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Inhibitory effects of ethacrynic acid analogues lacking the α,β-unsaturated carbonyl unit and *para*-acylated phenols on human cancer cells

Zack E. Bryant [a], Romy F. J. Janser [a], Medina Jabarkhail [a], Melissa S. Candelaria-Lyons [b], Brittni B. Romero [b], Severine Van slambrouck [b,†], Wim F. A. Steelant [b,c], Ingo Janser [a,*,†]

[a] *Laboratory of Organic Synthesis, Catalysis & Biochemistry (LOSCB), Department of Chemistry, New Mexico Institute of Mining and Technology, 801 Leroy Place, Socorro, NM 87801, USA*
[b] *Laboratory of Biochemical and Biomedical Research, Department of Chemistry, New Mexico Institute of Mining and Technology, Socorro, NM 87801, USA*
[c] *School of Science, Technology and Engineering Management, St. Thomas University, Miami Gardens, FL 33054, USA*

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ABSTRACT

A series of ethacrynic acid analogues, lacking the α,β-unsaturated carbonyl unit, was synthesized and subsequently evaluated for their ability to inhibit the migration of human breast cancer cells, Hs578Ts(i)8 as well as of human prostate cancer cells, C4-2B. These cell lines provide a good model system to study migration and invasion, since they represent metastatic cancer. Our studies show that ethacrynic acid analogues with methyl substituents at the aromatic ring demonstrate no inhibitory effect on the migration of both cancer cell lines, whereas a precursor in the synthesis of these ethacrynic acid analogues (**II-1**, a *para*-acylated *m*-cresol) is an excellent inhibitor of the migration of both cancer cell lines.

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Ethacrynic acid (**EA**) (Fig. 1) is a loop diuretic and is used to treat high blood pressure and the swelling caused by diseases like liver, kidney, and congestive heart failure.[1,2] **EA** possesses an α,β-unsaturated carbonyl unit (a Michael acceptor) and can be attacked by nucleophiles (e.g., sulfur-atoms) at the β-carbon. This α,β-unsaturated carbonyl moiety is often employed in the design of SH-enzyme inhibitors (e.g., cysteine proteases).[3–6] **EA** inhibits the enzyme by binding to the cysteinyl residue in the active site by means of a Michael-like addition and is therefore a good glutathione *S*-transferase P1-1 (GST P1-1) inhibitor.[7] Several research groups have synthesized **EA** analogues in order to increase the inhibitory effect on GST P1-1. GST P1-1 is also of importance in controlling multidrug resistance (MDR), proliferation and apoptosis processes and hence can be considered a target for cancer treatment. Along this line, it has been demonstrated that **EA** exerts anti-proliferative effects against tumor cells, but only at higher concentrations (60–100 μM).[2,8–10] Additionally, it has also been shown that **EA** is cytotoxic towards primary chronic lymphocytic leukemia (CLL) cells.[11] However, the relative lack of potency and the diuretic properties do not support the use of **EA** as a chemotherapeutic agent.[12]

Recently, we demonstrated that **EA** analogues lacking the α,β-unsaturated carbonyl unit are able to inhibit the migration of human breast cancer cells, MCF-7/AZ, at non toxic concentrations.[13] This is, insofar, a very promising result, because an important aspect of cancer treatment is the prevention of metastases formation since the metastatic spread of primary tumors to distant sites constitutes the most lethal aspect of cancer. The exact mechanisms of local invasion and the formation of metastases are still not completely understood and remain an important field of research.[14] Intrigued by our very promising results, we were eager to further develop and evaluate biologically active ethacrynic acid analogues lacking the α,β-unsaturated carbonyl unit.

Our previous studies show that the most potent candidates to inhibit the migration of human breast cancer cells, MCF-7/AZ, are **EA** analogues with one or two methoxy groups attached to the aromatic system but lacking the α,β-unsaturated carbonyl unit. We hypothesized that the increase in electron density of the aromatic system, caused by electron donating groups like the methoxy group, is responsible for the anti-migratory properties of the corresponding compounds.[13] To further investigate this hypothesis, we were interested in synthesizing **EA** analogues with one or two methyl groups attached to the aromatic system. Methyl groups are only very weak electron donating substituents in comparison to the strong electron donating methoxy groups.

