ANTICANCER RESEARCH *40*: 229-238 (2020)
doi:10.21873/anticanres.13944

In Vitro Analysis of the Combinatory Effects of Novel Aminonaphthoquinone Derivatives and Curcumin on Breast Cancer Progression

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Abstract. *Background/Aim: We previously reported the potential of aminonaphthoquinone derivatives as therapeutic agents against breast and other oestrogen-responsive tumours when combined with curcumin. This study aimed at screening of novel aminonaphthoquinone derivatives (Rau 008, Rau 010, Rau 015 and Rau 018) combined with curcumin for cytotoxic, anti-angiogenic and anti-metastatic effects on MCF-7 and MDA-MB-231 breast cancer cells. Materials and Methods: Cytotoxic and anti-angiogenic effects were analysed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and enzyme-linked immunosorbent assay; while anti-metastatic effects were measured using adhesion assay, Boyden chambers and Matrigel. Results: Curcumin combined with Rau 008 elicited marked cytotoxic effects in MCF-7 cells compared with the individual treatments, whereas when it was combined with Rau 015 and with Rau 018, it displayed similar effects in MDA-MB-231 cells. The anti-angiogenic effect of Rau 015 plus curcumin in MCF-7 cells and Rau 018 plus curcumin in MDA-MB-231 cells was more effective than individual treatments, while the metastatic capability of MDA-MB-231 cells was significantly reduced after treatment with the aminonaphthoquinone-curcumin combinations. Conclusion: Aminonaphthoquinones may offer significant promise as therapeutic agents against breast cancer, particularly when combined with curcumin.*

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Key Words: Aminonaphthoquinone, curcumin, combination, cytotoxic, anti-angiogenic, anti-metastatic.

Cancer elicits great suffering and economic loss world-wide (1). In 2018, the world-wide affliction of cancer rose to an estimated 18 million new cases with a concomitant 9.6 million deaths resulting from cancer (2). Worldwide, in 2018, the most common cancer types diagnosed were those of the lung, followed by breast and prostate (2). Breast cancer is the most commonly diagnosed cancer in women worldwide (3, 4), with approximately two-thirds of all breast cancer cases classified as oestrogen receptor-positive or ER^+ (5).

The leading cause of death among women with breast cancer is attributed to metastasis (6), which involves the adhesion of tumour cells to the extracellular matrix (ECM) and subsequent invasion and migration of these cells within the bloodstream to distant sites, leading to the formation of secondary tumours in non-breast tissue (7-9). Metastasis relies predominantly on angiogenesis, a tightly-regulated process that involves the formation of new blood vessels from existing vasculature (10). Angiogenesis plays a vital role in biological processes such as reproduction, embryonic development and wound healing. This includes tumour development and progression, since it provides both a vascular supply as well as nutrients (such as growth factors and oxygen) to the growing tumour (11). The crucial role of angiogenesis in the development and progression of cancer makes it an important target in the treatment of cancer (12). Vascular endothelial growth factor (VEGF) is the main inducer of tumour angiogenesis and amplifies the expression of local proteases that degrade the ECM, in addition to being the most effective factor that promotes vasodilation of the existing vessels and increases the permeability of the vessel wall (12-14).

Although metastasis may be directed to several tissues, the most common site of breast cancer metastasis is the bone, as certain hormone-responsive breast tumours have a greater propensity to metastasize to bone than to the viscera (15-17).

The selective oestrogen modulator, tamoxifen, is extensively used to treat all stages of ER^+ breast cancer (18); however, the long-term efficacy of selective oestrogen modulators is limited by disease recurrence and tumour resistance (19). Moreover, tamoxifen acts agonistically in endometrial tissue, which is linked to an increased incidence of endometrial cancer (20, 21).

While some targeted therapies are associated with enhanced therapeutic effects, in most cases, these effects are not sustainable when used as monotherapy, due to the development of drug resistance or clinical relapse (22, 23). Combination therapies are thus often used to treat cancer more effectively, since the action of multiple drugs may involve different mechanisms or modes of action, and can be directed at multiple targets, thereby resulting in higher therapeutic efficacy (24).

One avenue of cancer research that has shown promise is that of the plant phenols, since these compounds target multiple pathways to achieve cellular death (25). A well-studied example is curcumin, the active constituent of turmeric (25, 26). Curcumin is notable for its widely accepted pharmacological safety and broad range of biological activities that include antioxidant, anti-inflammatory, antiviral, antifungal, antibacterial, anticancer, antidiabetic, and neuroprotective properties (27-33). Despite several reports challenging the efficacy of curcumin as a potential anticancer agent (34-36), curcumin inhibits various cell proliferation signalling pathways that are activated during cancer progression (37), while increasing the expression of various tumour-suppressor and pro-apoptotic factors (38, 39). Moreover, curcumin inhibits angiogenic factor expression (40) and restricts metastasis by targeting several adhesion-, invasion- and migration-related factors (41). Nonetheless, its therapeutic use is limited as it is insoluble in water and undergoes photodegradation, leading to low bioavailability (42). In this regard, several formulations have been designed with curcumin, as described by Prasad *et al*., where the delivery, bioavailability and metabolism of curcumin and its formulations are explored (43).

