Original Paper

Biological Evaluation of Two 1,4-Naphthoquinone Derivatives Against a Breast Human Adenocarcinoma Cell Line

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ABSTRACT: Two novel 1,4-naphthoquinone derivatives containing salicylic acid and procaine moieties were synthesized and evaluated for their anticancer activity in vitro. The antiproliferative effect was assayed against MDA-MB-231 cells, a human breast adenocarcinoma cell line, using CellTiter-Glo® Luminescent Cell Viability Assay. Both compounds tested proved a growth inhibition effect on this cell line in a dose-dependent manner. Our results showed that the compound with procaine effectively reduces breast cancer MDA-MB-231 cells viability and proliferation at higher concentration while that with salicylic acid had an inhibitory effect at lower concentrations and might be tested as an anticancer agent.

KEYWORDS: *1,4-naphthoquinone derivatives, anticancer activity, human breast cancer cells*

Introduction

Breast cancer is a major health problem more commonly observed in the developed countries were represents the leading cause of death in women aged 40-59 years, more than a million new cases being detected annually [1,2]. Despite all the progress made in its therapeutically approach, cancer is still a life-threatening disease and remains a serious menace to human health. Chemotherapy is vital to cancer treatment and therefore, it is a continuous need to develop more effective antitumor agents. Because the majority of currently used anticancer drugs have originated from natural products, naphthoquinones isolated from natural sources and derivatives are a group that has attracted interest of many researchers [3].

The 1,4-naphthoquinone pharmacophore is a core structure present in numerous naturally occurring bioactive quinones as well as some synthetic compounds. Literature revealed that natural and synthetic naphthoquinone derivatives are biologically active compounds that exhibit significant antibacterial [4-6], antiviral [7-9], antifungal [6,7,10,11], antiparasitic [12,13], antiprotozoal [14], anti-inflammatory [15-17], and anticancer properties [18-21].

Natural and synthetic 1,4-naphthoquinone derivatives are known to display antitumor activity in several drugs, such as anthracyclines (doxorubicin for solid tumors, daunorubicin for leukemia) and mitoxanthrone [22]. For this reason, the development of small molecules with anticancer activity bearing the 1,4-naphthoquinone moiety remains the focus of future research in the field.

Multiple epidemiological studies have demonstrated a correlation between regular aspirin use and reduced risk of colorectal carcinoma [23]. Aspirin is the acetylsalicylic acid and was postulated that its cancer preventive action is due to the main metabolite, the salicylic acid. In the last decade, some authors reported that the local anesthetic procaine revealed to be a DNA-demethylating agent possessing growth-inhibitory effect in a human breast cancer cell line [24] and on human hepatoma cells *in vitro* and *in vivo* [25]. Therefore, in this study we prepared two novel 2,3-disubstituted-1,4-naphthoquinones containing residues of salicylic acid and procaine and evaluated their antiproliferative activity against a human breast cancer cell line.

Material and Methods

Chemicals and reagents

The compounds tested were naphthoquinone derivatives synthesized from 2,3-dichloro-1,4-naphthoquinone with substitution of chloride atom from position 2 with salicylic acid and procaine residues following the protocol previously described [26]. The reagents and the

solvents used for their synthesis were of analytical grade and purchased from Merck Co. The degree of purity and confirmation of structures were determined according to [27,28].

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin were obtained from Sigma-Aldrich and dimethyl sulfoxide from Merck Co. CellTiter-Glo® Luminescent Cell Viability Assay was purchased from Promega.

Cell culture

Human breast epithelial cell line MDA-MB-231 was used in this experiment. This is a standardized estrogen receptor-negative line derived from a metastatic carcinoma and described after the isolation for the first time from the pleural effusion of a 51-year old Caucasian female [29]. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and 100units/mL penicillin/streptomycin and incubated at 37^{o}C in a humidified incubator with a 95% air/5% carbon dioxide atmosphere. Cells were grown in 6-well plates at 70-80% confluence and then treated with various concentrations of each naphthoquinone derivative dissolved in DMSO and incubated for 48 hours. Untreated control cells were incubated under the same conditions with DMSO.

Cell viability and proliferation

The viability of cells incubated with the compounds tested was evaluated using the CellTiter-Glo® Luminescent Cell Viability Assay. This is a homogeneous method to determine the number of viable cells based on the quantification of the ATP present. Adding the CellTiter-Glo® substrate solution causes cell lysis and generation of a luminescent signal proportional to the amount of ATP which in turn is proportional to the number of metabolically active cells present in culture. Briefly, 100µL of CellTiter-Glo® reagent mixture was added directly to cells without removing the medium.