The preparation of the various ethacrynic acid analogues, lacking the α,β-unsaturated carbonyl unit, was accomplished by

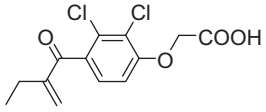

Figure 1. The chemical structure of ethacrynic acid (**EA**).

* Corresponding author. Tel.: +1 575 835 6948; fax: +1 575 835 5364.
E-mail address: ijanser@nmt.edu (I. Janser).
† These authors contributed equally.

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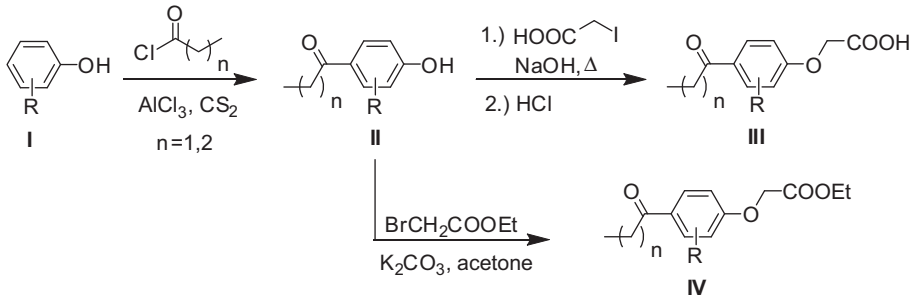


Scheme 1. Synthetic pathway for the synthesis of **EA** analogues.

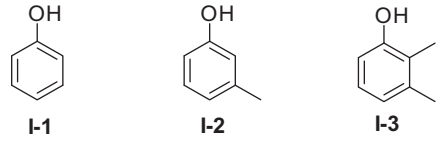
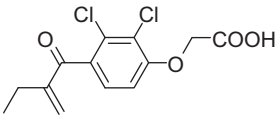
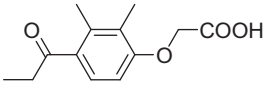
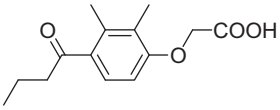
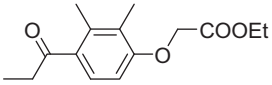
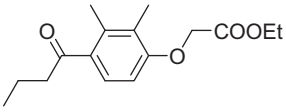
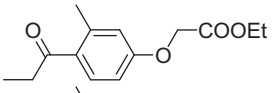
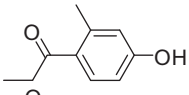
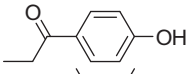
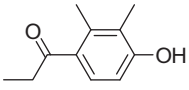


Figure 2. Phenol **I-1** and two substituted phenols **I-2–3**.

a two-step reaction shown in Scheme 1. The Friedel–Crafts acylation of the phenol **I-1** or the substituted phenols **I-2–3** (Fig. 2), respectively, with acyl chloride, was performed in the presence of powdered aluminum chloride ($AlCl_3$) in carbon disulfide, to generate the corresponding acylated compounds **II**. In order to obtain a higher ratio of the *para*-acylated versus the *ortho*-acylated product (with respect to the hydroxyl group), the reaction was carried out at 0 °C. After separation of the obtained isomers on silica gel, the *para*-acylated phenol-analogues **II** were treated with 2-iodocarboxylic acid in the presence of sodium hydroxide to afford the corresponding sodium salts. Acidification of these salts with hydrochloric acid yielded the corresponding compounds **III**. In order to obtain the phenoxy acetic acid esters **IV**, the *para*-acylated phenols **II** were treated with ethyl bromoacetate and refluxed in acetone in the presence of potassium carbonate as the base.

In the present study, we used the Hs578Ts(i)8 breast cancer cell line and the C4-2B prostate cancer cell line, which are ideal model systems to study the effect of the synthesized compounds on

Table 1
The inhibitory effect of ethacrynic acid (**EA**)

Code	Structure	Inhibition (%)		OD_{80}/IC_{20} (μM)	
		C4-2B	Hs578Ts(i)8	C4-2B	Hs578Ts(i)8
EA		None	None	10	80
III-1		None	None	>100	>100
III-2		None	None	>100	>100
IV-1		None	None	>100	>100
IV-2		None	None	50	>100
IV-3		23	19	>100	>100
II-1		65	86	>100	>100
II-2		25	28	>100	>100
II-3		None	17	>100	>100