Studies involving aminonaphthoquinones have yielded promising results (44-46). In this study, four novel, synthetic aminonaphthoquinone derivatives (46), coded Rau 008, Rau 010, Rau 015 and Rau 018 (Figure 1), were analysed in combination with curcumin for their potential as cytotoxic, anti-angiogenic and anti-metastatic agents against breast cancer. Previous studies of these compounds combined with curcumin showed significant anticancer effects in ER-dependent and ER-independent breast cancer cells, including other oestrogen-responsive tumour cell lines (46).

This study aimed to determine the combinatory effects of the Rau compounds with curcumin on the progression of ER^+ (MCF-7) and ER^- (MDA-MB-231) breast cancer cells. In this regard, we examined the effect of the combinations on: i) Cell viability, ii) levels of VEGF iii) the ability of MCF-7 and MDA-MB-231 breast cancer cells to adhere predominant ECM proteins (47), and the iv) invasive and v) migratory potential of MDA-MB-231 breast cancer cells, which is considered to be highly metastatic (48, 49).

Materials and Methods

Cell culture and maintenance. The cell lines (MCF-7 and MDA-MB-231) were obtained from the National Institute of Biomedical Innovation (Osaka, Japan). Cells were grown in complete growth medium consisting of Dulbecco's minimal essential medium (DMEM; Hyclone Ltd., Northumberland, UK) supplemented with 10% foetal bovine serum (Hyclone Ltd.). The cells were subcultured every 3-4 days and were incubated at 37˚C in a humidified incubator with 5% CO_2 and 95% air.

Preparation of compounds and controls. The synthesis of the Rau compounds (45), including the spectroscopic analysis of Rau 008 (50), Rau 010 (45), Rau 015 (51) and Rau 018 (45), have been reported previously. Drug interactions between the Rau compounds and curcumin in MCF-7 breast cancer cells were initially investigated in our laboratory (46), using combination index (52-55) and isobologram (56) approaches. Accordingly, drug combinations displaying potential synergistic or additive interactions (46) were selected for further analyses in this study.

Stock solutions of the Rau compounds, curcumin (Sigma-Aldrich, Munich, Germany) and tamoxifen (Sigma-Aldrich) were prepared at 100 mM in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Similarly, 17β-oestradiol (Sigma-Aldrich) was prepared using absolute ethanol (Sigma-Aldrich). The final well concentration of DMSO or ethanol in treated or control samples was 0.15% v/v, which did not affect cell growth (46). Dilutions (at final well concentrations ranging from 15 to 120 μM) of agents were prepared fresh from stock solutions with complete growth medium.

Cell viability studies. The cytotoxicity of the test compounds and controls was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (57). Cells were harvested after reaching 80% confluence and seeded at a density of 1×10^4 cells per well in complete growth medium, in 96-well tissue culture plates. After an overnight incubation at 37˚C, cells were treated with the Rau compounds/controls (tamoxifen or 17β-oestradiol), alone and in combination with curcumin, at final well concentrations ranging from 15 to 120 μM. Vehicle controls (0.15% DMSO or 0.15% ethanol), as well as an untreated control, were included. The cells were incubated at 37˚C for 24 h, followed by removal of the conditioned media and subsequent storage at −80˚C for VEGF determinations. Thereafter, MTT (0.5 mg/ml, 100 μl; Duchefa Biochemie, Haarlem, the Netherlands) was added to each well and the plates were incubated for 3 h at 37˚C. The purple formazan product formed was solubilised in 100% DMSO (200 μl) and the absorbance read at 540 nm using a microtitre plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA), against a DMSO blank. Data were normalized to the cell number using a cell number standard curve and the proportion of viable cells was expressed as a percentage of the 0.15% DMSO or 0.15% ethanol (for 17β-oestradiol) vehicle-treated controls.