The content was mixed for 2 minutes to allow lysis and the plate was than incubated for 10 minutes at room temperature to stabilize the luminescent signal. Evaluation of cell viability is based on the activity of Ultra-Glo-luciferase, enzyme that catalyzed the oxidation of luciferin in the presence of ATP released by metabolically active cells. Luminescence was recorded using a GloMax®-Single-Tube Luminometer. For each independent experiment the assays for both compounds were performed in triplicate. From a previous attempt with naphthoquinone derivatives we established the time of incubation at 48h and the range of concentrations (1, 2.5, 5, 10µM) to be tested.

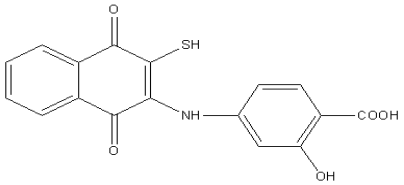
Statistical Analysis

The experimental values are shown as mean±SD. Data are statistically evaluated by the Student's t-test, the level of statistical significance in our study was settled p <0.05.

Results

For the synthesis of 1,4-naphthoquinone derivatives containing salicylic acid and procaine we obtained first 2-chloro-3-N-substituted-1,4-naphthoquinones in one-step reaction between 2,3-dichloro-1,4-naphthoquinone and 4-aminosalicylic acid or procaine. From these intermediaries we carried out the synthesis of 2-mercapto-3-N-substituted-1,4-naphthoquinones by a three-step-reaction previously described [26]. Table 1 presents some physico-chemical features of the new synthesized compounds.

Table 1. Physico-chemical features of tested compounds

Structural formula	Molecular formula	Molecular weight (g/mol)	Color	Melting point (^{o}C)	η (%)
 NQ-PAS 4-(1,4-dihydro-2-mercapto-1,4-dioxonaphthalen-3-ylamino)-2-hydroxybenzoic acid	$C_{17}H_{11}O_5NS$	341.23	Black powder	101	83.51

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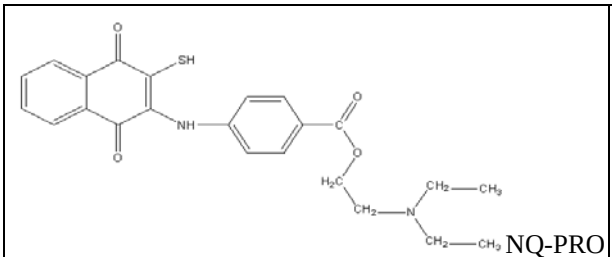 NQ-PRO 2-(diethylamino)ethyl 4-(1,4-dihydro-2-mercapto-1,4-dioxonaphthalen-3-ylamino)benzoate	$C_{23}H_{24}O_4N_2S$	424.28	Red-brown powder	155	71.22

In our experiments, MDA-MB-231 cells were seeded, allowed to adhere and treated for 48 hours with various concentrations (1, 2.5, 5, respectively 10µM) of compounds NQ-PAS and NQ-PRO prepared from stock solutions of 10mM and vehicle control (DMSO). We compared the growth of cells treated with that of untreated cells (control). Mean and standard deviation are considered for the values obtained in two independent experiments with the assays performed in triplicate (Table 2).

Table 2. Effect on cell viability of 1,4-naphthoquinone derivatives expressed in units of luminescence (RLU)

	NQ-	NQ-
1µM	1634565±11657	1456820±22233
2.5µ	1508710±28822	1366072±29337
5µM	470654±37272.	738877.5±1859
10	337887±29146.	613785±14535.

*Each value represents the mean±SD. Control obtained using DMSO (1888650±278741.5 RLU)

We observed that both naphthoquinone derivatives reduce cell proliferation of MDA-MB-231 cells in a dose-dependent manner. (Fig.1)

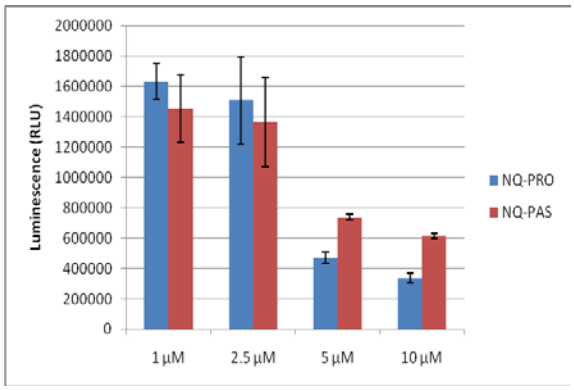

Fig.1. Effect of treatment with the different concentrations of naphthoquinone derivates on viability of MDA-MB-231 cells, evaluated through the activity of luciferase stimulated by ATP released by viable cells (NQ-PRO-derivate with procaine; NQ-PAS-derivate with salicylic acid); mean values are plotted. Error bars represent standard deviation of the means

