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Potent anti-proliferative actions of a non-diuretic glucosamine derivative of ethacrynic acid

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ABSTRACT

Ethacrynic acid (EA), a known inhibitor of the neoplastic marker glutathione *S*-transferase P1 and other GSTs, exerts a weak antiproliferative activity against human cancer cells. The clinical use of EA (Edecrin) as an anticancer drug is limited by its potent loop diuretic activity. In this study, we developed a non-diuretic 2-amino-2-deoxy-D-glucose conjugated EA (EAG) to target tumors cells via the highly expressed glucose transporter 1 (GLUT1). Cell survival assays revealed that EAG had little effect on normal cells, but was cytotoxic 3 to 4.5-fold greater than EA. Mechanistically, the EAG induced selective cell death in cancer cells by inhibiting GSTP1 and generating abundant reactive oxygen species. Furthermore, EAG induced $p21^{cip1}$ expression and a G2/M cell cycle block irrespective of the p53 gene status in tumor cells. These data encourage the development of new EA analogs.

Low levels of reactive oxygen species (ROS) generated as a result of mitochondrial electron transport activity, NADH oxidase and cytochrome p450 enzyme activities in normal cells function in cell physiological signaling, mitogenesis and angiogenesis. Human cancers, however, harbor higher levels of ROS, including H_2O_2 due to their increased metabolic activity, cytokine production and various gene alterations.[1] Because the ROS are capable of damaging crucial cellular macromolecules, including DNA, cancers adapt to oxidative stress by upregulating the antioxidant systems such as glutathione to counteract the detrimental effects of the oxygen free radicals, H_2O_2, nitrosative and other stresses.[2] This dependency might not be shared by many nontransformed cells, whose lower basal ROS levels and/or elevated antioxidant capacity could provide resistance to treatments that impair ROS metabolism. Therefore, the elevation of oxidative stress preferentially in cancer cells by depleting glutathione or generating ROS is a logical therapeutic strategy for the development of anticancer drugs.[3] Consistent on this hypothesis, various small molecules having disulfide, α,β-unsaturated carbonyl, sulfonate, or other electrophilic functional groups, have previously been reported to elevate ROS levels and induce cancer cell death by depleting glutathione levels.[4]

Ethacrynic acid (EA) is a loop diuretic used to treat hypertension treatment.[5] EA possesses an α,β-unsaturated carbonyl unit (a Michael acceptor) and can be attacked by nucleophiles (e.g., sulfur-atom containing glutathione) at the β-carbon, thereby induces oxidative stress in the cells.[6] The α,β-unsaturated carbonyl moiety of EA also participates in the inhibition of glutathione *S*-transferase P1-1 (GSTP1) by binding to the cysteinyl residue in the active site via a Michael-like addition.[7] GSTP1 is highly expressed in human malignancies and plays both catalytic and non-catalytic roles as a major determinant of tumor resistance to alkylating agents, cisplatin and other anticancer drugs.[8] While the conjugation of the electrophilic drugs with GSH by GSTs directly inactivates the drugs, the ability of GSTP1 to form protein complexes with the stress-related and apoptosis-inducing kinases (JNK, ASK1 etc.) restrains the cell signaling in non-stressed cells.[9] Along this lines, it has been demonstrated that EA exerts anti-proliferative effects against tumor cells, albeit at higher concentrations, but enhances the therapeutic efficacies of several anticancer agents. Nevertheless, the relative lack of potency and the potent diuretic properties diminish the use of EA as a chemotherapeutic agent.[10] Structure and activity relationship studies have revealed that the ability of EA to inhibit GSTP1 activity results from its α,β-unsaturated carbonyl group and that the main diuretic side effect of EA results from its carboxyl group.[11] Thus, several groups developed EA analogs by modifying acid group into ester, amide, oxazole, thiazole and others.[12]

Cancers of diverse origins exhibit a marked demand for glucose for sustaining elevated rates of glycolysis and metabolite requirements. Glucose transporter 1 (GLUT1) is a major mediator of

