor bearing mice for 7 days.

For LLC tumors, the dorsal skin of the C57/BL6 mice was shaved and 200 μ L of cells at a concentration of 1×10^7 cells /ml, were injected subcutaneously into the right flank of mice. When tumors reached a size of 100mm^3 (~ 10 days) mice were randomly divided into vehicle, DA and sunitinib treatment groups (17, 18). For CFU-GM assay, DA was administered for 7 days (50mg/kg), 5-FU was administered at a dose of 20 mg/kg once daily for 5 consecutive days (18) either alone or after DA treatment of normal mice for 7 days and then BM was collected.

Tumor growth and angiogenesis

For orthotopic HT29, tumors were collected and growth was determined by weighing the tumor mass (18). For LLC tumors, growth was determined by caliper measurement of the largest diameter and its perpendicular and tumor volume was calculated (12). Tumor angiogenesis was determined by immunohistochemical staining of CD31 positive vessels (11, 12)

Hematology and Biochemical Assays

Blood was collected by cardiac puncture after anesthetizing the mice. Complete blood counts with 6-part white blood cell differential were performed on a portion of EDTA anti-coagulated whole blood (FORCYTE Autosampler10, Oxford Science, Inc., Oxford, CT). Following coagulation of the remaining whole blood at room temperature for 30 min., the clotted blood was centrifuged at 3000 rpm for 5-10 min. at 4C. Biochemical profiles were performed on serum samples (VetACE, Alfa Wasserman, West Caldwell, NJ)

Measurement of Blood Pressure

Systolic, Diastolic and Mean blood pressures (BP) were measured in untreated, DA and sunitinib treated mice using a computerized tail-cuff system (CODA System, Kent Scientific). Prior to treatment mice were trained daily and the BP were recorded at the end of treatment. This method of BP measurement corresponds with direct measurements of intra-arterial pressure (19).

Colony Forming Assay

Mouse BM colony forming assay was performed using Methocult products for CFU assays (StemCell Technologies). BM was collected from femurs and tibia in Iscove's MDM from normal, 5 FU, DA and DA+5FU treated normal mice. Cells were seeded in culture dishes with 1×10^5 cells/ml MethoCult M3434 medium (StemCell Technologies, Inc.) (total volume, 1.1 ml) and incubated for 12 days at 37° C with 5 % CO₂ in air and 95 % humidity. CFU-GM colonies were identified and counted using an inverted microscope (20).

Statistics

For statistical analysis the differences among the groups were evaluated by ANOVA and unpaired student's test or Dunn's multiple comparison tests. Mean and SE were calculated and $P < 0.05$ was considered significant (12)

Results and Discussion

Effect on tumor growth and angiogenesis

In HT29 colon (Fig1*a*) and LLC tumors (Fig1*b*), DA treatment showed significant reduction in tumor growth when compared with vehicle-treated controls. Angiogenic response as assessed by counting the numbers of CD31 positive vessels were also significantly less in DA treated tumors (Fig 1*c* and *d*).

Effect on Blood Pressure

Among the several toxicities that have been reported following the use of anti-VEGF agents, hypertension is most commonly observed with recent reports indicating that the incidence of hypertension following use of these agents varies between 20%–87% (8, 21). Therefore, detection and management of anti-VEGF therapy induced hypertension has become a major challenge. Hence while testing the possible side effects which the anti-angiogenic dose of DA might have, we first studied the effect of DA on the BPs of animals. After establishing baseline BPs, normal nude (Fig1*e*) and C57/BL6 (Fig1*f*) were injected with either DA (50mg/kg i.p.) or sunitinib (40mg/kg orally) or vehicle (PBS) for 7days. Following completion of treatment, no change in BP (systolic, diastolic and mean) was noted between DA treated and vehicle treated groups (Fig 1*e* and *f*). However, sunitinib caused significant increase in the BPs of the animals (Fig 1*e* and *f*). Similar effects were also observed in HT29 and LLC bearing animals (data not shown). These results indicated that the anti-angiogenic dose of DA does not cause hypertension.