EA analogues lacking the α,β-unsaturated carbonyl unit (**III-1–2**, **IV-1–3**), and *para*-acylated phenols (**II-1–3**) on the migration of human C4-2B prostate cancer cells and human Hs578Ts(i)8 breast cancer cells and their respective observed cytotoxicities expressed in OD_{80}/IC_{20} values.

migration and invasion of cancer cells, since they represent metastatic cancer.[15–17] The results from the in vitro cytotoxicity assays[18–20] (Table 1) reveal that 24 h treatment of human Hs578Ts(i)8 breast cancer cells and human C4-2B prostate cancer cells with compounds **II-1-3**, **III-1-2**, **IV-1**, and **IV-3** has no effect on the cell viability, even at high concentrations of 100 μM. Only compound **IV-2** reduces the cell viability of the human C4-2B prostate cancer cells by 20% at a concentration of 50 μM. In contrast to these results, concentrations of 10 μM (C4-2B) or 80 μM (Hs578Ts(i)8), respectively, result in a 20% reduction of the cell viability of the corresponding cell line, when they are treated with ethacrynic acid.

In accordance with our expectations, the in vitro wound healing assays[17,21] for compounds **III-1–2**, **IV-1–3** don't show any substantial effect on the migration of the two human cancer cell lines. In stark contrast to these results, compound **II-1** shows a significant inhibition of the migration of both human cancer cell lines, C4-2B and Hs578Ts(i)8. This compound inhibits the migration of the human C4-2B prostate cancer cells by 65% and the migration of the human Hs578Ts(i)8 breast cancer cells even by 86% (Figs. 3 and 4), without affecting the growth of these cell lines (data not shown).

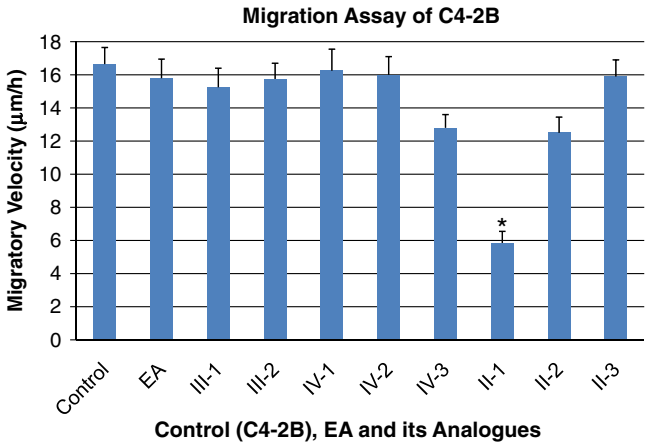


Figure 3. Migration assay of the human prostate cancer cell line, C4-2B, in the absence (control) or presence of ethacrynic acid (**EA**), its analogues (**III-1–2**, **IV-1–3**), and *para*-acylated phenols (**II-1–3**), * indicates p <0.05.

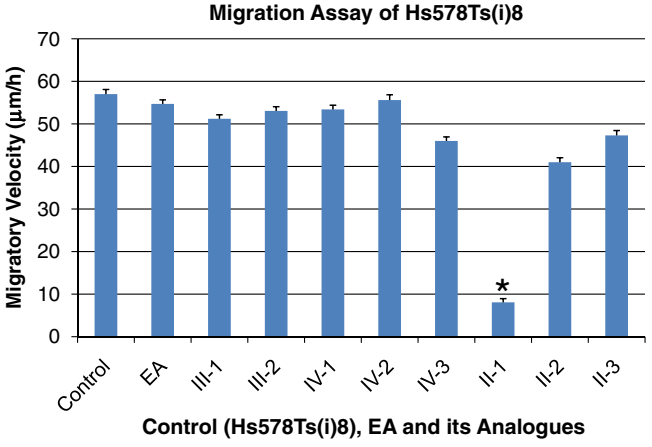


Figure 4. Migration assay of the human breast cancer cell line, Hs578Ts(i)8, in the absence (control) or presence of ethacrynic acid (**EA**), its analogues (**III-1–2**, **IV-1–3**), and *para*-acylated phenols (**II-1–3**), * indicates p <0.05.