Angiogenic studies. The concentration of VEGF in cell supernatants was quantified using Invitrogen human VEGF ELISA kit

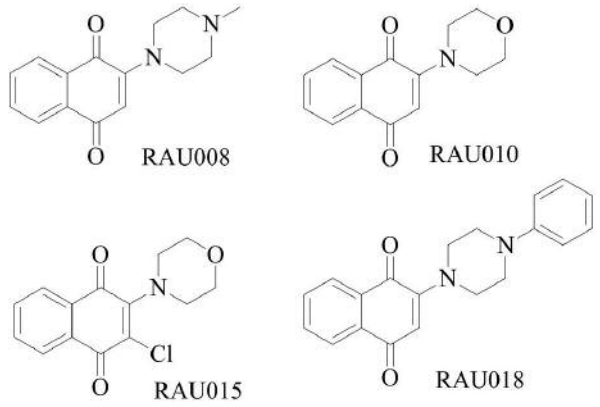

Figure 1. *Structures and name codes of the aminonapthoquinones used in the anticancer analyses (46).*

(Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Adhesion assay. The cell adhesion assay was performed as previously described (58), with slight modifications. Briefly, 96-well tissue culture plates were coated with 5 μg/ml fibronectin (B.D. Biosciences, San Jose, CA, USA), 5 μg/ml collagen (Sigma-Aldrich) or 5 μg/ml laminin (Sigma-Aldrich), and left overnight at 4˚C. The plates were rinsed with 0.1 M phosphate-buffered saline (PBS, pH 7.4) and blocked with 1% bovine serum albumin (BSA, in 0.1 M PBS, pH 7.4) for 30 min at 37˚C. Cells (1×10^5 cells/well) in 100 μl serum-free DMEM were added to pre-coated wells and incubated with the Rau compounds/controls, alone and in combination with curcumin, at final well concentrations ranging from 15 to 120 μM for 2 h at 37˚C. Non-adherent cells were removed by rinsing with PBS. Adherent cells were fixed with 95% ethanol for 5 min, rinsed three times with PBS and stained with 100 μl crystal violet solution (0.5% w/v crystal violet in 20% ethanol) for 10 min. Excess stain was removed with distilled water and extraction solution (100 μl 10% acetic acid) was added to each well. The absorbance was read at 590 nm using a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) against the extraction solution blank. The number of attached cells was ascertained by interpolation from a cell number standard curve and expressed as a percentage of the 0.15% DMSO or 0.15% ethanol (for 17β-oestradiol) vehicle-treated control.

Invasion assay. The effects of the various treatments on the invasive ability of MDA-MB-231 cells was determined using CytoSelect™ 96-Well Cell Invasion Assay kit (Cell Biolabs Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Cell migration analysis. A Boyden chamber migration assay was performed as previously described (59, 60), with slight modifications. For this assay, Transwell cell culture inserts (6.5 mm diameter, 8 μm pore) in a 24-well format were used (Corning Costar, NY, USA). Confluent MDA-MB-231 cells, which were serum-starved for 24 h, were harvested and resuspended at 1×10^6 cells/ml in serum-free DMEM. Medium (500 μl) containing 10% FBS (which served as the chemoattractant) was added to the lower well of the migration plate. The test compounds were prepared using serum-free DMEM in a 96-well plate (100 μl) to which 100 μl cell suspension (1×10^6 cells/ml) was added. The cell suspension and test compounds (at final well concentrations ranging from 15 to 120 μM) were thoroughly mixed before transferring 100 μl aliquots to the inside of each insert. After a 6 h incubation at 37˚C, the medium was carefully aspirated from the inside of the insert, while non-migratory cells were carefully removed with wet cotton-tipped swabs. Migratory cells on the lower side of the insert membrane were fixed with ice-cold 95% ethanol and left at room temperature for 10 min. The inserts were washed three times with 0.1 M PBS (pH 7.4), followed by staining with 200 μl 0.5% crystal violet solution and subsequent incubation at room temperature for 10 min. The stained inserts were washed thrice in distilled water, allowed to air dry and transferred to a new well containing 200 μl extraction solution (10% acetic acid) for a further 10 min. Thereafter, the inserts were removed and the absorbance of the solubilised crystal violet was read at 590 nm using a microtitre reader (Bio-Tek Instruments Inc., Winooski, VT, U.S.A.) against the extraction solution blank. Results were normalized to cell number, and the number of migrated cells following treatment was calculated as a percentage of the 0.15% DMSO or 0.15% ethanol vehicle control.

Statistical analysis. Data are presented as the mean±SD of three experiments (n=3). Differences between experimental groups and controls were analysed for significance using Student's *t*-test. Values of $p<0.05$ were considered statistically significant.

Results

Cell viability analyses. The effect of the various treatments on cell viability was analysed using the MTT assay. In MCF-7 cells, all combination treatments reduced cell viability significantly in relation to the vehicle control ($p<0.05$) (Figure 2). The inhibition elicited by Rau 008–curcumin was more effective than Rau 008 ($p=0.0182$) or curcumin alone ($p=0.0027$). Similarly, tamoxifen–curcumin proved more cytotoxic than either agent alone ($p<0.05$, respectively). Furthermore, the cytotoxicity exerted by Rau 008–curcumin, Rau 015–curcumin, Rau 018–curcumin and tamoxifen–curcumin appeared more significant than that of 17β-oestradiol–curcumin ($p<0.05$, respectively). The viability of MDA-MB-231 cells (Figure 2) was significantly reduced following treatment with the Rau–curcumin combinations ($p<0.05$, compared to the vehicle control). Moreover, the inhibitory effect of these combinations was more marked compared to treatment with tamoxifen–curcumin or 17β-oestradiol–curcumin ($p<0.005$, respectively). Notably, combinatory treatment with Rau 015–curcumin or Rau 018–curcumin resulted in enhanced cytotoxic effects compared to treatment with the individual compounds ($p<0.05$, respectively). However, treatment with 17β-oestradiol–curcumin did not significantly affect cell viability in relation to the vehicle control ($p=0.2247$).