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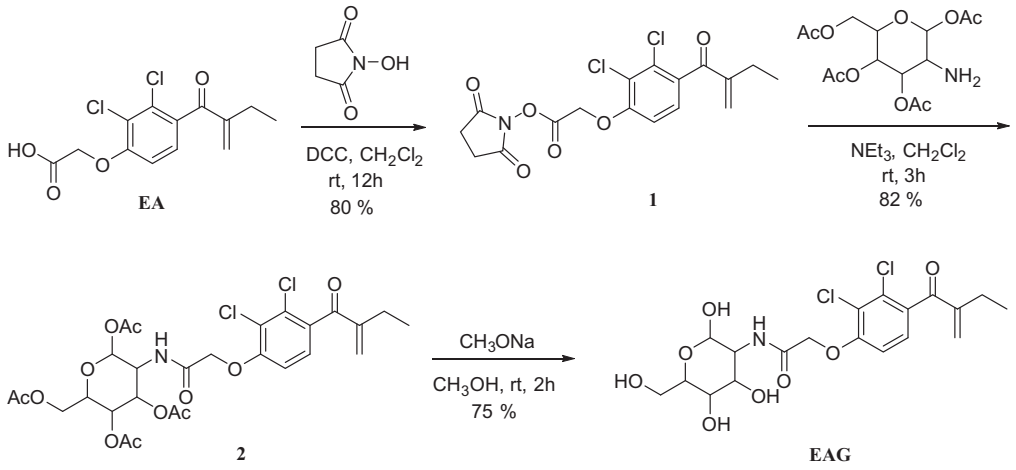
glucose uptake into the cancers through a facilitative diffusion.[13] Consequently, an increased supply for glucose in tumors translates to an enhanced expression and activity of GLUT1, which can be exploited as a targeting strategy. Thus, glycoconjugation, the linking of a drug to glucose, particularly, 2-amino-2-deoxy-D-glucose (2DG), which is recognized by and transported into the cells by GLUT1 has emerged as a viable approach for tumor targeting. The above strategy has been exploited in preclinical and clinical imaging for tumor diagnosis, staging, and monitoring of therapeutic response.[14] In the present study, we developed a new glucosamine conjugated EA, which shows excellent anticancer activity by targeting the oxidative stress response along with GSTP1 inhibition without the unwanted diuretic activity.

The preparation of ethacrynic acid-glucosamine conjugate (EAG) was accomplished by a three-step reaction as presented in Scheme 1. Commercially available EA was first converted in to NH-ester with *N*-hydroxy succinamide using DCC and activated EA **2** was reacted with 2,3,4,6-tetra-*O*-acetyl-beta-D-glucosamine (2-DG) hydrochloride in the presence of base diisopropylethylamine to yield acetyl protected EAG intermediate **3**. Deprotection of acetyl groups by using sodium methoxide gave the final product EAG in good yield.



Scheme 1. Synthesis of EAG.