Driven by these highly promising results, we also tested compounds **II-2** and **II-3** for their ability to inhibit the migration of the human C4-2B prostate cancer cells and the human Hs578Ts(i)8 breast cancer cells. Both compounds are able to inhibit the migration of the respective cancer cells, but only in a range of 17–28% (Figs. 3 and 4). Although, compounds **II-2** and **II-3** are very similar in structure to compound **II-1** (they only differ in the amount of methyl groups attached to the aromatic system) they are very different in their ability to inhibit the above mentioned cancer cells.

The data from the wound healing assays reveal that ethacrynic acid analogues, lacking the α,β-unsaturated carbonyl unit, and only weak electron donating groups attached to the aromatic ring (**III-1–2** and **IV-1–3**), are not able to significantly inhibit the migration of either the human C4-2B prostate cancer cell line nor the human Hs578Ts(i)8 breast cancer cell line. These results were expected, since we already tested several ethacrynic acid analogues with no or one methyl group attached to the aromatic system, which showed no potential to inhibit the migration of human breast cancer cells, MCF-7/AZ.[13] However, to our surprise and delight, the *para*-acylated *m*-cresol **II-1** shows a very high activity towards the inhibition of human C4-2B prostate cancer cells and human Hs578Ts(i)8 breast cancer cells. Comparing these results with the results obtained for compounds **II-2** and **II-3** (Table 1), which are all very similar in structure, it becomes obvious that only slight changes in the substitution pattern of the aromatic ring can cause a significant change in the observed biological activity. It is clear that compound **II-1** is an excellent inhibitor for the migration of the two human cancer cell lines, C4-2B and Hs578Ts(i)8, and it will be worth to further investigate the activity of this compound towards other cancer cell lines with an eye to the mechanism of action.

In summary, we have synthesized five different **EA** analogues, lacking the α,β-unsaturated carbonyl unit, which didn't show any pronounced activity towards the inhibition of the migration of the two cancer cell lines, C4-2B (prostate cancer) and Hs578Ts(i)8 (breast cancer). In sharp contrast, the *para*-acylated *m*-cresol **II-1** revealed a very pronounced activity towards the inhibition of both cancer cell lines. This compound inhibits the migration of the human C4-2B prostate cancer cells by 65% and the migration of the human Hs578Ts(i)8 breast cancer cells even by 86%. Further studies of these compounds against other cancer cell lines (e.g., the human breast cancer cells, MCF-7/AZ) are currently underway in our laboratory.

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18. The cytotoxicities of ethacrynic acid and the analogues were tested in accordance to Sigstedt et al.[20] Mitochondrial dehydrogenase activities were measured by an MTT-reagent. Cells were seeded in 96-well plates at an initial density of 1.5×10^4 cells in 200 μL of the appropriate culture medium, supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin. After 24 h of incubation, at 37 °C in a humidified atmosphere containing 5% CO_2, cells were treated with eight different concentrations (1, 5, 10, 20, 40, 60, 80, and 100 μM) of EA and various EA analogues in culture medium. After 24 h of incubation, 100 μL medium was removed prior to the addition of the MTT reagent. Three independent experiments were carried out for each concentration, to determine the mean optical density (OD) referring to cell viability. OD_{80}/IC_{20} values were determined from the respective graphs. These values represent the concentrations of the analogues, required for 20% inhibition in vitro or leaving approximately 80% of the cells viable.
19. All compounds were tested in a concentration range of 1–100 μM, and no toxicity of 50% was observed for any of the compounds, which means that the concentration to kill 50% of the cells is a lot higher than 100 μM. The highest toxicity observed was 10–15% at 100 μM, even after 72 h incubation.
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21. Cells were grown in 6-well plates in the appropriate medium until confluence and then washed twice with phosphate buffered saline (PBS). A gap of approximately 1500 μm (the wound) was created in the cell monolayer and images were captured. Subsequently, 3 mL of medium, in the presence or absence of ethacrynic acid or EA analogues, was added. The respective concentrations of these solutions were determined in the 24 h MTT assay. After 24 h of cell migration in order to close the wound, images were once again captured, and compared to the respective previous images in order to quantify the migration rate, which is expressed as migratory velocity (μm/h). At least three independent experiments were performed. Student's *t*-test was used for the analysis of data with $p < 0.05$ considered significant.