Liver function tests

Severe hepatic toxicity has been reported in patients receiving currently available anti-VEGF drugs (22). Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) elevations were reported in cancer patients treated with these angiogenic inhibitors. Transaminase elevations usually occur early during the treatment and they are reversible with patients generally being asymptomatic. However, such elevations may also lead to severe liver damage, which in turn may lead to interruptions in treatment and significant morbidity (22). We therefore tested whether DA can cause hepatic toxicity in normal, HT29 and LLC tumor bearing mice. Our results demonstrate that there were no abnormal changes in the serum ALT (Fig2*a*) and AST levels (Fig2*b*) in DA treated normal and tumor bearing mice. In addition, there was also no elevation of serum bilirubin level (Fig2*c*) after DA treatment, thereby indicating that unlike other anti-angiogenic drugs, DA administration does not adversely affect liver functions. However, in contrast to DA, significant increase in these two liver enzymes was noted in normal mice treated with sunitinib. ALT and AST levels were also increased in sunitinib treated tumor bearing animals in comparison to

untreated controls (Fig 2a and b). We did not detect any significant changes in the serum bilirubin levels among the different groups (Fig 2c)

Renal effects

The association between VEGF inhibitors and renal damage has been commonly reported (21). We therefore tested whether DA treatment has any impact on renal functions of animals. Our results demonstrate that serum blood urea nitrogen (BUN) (Fig 2d) and creatinine (Fig 2e) levels were normal after DA treatment in normal, HT29 and LLC tumor bearing mice. On the contrary, serum BUN was increased in sunitinib treated animals (Fig 2d). No significant differences were noted in the serum creatinine levels between the groups (Fig 2e).

Hematological effects

Myelosuppression and thrombocytopenia are common problems in cancer patients receiving anti-angiogenic drugs (23). Drugs that inhibit the actions of VEGFA can cause myelosuppression and thrombocytopenia as VEGFRs expressed in hematopoietic cells play a role in myelopoiesis and thrombopoiesis (23). However, the degree of suppression varies between drugs, with some causing both thrombocytopenia and neutropenia (23). We therefore determined the platelet (Fig 3a) and neutrophil (NE) (Fig 3b) counts in normal and tumor bearing mice treated with DA. Administration of DA did not cause thrombocytopenia in normal, HT29 human colon cancer and LLC bearing mice (Fig 3a). In addition, DA administration also did not induce neutropenia in these animals (Fig 3b). It is also important to note here that there were also no significant changes in the hemoglobin levels of the animals treated with DA (Fig 3c). Sunitinib in contrast caused decrease in both platelet and neutrophil counts (Fig 3a and b). We did not find any significant differences in hemoglobin (Hb) levels between the different groups (Fig 3c).

Furthermore, we had previously reported that combination treatment of DA with 5FU can significantly inhibit colon cancer growth in mice (12). Because one of the serious side effects of 5FU alone and combining anti-VEGF drugs with 5FU is low NE count in colon cancer patients (24), we therefore examined whether administration of DA together with 5FU has any effect on NE counts in colon cancer bearing mice. Mice receiving only 5FU injections had significantly lowered NE counts (Fig 3d). However, when these tumor bearing animals were treated with DA followed with 5FU, DA was able to prevent this suppressive action of 5FU (Fig 3d).

Effect on CFU-GM colony formation by dopamine

Thereafter to elucidate the mechanism by which DA prevented 5-FU induced myelosuppression, we determined the number of colony forming unit-granulocyte macrophage (CFU-GM) colonies in normal, DA, 5FU and DA+5FU treated normal mice because there are several reports which indicate that 5-FU administration suppresses the CFU-GM colony forming cells in the BM (25). Our results indicated that treatment with 5-FU resulted in significantly lower CFU-GM colonies in comparison to normal (Fig 3e). On the contrary, the number of CFU-GM colonies formed on treatment with DA and then with 5FU was significantly more than that formed by 5FU alone; thereby indicating that DA

could inhibit 5FU mediated suppression of CFU-GM colony forming units in the BM (Fig3e).