Influence of combinatory treatments on the induction of angiogenesis. To determine the effect of the combinatory

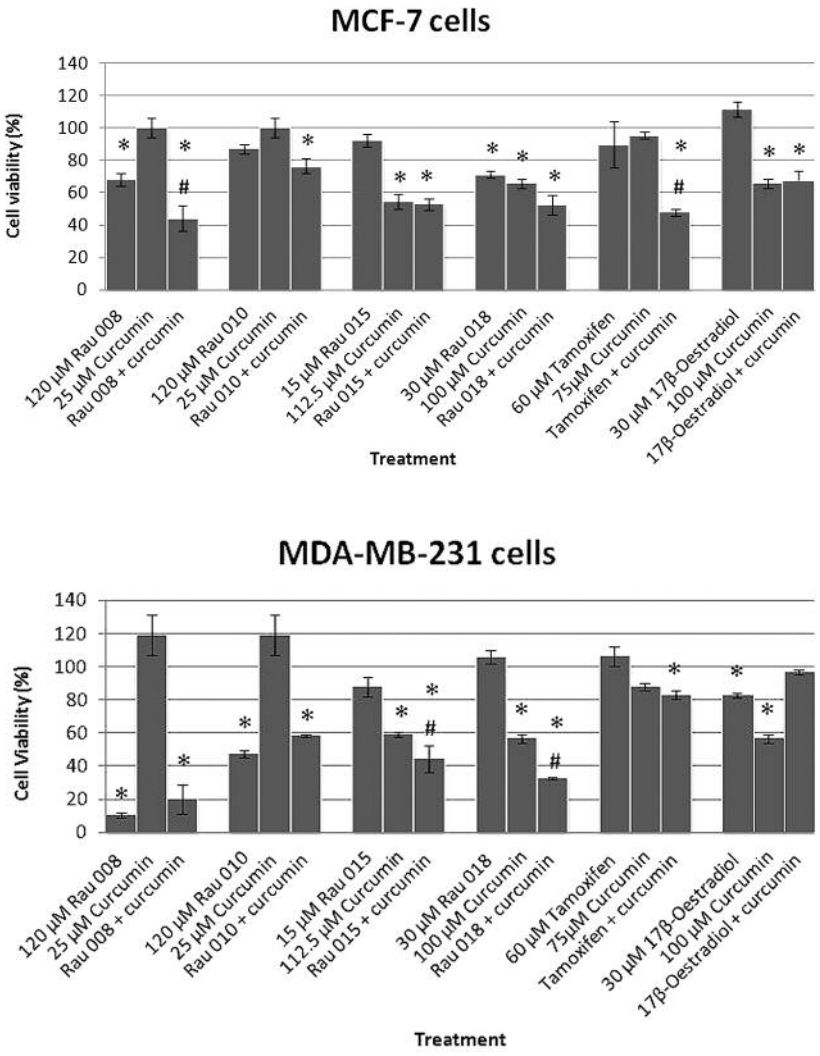

Figure 2. *Cytotoxicity of the various treatments in MCF-7 and MDA-MB-231 breast cancer cells. The number of viable cells is presented as a percentage of the vehicle control (0.15% dimethyl sulfoxide or 0.15% ethanol). The viability of the cells treated with vehicle control was not significantly different compared to that of the untreated control ($p>0.05$), and was therefore set at 100% (not shown). Data are reported as mean±S.D. values (n=3). Significantly different at $p<0.05$ relative to: *corresponding vehicle control; #individual treatment.*

treatments on the induction of angiogenesis in breast cancer cells, the VEGF level was determined. In MCF-7 cells, Rau 015–curcumin and tamoxifen–curcumin reduced the VEGF level significantly compared to the DMSO vehicle control ($p<0.05$), and exerted an enhanced anti-angiogenic effect in relation to individual treatment ($p<0.05$, respectively) (Figure 3). It is noteworthy that treatment with Rau 015 (15 μM), Rau 018 (30 μM) and 17β-oestradiol (30 μM) significantly increased the VEGF level compared to the relevant vehicle controls ($p<0.05$), indicating that these compounds may promote angiogenesis in ER^+ breast cancer. In MDA-MB-231 cells, all Rau–curcumin combinations reduced the level of VEGF ($p<0.05$), with Rau 018–curcumin inhibiting the VEGF level more effectively than Rau 018 and curcumin alone ($p<0.05$, respectively). Notably, treatment with 60 μM tamoxifen or 30 μM 17β-oestradiol significantly increased the VEGF level compared to the relative vehicle controls ($p<0.05$), whereas the effect of tamoxifen–curcumin and of 17β-oestradiol–curcumin on the VEGF level was not significant ($p>0.05$, respectively) (Figure 3).