The effect of these was alike at low concentration, with a better activity for NQ-PAS, but it increase for NQ-PRO at higher concentrations. Our results show that treatment of cells with NQ-PRO induced dropping of viability to 86.55% for the 1µM concentration, 79.88% for 2.5µM, 25% and 18% for 5µM and 10µM respectively ($p<0.05$) when compared to control. (Fig.2) For the compound NQ-PAS, the viability was more influenced at lower concentrations when compared to control (77.14% for 1µM, 72.33% for 2.5µM, 39.12% and 32.5% for 5µM and 10µM, respectively ($p<0.05$). (Fig.2)

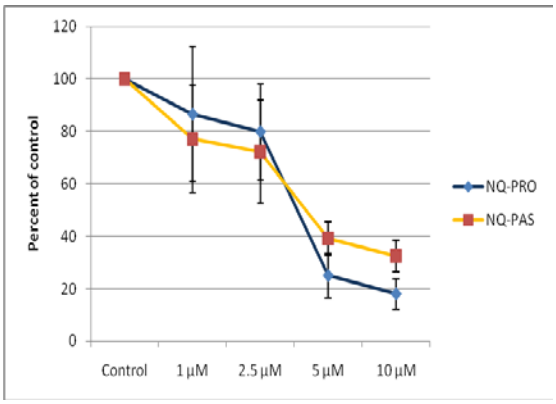

Fig.2. Percent of dropping of viability of MDA-MB-231 cells compared to control. The cells were exposed to various concentrations of the two naphthoquinone derivatives (1µM, 2.5µM, 5µM and 10µM) for 48 hours (for all, p<0.05). Error bars represent standard deviation of the means

Discussion

A time- and dose-dependent variation of cell viability inhibition was reported also by other groups because the anticancer activity of natural and synthetic compounds bearing the 1,4-naphthoquinone skeleton has been intensively reported [18-21,30]. Vitamin K and various analogues with alkyl or alkenyl side chains, incorporating hydroxyl, tiol or amino groups proved their efficiency on several tumor cell lines [24,31-33]. Several mechanisms are postulated to explain their effect on cell activity.

It was reported that 1,4-naphthoquinones interfere with the electron transport and oxidative phosphorylation, are important for enzyme inhibition, and DNA cross linking [30]. The quinone moiety participates in the cell redox cycle releasing reactive oxygen species and leading to oxidative stress; on the other hand *in situ* reduction could occur under hypoxia leading to conjugated intermediates which are powerful DNA-alkylating agents [24]. In such conditions various signaling pathways could be activated in cancer cells.

For the compound NQ-PRO tested, the inhibitory effect due to naphthoquinone moiety could be supported by the potential action as DNA-demethylating agent of procaine residue being known that methylation-associated silencing of tumor suppressor genes is a hallmark of human cancer [24]. Research data emerging from the past 10 years have consolidated the effect of salicylic acid of primary metabolite of aspirin as a chemopreventive agent [34]. In their study, Dachineni and coworkers demonstrated that salicylic acid down-regulate cyclin A2/CDK2 proteins in various cancer cell lines suggesting a novel mechanism of action in chemoprevention. Their experiments revealed that salicylic acid directly binds to CDK2 and alter its conformation. For this reason, the inhibitory effect of the compound NQ-PAS could be explained by a potential action on cell cycle regulators related to salicylic acid residue that has been proved to reduce proliferation in MDA-MB-231 cells.

Conclusions

In summary, we have tested the anticancer activity of two 2,3-disubstituted-1,4-naphthoquinones containing procaine and salicylic acid moieties. In term of efficiency, the compound with salicylic acid residue showed a superior effect on cell viability at low concentrations. The mechanism of cell growth inhibition for these compounds remains the target of future investigations.