In summary, there are now several reports which indicate that anti-VEGF agents can cause hypertension, abnormal AST, ALT and BUN levels suggesting hepatic and renal damage (8, 9). In addition, these drugs can also cause neutropenia and thrombocytopenia (8, 9). On the contrary, our results demonstrate that the anti-angiogenic dose of DA is devoid of serious toxicities usually observed with the use of anti-VEGF agents in the clinics such as sunitinib (23).

Importantly, our experiments also indicate that DA can ameliorate neutropenia induced by commonly used anti-cancer drugs, such as 5FU. This information together with the safety profile of DA known due to its use in the treatment of other disorders demonstrate that this small molecule can not only be used as an effective anti-angiogenic drug, it may also be utilized to prevent bone-marrow suppression observed with 5FU when used in combination with anti-VEGF agents for the treatment of colon cancer in the clinics. Finally, the cost of treatment with DA is significantly less and this in turn is also very important in perspectives of health economics for proper utilization of the funds allocated for the treatment of cancer.

Acknowledgements

Grant Sponsor: NIH/NCI, R01 CA124763 to S.B. and CSIR 21(0895)/12EMR-II to PSD. We also thank the Comparative Pathology and Mouse Phenotyping Shared Resource of the Ohio State University for hematological and clinical chemistry tests.

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What's new?

This report for the first time indicates that the anti-angiogenic dose of DA does not cause any untoward side effects commonly observed with the presently used anti-VEGF agents. In addition, DA can also prevent 5FU induced neutropenia in tumor bearing animals. Therefore this inexpensive drug which is being used in the clinics for many years for the treatment of cardiovascular and renal disorders may also be safely administered as an anti-angiogenic agent for the treatment of malignant tumors.