Influence of combinatory treatments on the ability of MCF-7 and MDA-MB-231 cells to adhere to different ECM substrates. To determine if the combinations impeded the ability of MCF-7 and MDA-MB-231 cells to adhere to different ECM substrates, an adhesion assay was performed.

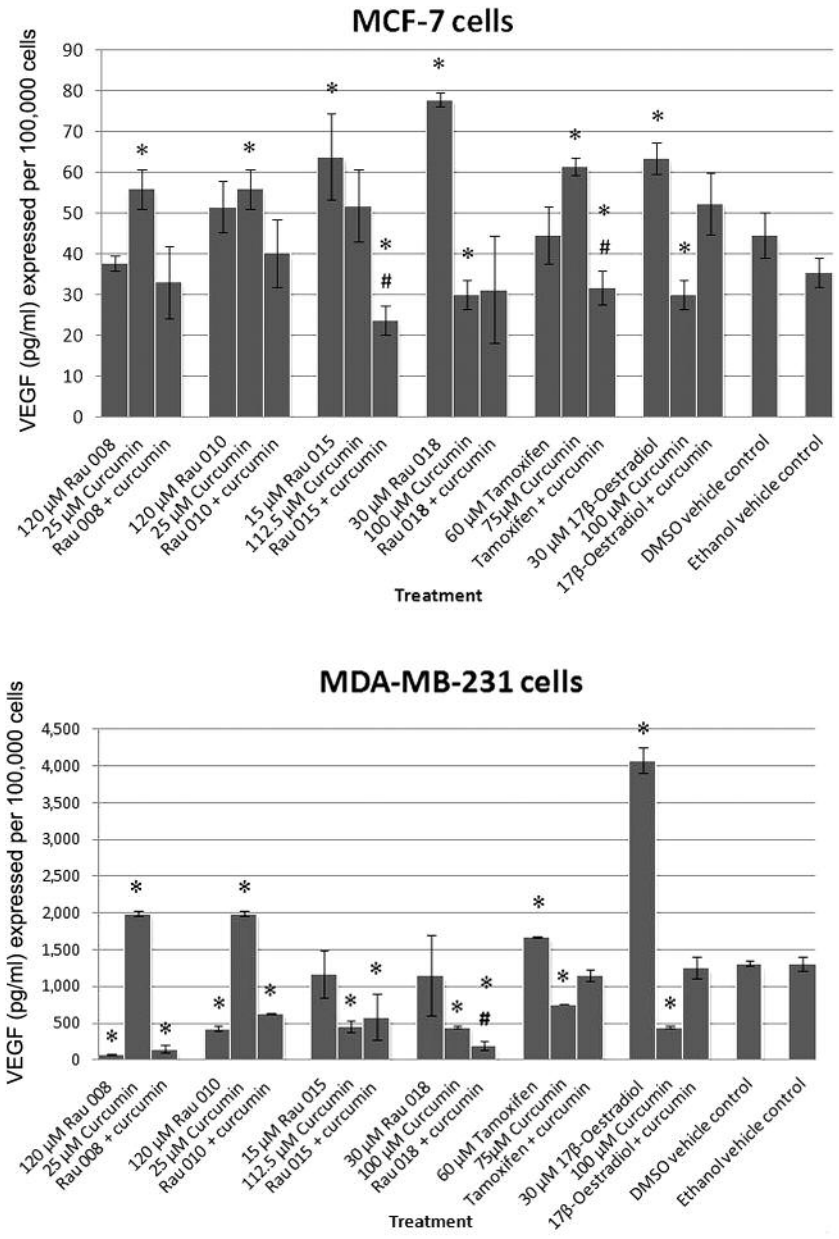

Figure 3. *Effect of treatments on vascular endothelial growth factor levels (VEGF) in MCF-7 and MDA-MB-231 cells. VEGF levels were normalised by cell number and are reported as the mean±S.D. values (n=3). Significantly different at p<0.05 relative to: *corresponding vehicle control; #individual treatment.*

As indicated in Table I, Rau 015–curcumin, Rau 018–curcumin, tamoxifen–curcumin and 17β-oestradiol–curcumin significantly reduced the attachment of MCF-7 cells to fibronectin, collagen and laminin in relation to the vehicle control ($p<0.05$), however, these inhibitory effects were similar to those of curcumin alone ($p>0.05$, respectively). Furthermore, these combinations were more effective at reducing the attachment of MCF-7 cells to the different ECM substrates than treatment with Rau 008–curcumin and Rau 010–curcumin ($p<0.05$). In MDA-MB-231 cells, all combination treatments significantly reduced cell attachment to the different ECM substrates ($p<0.05$) (Table I). It is evident that the anti-adhesive effects displayed by Rau 010–curcumin to fibronectin, Rau 018–curcumin to collagen, and Rau 008–curcumin or 17β-oestradiol–curcumin to laminin was more effective than individual treatment ($p<0.05$).