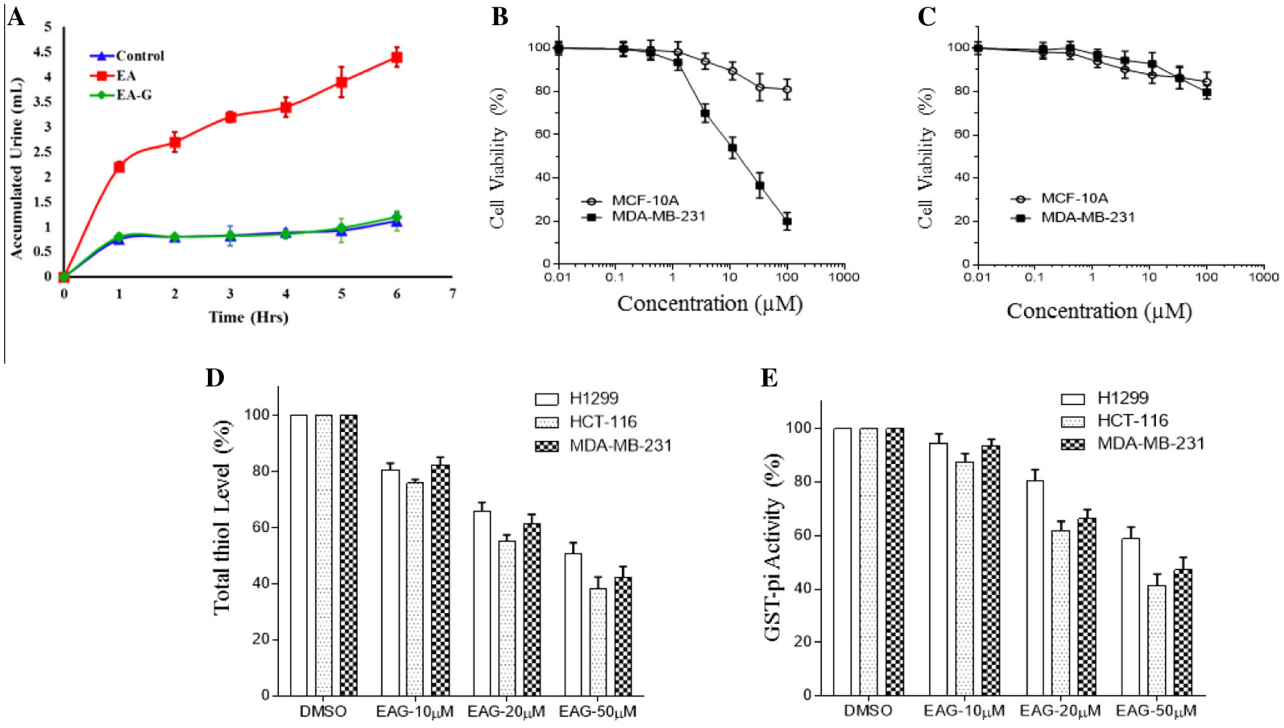


Figure 1. (A) Time course of diuresis in mice treated with EA or EAG at 10 mg/kg. Control group received the vehicle alone. Mice were placed on an elevated grid floor and isolated. Narrow graduated cylinders were placed underneath each mouse to collect the voided urine. (B) Cytotoxicity of EAG against MCF10A normal breast epithelial cells and cancerous MDA-MB-231 cells. (C) Cytotoxic effect of 2-DG against MCF10A and MDA-MB-231 cells. MTT assays were used. (D) Thiol depletion in cancer cell lines by EAG. The H1299, HCT116 and MDA-MB-231cells were exposed to EAG at concentrations shown for 3 h, trypsinized the total thiol levels in cell extracts were quantified using the Ellman's reagent. (E) Measurement of GSTP1 activity in H1299, HCT116 and MDA-MB-231cells after EAG treatment. Cells were exposed to EAG at concentrations specified for 3 h. The resulting cell extracts were used for the spectrophotometric assay using CDNB as the substrate. The kinetics of GSH-CDNB conjugation reactions were followed over 5 min and linear parts of the curves were used for quantitation.

As a first step of biological evaluation, we tested the effect of EAG on toxicity in mice. The animals given single doses of EAG at 120 mg/kg per week for two weeks were devoid of any ill effects. No tremors, lethargy, paralysis, stress, weight loss or diarrhea were observed. Next, we examined the diuretic activity of EAG and EA on female CD-1 mice, using the procedure of Hailu et al.[15] The mice were deprived of food for 12 h prior to drug administrations. Test compounds were prepared in PEG/EtOH/H_2O (54:14:32) and injected IP at 10 mg/kg. The animals were then divided into three groups, namely, the vehicle control, EAG- treated and EA-treated. The mice were placed in separate metabolic cages with free access to water. Urine was collected 0–6 h after drug injections in graduated tubes kept directly under the cage manifolds. The urine volumes shown in Fig. 1A indicate that EAG did not induce any diuretic effect over a 6 h test period compared to the progressive increase (up to 6-fold) of urine production seen with the EA. Furthermore, the urine output during the 6–24 h period was similar between the EAG and untreated controls (not shown). The results confirm that the side chain acid group of EA is indeed a major contributor to the diuretic property of the molecule.

Next, we evaluated the antiproliferative activity of EAG against a panel of ten different human cancer cell lines from lung (H1299), brain (SF188, GBM10), breast (SKBR-3, MDA-MB-231, MDA-MB-468, MCF7), colon (HT29 and HCT116) and fibrosarcoma (HT1080) along with the normal breast epithelial cells (MCF10a) using cell survival assays. The data shown in Table 1 indicates that EAG inhibited the growth of all cancer cell lines more potently (3 to 4.5-fold) than EA. Among the different cell lines tested, EAG showed a greater potency toward SF188 glioblastoma and HCT116 colon cancer cells. Overall, the EAG was equally potent

Table 1
In vitro cell growth inhibitory effects of EA and EAG on human cancer cell lines[a]