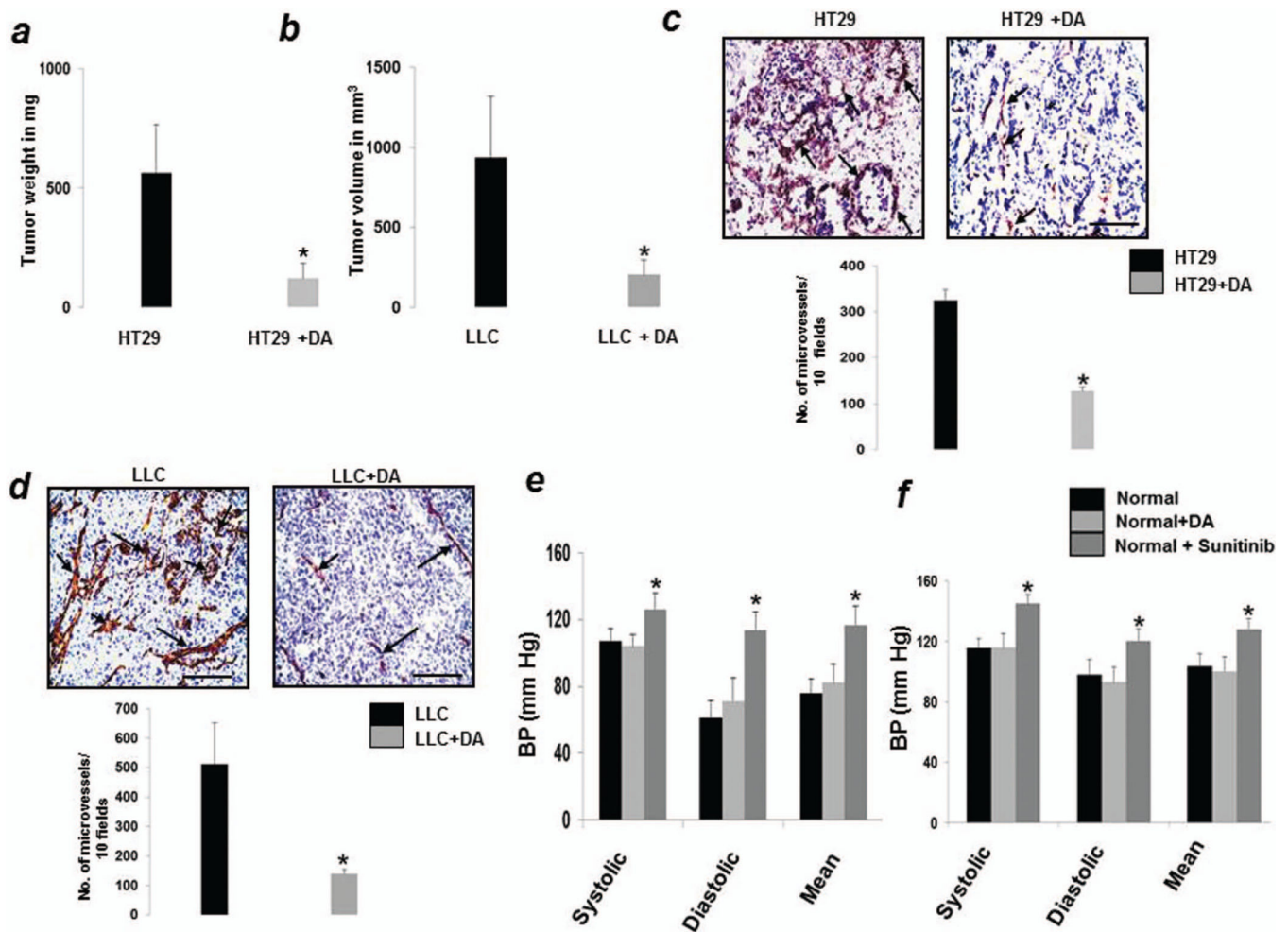