Table I. *Effect of the compounds and their combinations with curcumin on the ability of MCF-7 and MDA-MB-231 cells to adhere to various substrates. Experimental details are presented in the Materials and Methods section. The number of attached cells was expressed as a percentage of the 0.15% dimethyl sulfoxide or 0.15% ethanol vehicle control and is reported as the mean±S.D (n=3). The vehicle control did not affect cell adhesion significantly compared to the untreated control (p>0.05, data not shown), and the percentage of attached cells was therefore considered to be 100%. Significantly different at p<0.05 relative to: *relevant vehicle control; #individual treatment.*

	MCF-7 cells			MDA-MB-231 cells		
Treatment	Fibronectin	Collagen	Laminin	Fibronectin	Collagen	Laminin
120 μM Rau 008	74.04±5.09*	82.57±5.37	90.83±4.20	15.26±8.91*	76.98±4.66*	100.68±15.32
25 μM Curcumin	88.36±9.15	104.82±5.96	101.85±7.74	71.77±14.96*	95.77±5.98	92.86±21.65
Rau 008 + curcumin	82.59±1.88*	94.81±3.20	101.45±4.19	41.55±14.72*	85.22±5.52*	56.12±4.33*#
120 μM Rau 010	86.54±1.36*	106.80±7.98	112.67±4.50	64.33±6.12*	55.61±19.10*	91.16±13.59
25 μM Curcumin	88.36±9.15	104.82±5.96	101.85±7.74	71.77±14.96*	95.77±5.98	92.86±21.65
Rau 010 + curcumin	80.98±2.69*	95.30±2.26	102.77±3.00	22.75±3.88*#	59.61±11.36*	65.99±6.56*
15 μM Rau 015	80.70±3.99*	96.15±5.91	86.81±3.35	92.11±11.17	81.47±10.05*	35.42±4.51*
112.5 μM Curcumin	29.82±1.99*	40.15±1.88*	32.91±47.45*	76.97±2.79*	65.42±1.62*	31.77±5.02*
Rau 015 + curcumin	26.32±2.41*	44.00±4.81*	40.85±6.00*	73.03±2.79*	71.19±5.60*	42.97±1.10*
30 μM Rau 018	79.30±4.97*	87.29±9.10	77.85±6.03*	112.03±13.29	81.03±1.45*	64.06±6.63*
100 μM Curcumin	24.39±3.50*	42.24±7.78*	33.80±2.40*	53.20±15.68*	94.41±0.66	28.91±1.10*
Rau 018 + curcumin	26.14±1.10*	43.89±4.62*	36.11±4.72*	61.56±8.91*	55.35±1.50*#	28.65±8.02*
60 μM Tamoxifen	36.54±4.02*	39.49±10.23*	37.60±3.09*	5.49±0.69*	51.68±4.60*	22.45±2.89*
75 μM Curcumin	40.17±1.03*	48.46±2.78*	36.94±2.18*	51.80±9.93*	52.75±5.38*	94.90±12.99
Tamoxifen + curcumin	27.35±3.88*	46.35±5.77*	35.49±3.96*	48.43±7.49*	68.52±11.24*	51.02±8.66*
30 μM 17β-Oestradiol	94.62±6.64	91.51±4.48	104.10±8.75	104.76±3.45	131.94±23.82	117.85±9.11
100 μM Curcumin	24.39±3.50*	42.24±7.78*	33.80±2.40*	53.20±15.68*	94.41±0.66	28.91±1.10*
17β-Oestradiol + curcumin	28.07±3.08*	41.69±1.69*	32.97±4.43*	63.00±22.06*	69.65±18.78*	14.06±2.21*#

Influence of combinatory treatments on the invasive ability of MDA-MB-231 breast cancer cells. The percentage of invasive cells for the untreated control (18.41±4.43%, not shown) was comparable with findings by Booden *et al*. (49) and Sieuwerts *et al*. (60), who established an invasive potential of approximately 22-23% for unstimulated MDA-MB-231 cells. The invasive potential of the DMSO and ethanol vehicle controls was 27.73±6.38% and 23.56±2.90%, respectively (Figure 4). These results were not significantly different compared to the untreated control ($p>0.05$, not shown), showing that 0.15% DMSO and 0.15% ethanol did not affect the invasive potential of the MDA-MB-231 cells. Combinations of Rau 010, Rau 015, tamoxifen and 17β-oestradiol with curcumin significantly reduced the invasive ability of MDA-MB-231 cells compared to the vehicle control ($p<0.05$), whilst tamoxifen–curcumin showed a more enhanced anti-invasive effect than tamoxifen and curcumin alone ($p<0.05$, respectively) (Figure 4).