Number	Tumor type	Cell lines	Cytotoxicity (IC_{50}), μM	
			EAG	EA
1	Lung	H1299	11.53 ± 2.51	44.29 ± 1.79
2	Brain	GBM10	15.2 ± 1.23	29.88 ± 2.12
3	Brain	SF188	12.29 ± 0.98	31.65 ± 1.26
4	Breast	SKBR-3	16.09 ± 1.68	34.21 ± 1.6
5	Breast	MDA-MB-231	12.17 ± 1.63	45.24 ± 4.88
6	Breast	MDA-MB-468	18.37 ± 1.92	81.45 ± 4.22
7	Breast	MCF7	10.12 ± 1.12	28.92 ± 3.25
8	Colon	HT29	21.73 ± 2.98	62.39 ± 3.67
9	Colon	HCT116	10.29 ± 1.79	30.22 ± 4.62
10	Fibrosarcoma	HT1080	16.34 ± 2.19	54.53 ± 4.26

[a] IC_{50}: compound concentration required to inhibit tumor cell proliferation by 50%. Data is expressed as mean ± SD from the cell survival curves of three independent experiments.

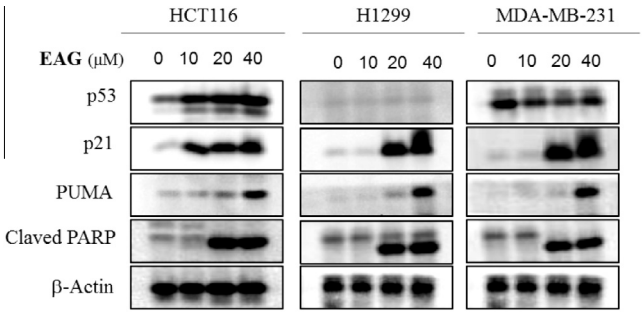


Figure 3. The effect of EAG on p53, p21, PUMA and PARP proteins was determined by western blot analysis in H1299 (p53-null), HCT116 (p53 wt) and MDA-MB-231 (p53 mutant) cells. β-Actin was used as a loading control.