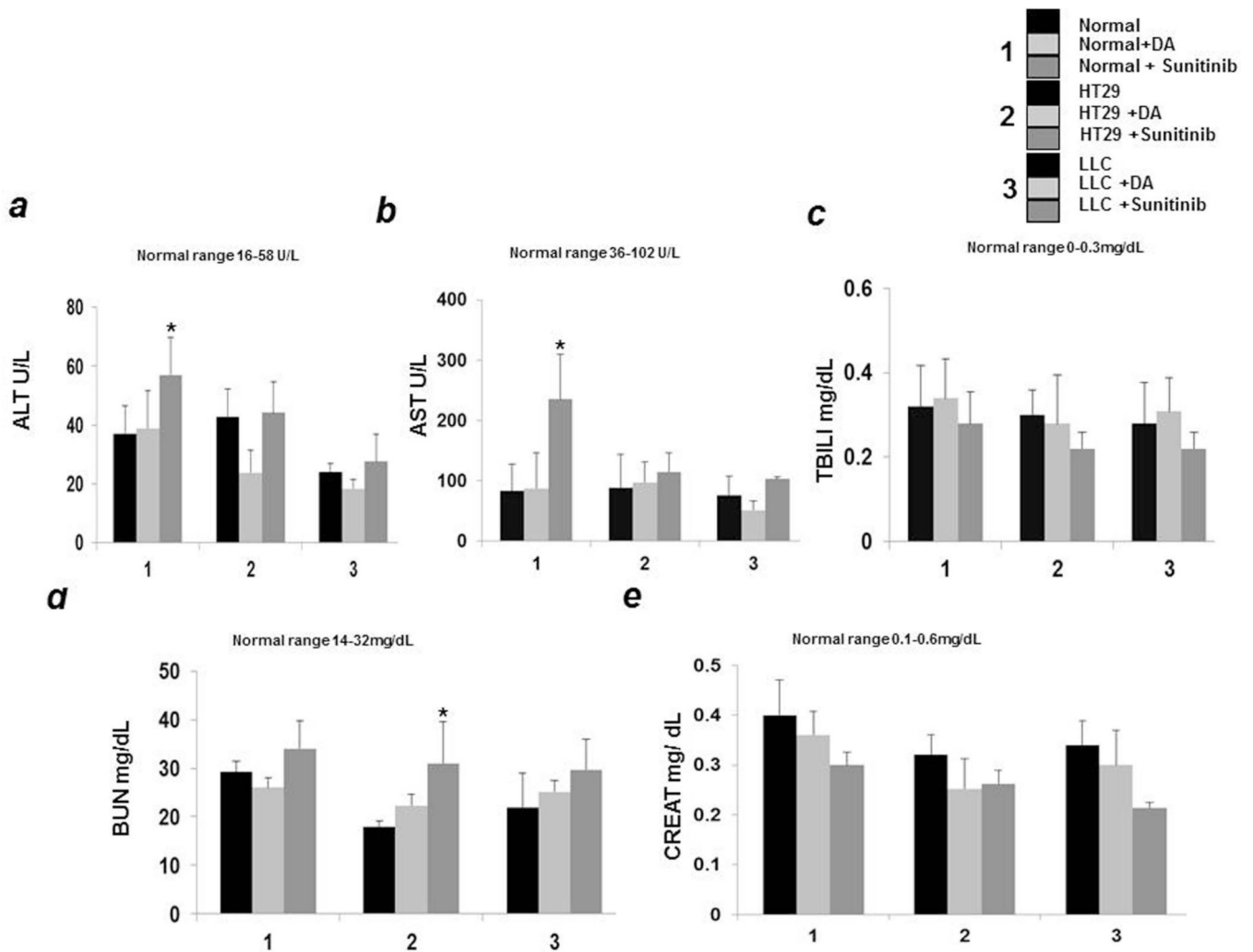