Effect of combinatory treatments on the migratory potential of MDA-MB-231 cells. All the test compounds (except 17β-oestradiol) significantly reduced the migratory potential of MDA-MB-231 cells ($p<0.05$, Figure 5). Individual treatment with the Rau compounds or curcumin was as effective as combined treatment ($p>0.05$), with a similar trend noted after treatment with tamoxifen or curcumin ($p>0.05$). Although the effect of 17β-oestradiol–curcumin was as effective as that of curcumin alone ($p>0.05$), it should be noted that this combination was highly effective at reducing the migration of MDA-MB-231 cells compared to the effect of 17β-oestradiol alone ($p<0.05$).

Discussion

The high incidence of adverse effects induced by the majority of currently available chemotherapeutics has motivated extensive investigations of alternative treatments, particularly combination therapies (24). In this study, we investigated the combination effects of novel aminonapthoquinone derivatives and curcumin on the progression of breast cancer cells. The combination ratios analysed were selected based on drug interactions between the Rau compounds and curcumin in ER+ breast cancer cells (46).

The results from this study suggest that the test agents may offer significant promise as therapeutic agents against both ER^+ and ER^- cancer, particularly when combined with curcumin. In this regard, it was shown that Rau 008 (120 μM), in combination with curcumin (25 μM), induced a marked cytotoxic effect in ER^+ breast cancer cells (Figure 2), in addition to reducing the attachment of ER^- breast cancer cells to laminin more effectively than individual treatment (Table I). Curcumin (25 μM) was shown to augment the anti-

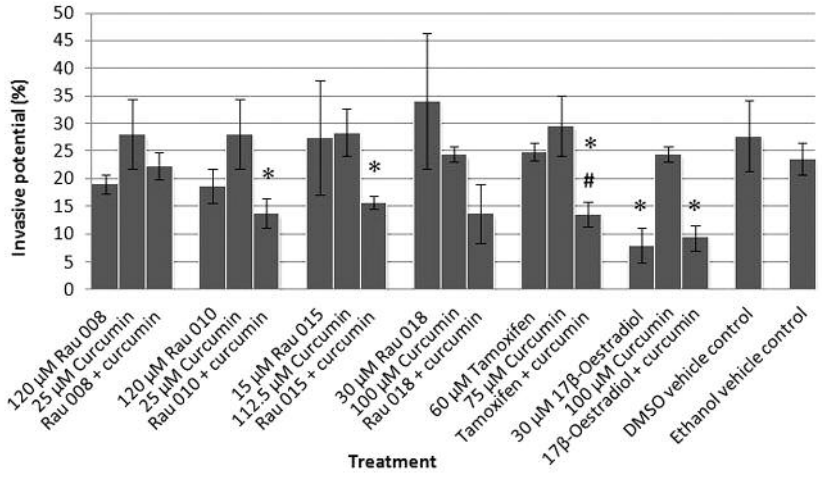

Figure 4. *Effect of treatments on the invasive potential of MDA-MB-231 cells. The number of invading cells present on the bottom side of the filter was expressed as a percentage of the total number of cells (sum of cells present in the basement membrane and on the bottom side of the filter) and is indicated as the invasive potential. Results are reported as the mean±S.D. values (n=3). Significantly different at p<0.05 relative to: *corresponding vehicle control; #individual treatment.*

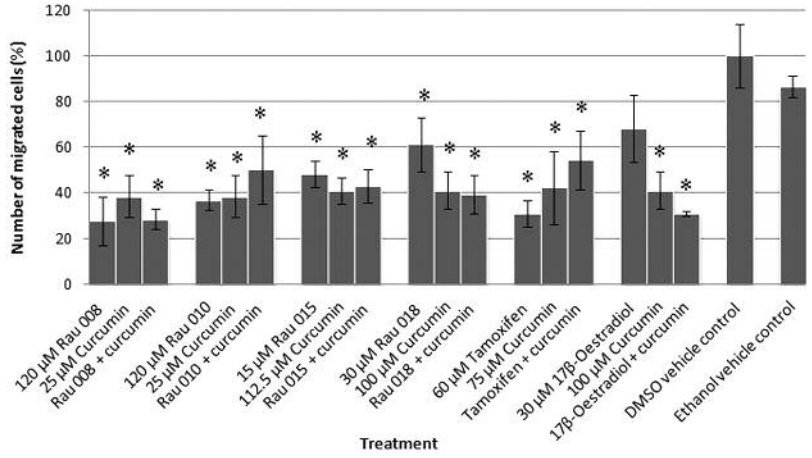

Figure 5. *Effect of treatments on the migratory potential of MDA-MB-231 cells. The number of migrated cells was expressed as a percentage of the 0.15% DMSO or 0.15% ethanol vehicle control and is reported as the mean±S.D (n=3). *Significantly different at p<0.05 relative to the corresponding vehicle control.*