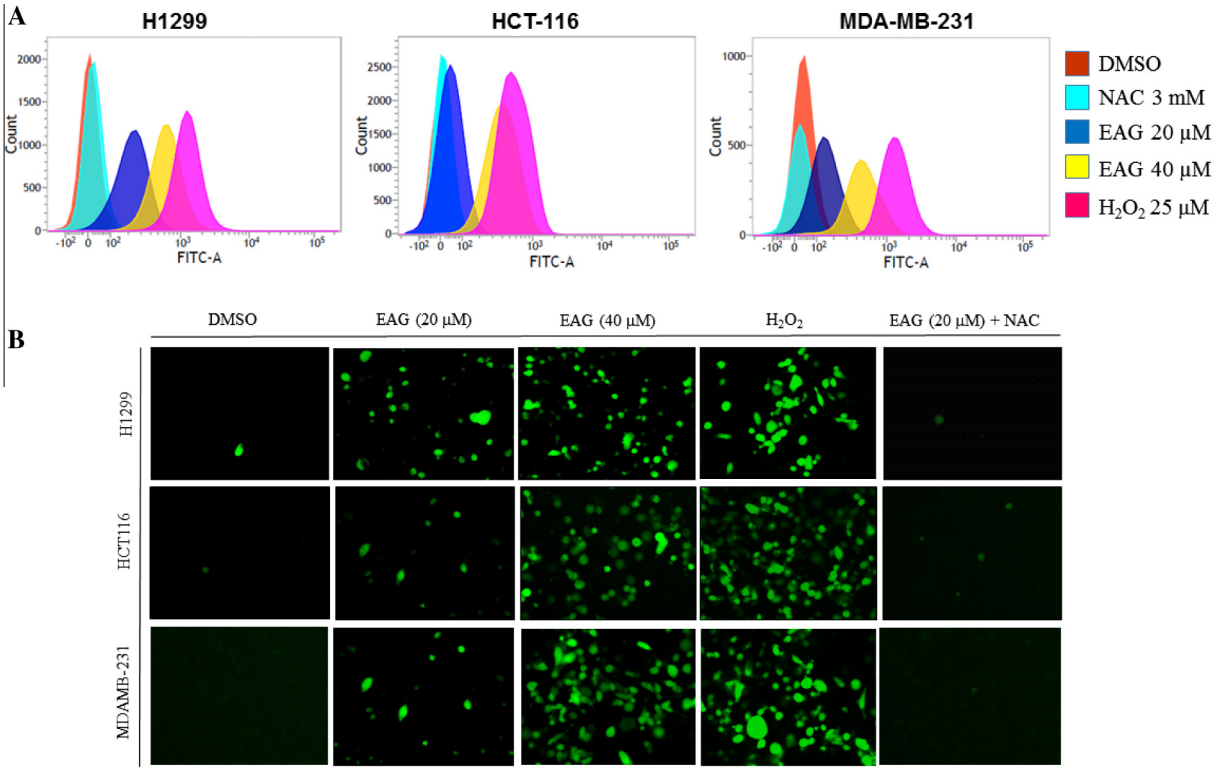


Figure 2. (A) Flow cytometric analyses of EAG-induced ROS elevation and its reversal by *N*-acetyl cysteine (NAC) in cancer cells. H1299, HCT116 and MDA-MB-231 cells were treated with or without EAG or 25 μM H_2O_2 for 3 h. In some cases, cells wert exposed to 3 mM NAC for 1 h prior to EAG treatment. The cell pellets were stained with 20 μM DCF-DA for 30 min prior to flow cytometry using a BD-Biosciences FACSVERSE instrument. (B) Representative Fluorescence microscopy images of H1299, HCT116 and MDA-MB-231cells treated for 3 h with different concentrations of EAG. H_2O_2 (25 μM) was used as positive control. In the last panel, cells were pretreated with 3 mM NAC before EAG exposure.

against all the cancer cell lines irrespective of their p53 gene status; the p53-null H1299 cells were also sensitive to the drug. EAG inhibited the proliferation of the normal breast epithelial cells (MCF10A) very slightly compared with the breast cancer MDA-MB-231 cells (Fig. 1B); the free 2DG ligand had no negative effect on cell growth on either cancerous or normal cells (Fig. 1C).

Structurally, the EAG shares characteristics of EA to conjugate with GSH and inhibit the GSTP1. EA can conjugate with GSH non-enzymically or enzymically and this conjugate is a more potent inhibitor of GSTP1.[16] Therefore, we measured the EAG-induced depletion of total thiols in three cell lines (H1299, HCT116, MDA-MB-231) using the Ellman's reagent. Fig. 1D shows that cells incubated with 0–40 μM EAG for 3 h lost the thiol content in a concentration-dependent manner, with 40 μM achieving a maximal 58% depletion. Extracts prepared from the EAG-treated cells were also subjected to GST activity using CDNB (1-chloro-2,4-dinitrobenzene) as the substrate. The catalytic activity of GSTs in these cells, again showed a significant and EAG-dose dependent inhibition with a maximal 60% inhibition in HCT116 cells following 50 μM drug treatment for 3 h (Fig. 1E). Consistent with this observation, the HCT116 cells were also more vulnerable to thiol depletion (Fig. 1D).

The ability of EAG to trigger ROS generation in cancer cells was studied by flow cytometry and confocal fluorescence microscopy (Fig. 2). Cells were treated with EAG (20 and 40 μM) and the level of ROS was measured using DCFH-DA (dichlorofluorescein-diacetate) as a fluorescent probe for intracellular ROS. The cell suspensions were analyzed using a BD Biosciences flow cytometer. EAG treatment for 3 h in H1299, HCT116 and MDA-MA-231 cells induced a significant accumulation of ROS as evident from the fluorescence shift compared with the untreated controls (Fig. 2A). Cells treated with 25 μM H_2O_2 served as positive control. Pretreatment of cells with N-acetylcysteine prior to EAG resulted in a fluorescence shift and signal attenuation to control levels, thus verifying the ROS production by EAG and its quenching by NAC (Fig. 2A). EAG-induced ROS generation was also confirmed by fluorescence microscopy (Fig. 2B). Again, 40 μM EAG induced intense green fluorescence at similar levels produced by 25 μM H_2O_2 in all three cell lines; as in the flow cytometry experiments, preincubation with N-acetylcysteine eliminated the EAG-generated fluorescence (Fig. 2B). Collectively, the observations made in Figs. 1 and 2 strongly suggest that EAG effectively depletes GSH to suppress the antioxidant systems and promote an oxidatively stressed cellular milieu.