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References

1. Hajighasemlou S, Farajollahi M, Alebouyeh M, Rastegar H, Taghizadeh Manzari M, Mirmoghtadaei M, Moayedi B, Ahmadzadeh M, Kazemi M, Parvizpour F, Gharibzadeh S. Study of the Effect of Silymarin on Viability of Breast Cancer Cell Lines. Adv Breast Cancer Res; 2014; 3(3):100-105.
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer Incidence and Mortality Worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer; 2015; 136(5):E359-E386.
3. Ramos-Peralta L, López-López LI, Silva-Belmares SY, Zugasti-Cruz A, Rodríguez Herrera R, Aguilar-González CN. Naphthoquinone: Bioactivity and Green Synthesis in: Mendez-Vilas A (Ed): The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs, FORMATEX Microbiology Series N°5; 2015; Vol.1:542-550, ISBN-13Vol.1:978-84-942134-6-5.
4. Tandon VK, Maurya HK, Mishra NN, Shukla PK. Design, synthesis and biological evaluation of novel nitrogen and sulfur containing hetero-1,4-naphthoquinones as potent antifungal and antibacterial agents. Eur J Med Chem; 2009; 44(8):3130-3137.
5. Kitagawa RR, Bonacorsi C, Da Fonseca LM, Vilegas W, Goncalves Raddi MS. Anti-Helicobacter pylori activity and oxidative burst inhibition by the naphthoquinone 5-methoxy-3,4-dehydroxanthomegnin from Paepalanthus latipes. Rev Bras Farmacogn; 2012; 2(1):53-59.
6. Ibis C, Tuyun AF, Ozsoy-Gunes Z, Bahar H, Stasevych MV, Musyanovych RY, Komarovska-Porokhnyavets O, Novikov V. Synthesis and biological evaluation of novel nitrogen- and sulfur-containinghetero-1,4-naphthoquinones as potent antifungal and antibacterial agents. Eur J Med Chem; 2011; 46(12):5861-5867.
7. Tandon VK, Singh RV, Yadav DB. Synthesis and evaluation of novel 1,4-naphthoquinone derivatives as antiviral,antifungal and anticancer agents. Bioorg Med Chem Lett; 2004; 14(11):2901-2904.
8. Sendl A, Chen JL, Jolad SD, Stoddart C, Rozhon E, Kernan M, Nanakorn W, Balick M. Two new naphthoquinones with antiviral activity from Rhinacanthus nasutus. J Nat Prod; 1996; 59(8):808-811.
9. Tandon VK, Yadav DB, Singh RV, Vaish M, Chaturvedi AK, Shukla PK. Synthesis and biological evaluation of novel 1,4-naphthoquinone derivatives as antibacterial and antiviral agents. Bioorg Med Chem Lett; 2005; 15(14):3463-3466.
10. Rahmoun NM, Boucherit-Otmani Z, Boucherit K, Benabdallah M, Villemin D, Choukchou-Braham N. Antibacterial and antifungal activity of lawsone and novel naphthoquinone derivatives. Méd Maladies Infect; 2012; 42(6):270-275.
11. Dzoyem JP, Tangmouo JG, Lontsi D, Etoa FX, Lohoue PJ. In vitro antifungal activity of extract and plumbagin from the stem bark of Diospyros crassiflora Hiern (Ebenaceae). Phytother Res; 2007; 21(7):671-674.