metastatic effect of Rau 010 (120 μM) in ER^- breast cancer cells by enhancing its anti-adhesive effect to fibronectin (Table I), in addition to reducing the invasive potential of these cells (Figure 4). Rau 015 (15 μM), in combination with curcumin (112.5 μM), caused increased cytotoxicity in ER- breast cancer cells (Figure 2), exerted a marked anti-angiogenic effect on ER^+ breast cancer cells (Figure 3) and attenuated the invasive potential of ER^- breast cancer cells (Figure 4). The sensitivity of ER^- breast cancer cells to Rau 018 (30 μM) was enhanced when combined with curcumin (100 μM) by exerting enhanced cytotoxic (Figure 2) and anti-angiogenic effects (Figure 3), as well as strong anti-adhesive effects to collagen (Table I). These results correlate with our previous finding that pointed to the influence of curcumin in the responsiveness of ER^+ and ER^- tumours towards these compounds (46).

It is important to highlight the fact that curcumin (75 μM) enhanced the effect of tamoxifen (60 μM) in ER^+ breast cancer (Figures 2 and 3), while also reducing the invasive potential of ER^- breast cancer more effectively than treatment with tamoxifen or curcumin alone (Figure 4). Our finding that tamoxifen lacked a significant cytotoxic effect

in MCF-7 cells (Figure 2) is comparable with findings from a combination study investigating the cytotoxic effects of tamoxifen with nordamnacanthal on the MCF-7 cell line (61). The authors found that treatment with tamoxifen alone for 24 h reduced the viability of MCF-7 cells by approximately 12%, however, this effect was not significant compared to the control. Conversely, a combination of nordamnacanthal and tamoxifen reduced MCF-7 cell viability by up to 77.0% (61). The significance of the anti-invasive effect of tamoxifen in breast cancer has been reported in another study investigating the effects of tamoxifen and a known anti-allergic drug, tranilast, where it was found that tamoxifen (2 μM), alone or in combination with tranilast (200 μM), reduced the invasive and metastatic capability of MCF-7 and MDA-MB-231 breast cancer cells by down-regulation of the expression level of the chemokine receptor protein C-X-C motif chemokine receptor 4 (CXCR4), and its ligand CXCL12 (62). This anti-invasive effect of tamoxifen does not correlate with our findings; however, this inconsistency might be due to differences in assay conditions. Nonetheless, our findings point to the efficacy of tamoxifen (60 μM) with curcumin (75 μM) against ER^+ breast cancer, as well as against ER^- breast cancer. The higher therapeutic efficacy of tamoxifen–curcumin compared to that of tamoxifen alone suggests that curcumin could be beneficial if used in addition to chemotherapeutic drugs, and this warrants further investigation.

The observation that a combination of 17β-oestradiol (30 μM) and curcumin (100 μM) reduced the attachment of ER^- breast cancer cells to laminin more effectively than individual treatment (Table I) indicates that this combination may serve as a therapeutically useful tool against ER-independent breast cancer, and also requires further investigation.

This study proposed that selected combinations of synthetic aminonaphthoquinone derivatives with curcumin might impede angiogenesis and metastasis, important factors affecting the progression of breast cancer. It was established that the combinations show promise as potential therapeutic agents against breast cancer and warrant further investigation. Future studies should involve mechanistic studies to identify putative targets of the relative drug combinations, as well as in-depth *in vivo* toxicity analyses to predict the efficacy of these treatments for therapeutic benefit. Furthermore, the absorption and bioavailability of curcumin should also be explored, so that curcumin-based combinations or derivatives can be used as an adjuvant to current chemotherapy regimens.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors' Contributions

Melanie C. Pereira designed the study, performed all the experiments, analysed the results and wrote the article. Hajierah Davids supervised the study and edited the article. Raushaan Mohammed synthesized the test compounds under the supervision of Willem A.L. Van Otterlo and Charles B. De Koning. Hajierah Davids, Willem A.L. Van Otterlo and Charles B. De Koning revised and approved the final article.

Acknowledgements

This study was funded by the National Research Foundation, NRF-Thuthuka Grant 87902, Pretoria, South Africa and Nelson Mandela University, Port Elizabeth, South Africa. C. de Koning and W. van Otterlo gratefully acknowledge NRF research funding and the University of the Witwatersrand and Stellenbosch University for support.

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Received November 27, 2019
Revised December 9, 2019
Accepted December 12, 2019