To investigate the mechanistic basis of EAG's antiproliferative effects and consequences of the redox stress created, we determined the levels of p53 and related apoptotic protein regulators. As shown in Figure 3, western blot analyses revealed that wt p53 expression along with $p21^{waf1/cip1}$ was significantly enhanced in HCT116 cells by EAG. Further, the $p21^{cip1}$ and PUMA, a p53 proapoptotic target, were induced in response to EAG, even in p53 null (H1299) and p53 mutant (MDA-MB-231) cells (Fig. 3). EAG treatment greatly enhanced the levels of the cleaved PARP (poly ADP-ribose polymerase) in all three cell lines. Overall, the results suggest that the redox imbalance triggered by EAG functions to prime the tumor cells toward apoptotic cell death. Further insight into the cytotoxic mechanisms of EAG was obtained by flow cytometric analyses of cell cycle progression. Accumulating data suggest that oxidative stress induced $p21^{cip1}$ inhibits cell cycle regulating cyclin-dependent kinases and orchestrates a G2/M phase arrest in cancer cells.[17] Consistent with this notion, the histograms displayed in Figure 4 show that EAG consistently induced a G2/M cell cycle arrest in a concentration dependent manner in HCT116, H1299 and MDA-MB-231cells.

In conclusion, we designed and synthesized a non-diuretic ethacrynic acid analog EAG with significant anti-cancer activity. EAG selectively targeted the cancer cells by downregulating the GSH/GST coupled antioxidant pathway and generating abundant ROS. The glucosamine linkage in EAG appeared to serve a dual purpose in blocking the diuretic activity and increasing the tumor

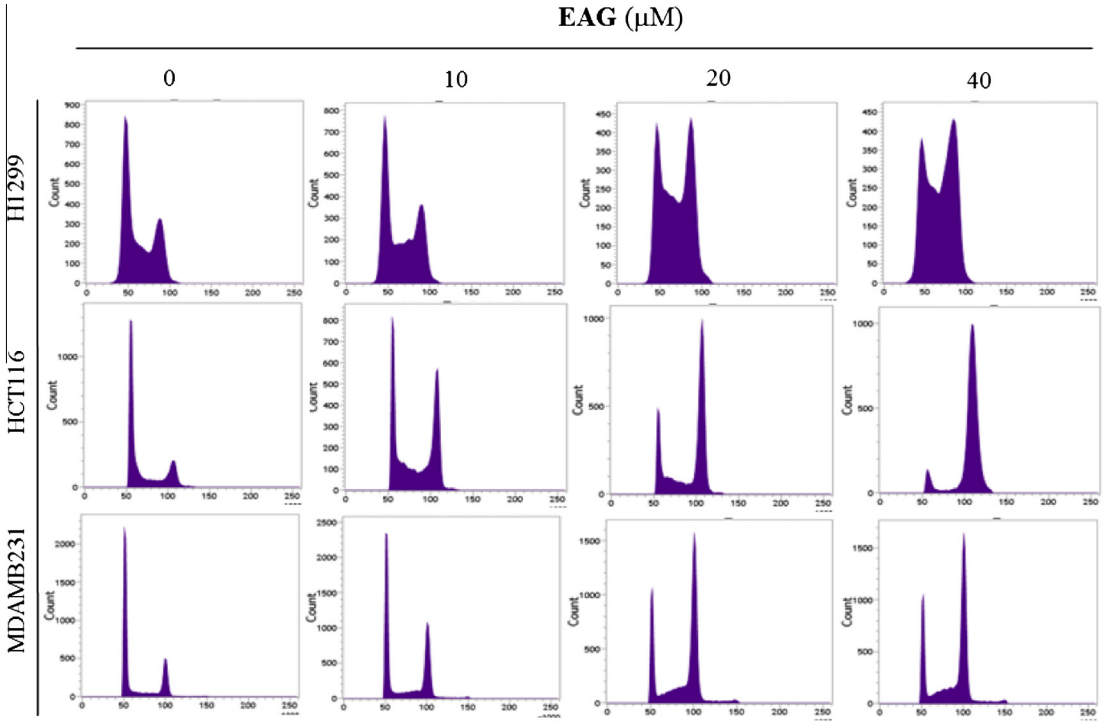


Figure 4. Induction of G2/M cell cycle arrest by EAG is not related to the p53 gene status. H1299, HCT116 and MDA-MB-231 cells were treated with EAG at concentrations shown for 24 h and subjected to flow cytometry by standard procedures described in Supporting information.

selectivity. The EAG-induced $p21^{cip1}$expression and the accompanying G2/M blockade occurred irrespective of the p53 gene status. Our findings encourage a preclinical evaluation of EAG and synthesis of new non-diuretic EA analogs for cancer treatment.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.04.060.

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