 10.12865/CHSJ.43.04.08

12. Hughes ML, Lanterib CA, O'Neil MT, Johnson JD, Gribble GW, Trumpower BL. Design of anti-parasitic and anti-fungal hydroxy-naphthoquinones that are less susceptible to drug resistance. Mol Biochem Parasitol; 2011; 177(1):12-19.
13. Johann L, Belorgey D, Huang HH, Day L, Chessé M, Becker K, Williams DL, Davioud-Charvet E. Synthesis and evaluation of 1,4-naphthoquinone ether derivatives as SmTGR inhibitors and new antischi stosomal drugs. FEBS J; 2015; 282(16): 3199-3217.
14. Pieretti S, Haanstra JR, Mazet M, Perozzo R Bergamini C, Prati F, Fato R, Lenaz G, Capranico G, Brun R, Bakker BM, Michels PA, Scapozza L, Bolognesi ML, Cavalli A. Naphthoquinone Derivatives Exert Their Antitrypanosomal Activity via a Multi-Target Mechanism. PLoS Negl Trop Dis; 2013; 7(1): e2012.
15. Luo P, Wong YF, Ge L, Zhang ZF, Liu Y, Liu L, Zhou H. Anti-inflammatory and analgesic effect of plumbagin through inhibition of nuclear factor-κB activation. J Pharmacol Exp Ther; 2010; 335(3):735-742.
16. Yang Y, Yang WS, Yu T, Yi YS, Park JG, Jeong D, Kim JH, Oh JS, Yoon K, Kim JH, Cho JY. Novel anti-inflammatory function of NSC95397 by the suppression of multiple kinases. Biochem Pharmacol; 2014; 88(2):201-215.
17. Soares AS, Barbosa FL, Rüdiger AL, Hughes DL, Salvador MJ, Zampronio AR, Stefanello MÉA. Naphthoquinones of Sinningia reitzii and Anti-inflammatory/Antinociceptive Activities of 8-Hydroxydehydrodunnione. J Nat Prod; 2017; 80(6):1837-1843.
18. Kanaan YM, Das JR, Bakare O, Enwerem NM, Berhe S, Beyene D, Williams V, Zhou Y, Copeland RL Jr. Biological evaluation of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquionone as an anti-breast cancer agent. Anticancer Res; 2009; 29(1):191-200.
19. Eldhose B, Gunawan M, Rahman M, Latha MS, Notario V. Plumbagin reduces human colon cancer cell survival by inducing cell cycle arrest and mitochondria-mediated apoptosis. Int J Oncol; 2014; 45(5):1913-1920.
20. Prachayasittikul V, Pingaew R, Worachartcheewan A, Nantasenamat C, Prachayasittikul S, Ruchirawat S, Prachayasittikul V. Synthesis, anticancer activity and QSAR study of 1,4-naphthoquinone derivatives. Eur J Med Chem. 2014; 84:247-263.
21. Wellington KW. Understanding cancer and the anticancer activities of naphthoquinones-a review. RSC Adv; 2015; 5:20309-20338.
22. Garcia Rodriguez LA, Huerta-Alvarez C. Reduced risk of colorectal cancer among long-term users of aspirin and nonaspirin nonsteroidal antiinflammatory drugs. Epidemiology; 2001; 12(1):88-93.
23. Paterson JR, Lawrence JR. Salicylic acid: a link between aspirin, diet and the prevention of colorectal cancer. QJM: International J Med; 2001; 94(8):445-448.
24. Villar-Garea A, Fraga MF, Espada J, Esteller M. Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. Cancer Res; 2003; 63(16):4984-4989.
25. Tada M, Imazeki F, Fukai K, Sakamoto A, Arai M, Mikata R, Tokuhisa T, Yokosuka O. Procaine inhibits the proliferation and DNA methylation in human hepatoma cells. Hepatol Int; 2007; 1(3):355-364.
26. Rău G, Creţu FM, Andrei AM, Pisoschi CG, Mogoşanu GD, Boroghină A, Baniţă IM, Stănciulescu EC. Synthesis and evaluation of antimicrobial activity of new 2-mercapto-3-substituted-1,4-naphthoquinones (I). Farmacia; 2015; 63(5):665-669.
27. Rău G, Iacobescu G, Meltzer V. A series of new azoderivatives-Synthesis and liquid cristak properties. Rev Roum Chim; 2005; 50(2):119-124.
28. Radu S, Rău G, Moanta A. Synthesis and characterization of some 4-[(chlorobenzyl)oxy]azogenzenes. REV CHIM-BUCHAREST; 2001; 52(11):619-622.
29. Pozo-Guisado E, Alvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernandez-Salguero PM. The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. Biochem Pharmacol; 2002; 64(9):1375-1386.
30. Wang SH, Lo CZ, Gwo ZH, Lin HJ4, Chen LG5, Kuo CD6, Wu JY7. et al. Synthesis and Biological Evaluation of Lipophilic 1,4-Naphthoquinone Derivatives against Human Cancer Cell Lines. Molecules 2015;20:11994-12015.
31. Lamson W, Plaza SM. The Anticancer Effects of Vitamin K. Altern Med Rev; 2003; 8(3):303-318.
32. Akiyoshi T, Matzno S, Sakai M, Okamura N, Matsuyama K.The potential of vitamin K3 as an anticancer agent against breast cancer that acts via the mitochondria-related apoptotic pathway. Cancer Chemother Pharmacol; 2009; 65(1):143-150.
33. Bonifazi EL, Ríos-Luci C, León LG, Burton G, Padrón JM, Misico RI. Antiproliferative activity of synthetic naphthoquinones related to lapachol. First synthesis of 5-hydroxylapachol. Bioorg Med Chem; 2010; 18(7):2621–2630.
34. Dachineni R, Ai G, Kumar DR, Sadhu SS, Tummala H, Bhat GJ. Cyclin A2 and CDK2 as Novel Targets of Aspirin and Salicylic Acid: A Potential Role in Cancer Prevention. Mol Cancer Res; 2016; 14(3):241-252.

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