Figure 1.

Dopamine (DA) treatment significantly reduced the growth of (a) orthotopic HT29 colon cancer and (b) subcutaneous lewis lung carcinoma (LLC) tumors as compared to untreated tumors. Tumor angiogenesis was also significantly reduced in dopamine treated (c) HT29 and (d) LLC tumors compared to respective untreated controls. In contrast to sunitinib, dopamine treatment had no significant effect on the diastolic, systolic and mean blood pressures of normal (e) athymic nude and (f) C57/BL6 mice. Results are mean \pm SE of three separate experiments; n=13 per group; * $P < 0.05$ when compared to respective controls.

**Figure 2.**

In contrast to sunitinib, dopamine (DA) treatment had no significant toxic effect on liver and renal functions compared to untreated controls as was determined by (a) alanine aminotransferase (ALT) (b) aspartate aminotransferase (AST) (c) total bilirubin (TBILI) (d) blood urea nitrogen (BUN) and (e) Creatinine (Creat) levels in serum of untreated and treated (DA and sunitinib) normal, HT29 and LLC tumor bearing mice. Results are mean \pm SE of three separate experiments; n=13 per group; * $P < 0.05$ when compared to respective controls.

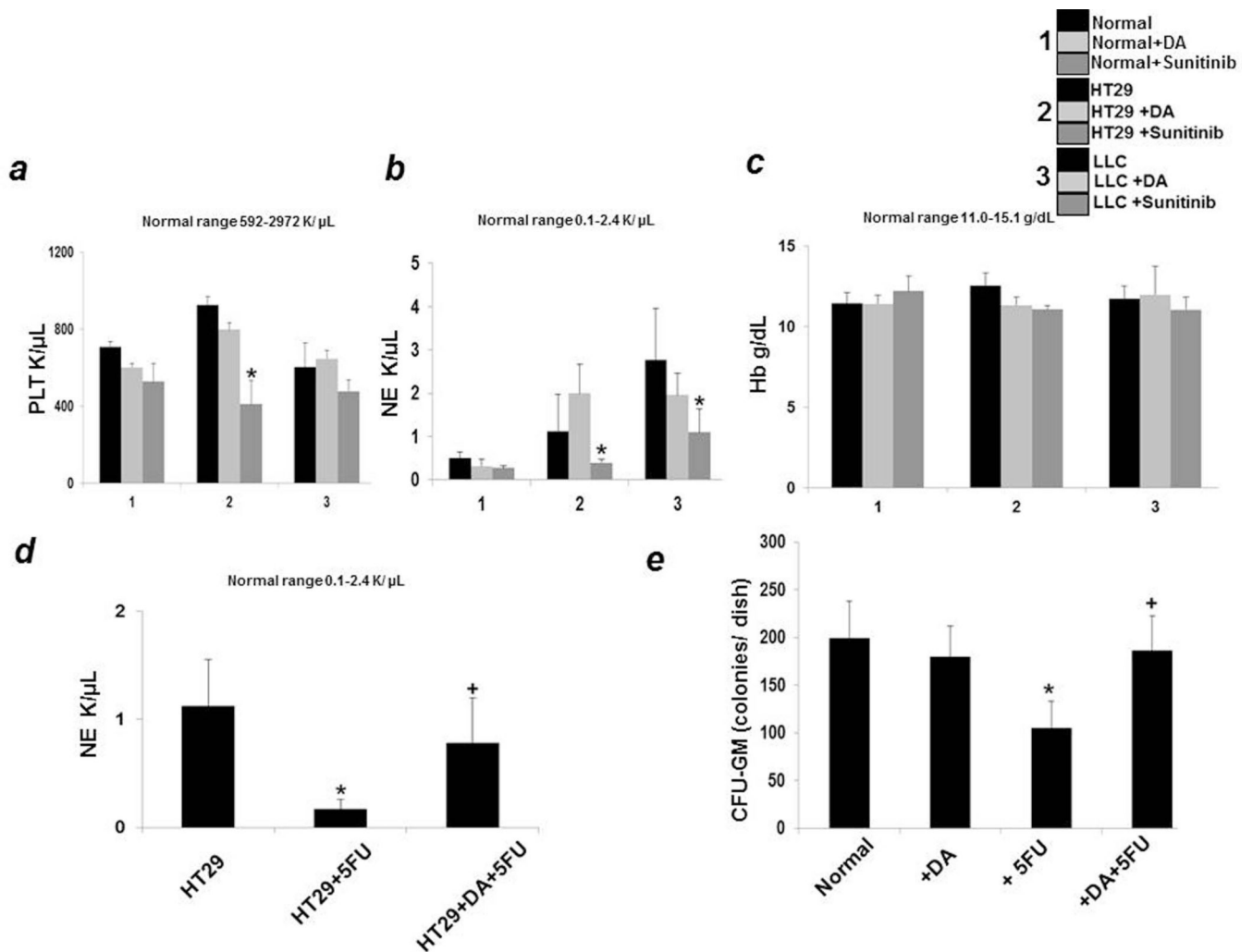


Figure 3.

In comparison to sunitinib, dopamine (DA) treatment showed no hematological toxicity as determined by measuring the (a) platelet (PLT) (b) neutrophil (NE) and (c) hemoglobin (Hb) levels in blood of DA and sunitinib treated normal, HT29 and LLC tumor bearing mice and comparing to untreated controls. (d). Combination treatment of DA and 5FU of HT29 tumor bearing mice prevented 5FU induced significant reduction of NE counts (* $P < 0.05$ when compared to control; + $P < 0.05$ when compared to 5FU treated group). (e) The number of CFU-GM colonies in culture significantly increased when bone marrow cells were collected from mice receiving combination treatment of DA and 5FU compared to 5FU alone. * $P < 0.05$ when compared to normal; + $P < 0.05$ when compared to 5FU treated group. Results are mean \pm SE of three separate experiments; n=13 per group.