Discovery of 1,4-Naphthoquinones as a New Class of Antiproliferative Agents Targeting GPR55

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S Supporting Information

ABSTRACT: A new series of 1,4-naphthoquinones, bearing various cyclic and aliphatic amines on C2, was designed and synthesized to identify antiproliferative agents for triple-negative breast cancer, which represents a clinical challenge without targeted therapies. Among naphthoquinones, **2a** and **3a** inhibited the proliferation of MDA-MB-231 cells ($EC_{50} = 1.6$ and $2.7 \mu\text{M}$, respectively), compared to primary human breast cells MCF10A. Furthermore, they did not affect the viability of peripheral blood mononuclear cells (PBMC), suggesting their potential safer use for cancer treatment. Recently, correlations have emerged between the expression of G protein-coupled receptor 55 (GPR55) and both triple-negative breast cancer development and invasion, making it a promising target for the development of targeted therapies. Based on this evidence, molecular docking studies supported the hypothesis of binding to GPR55, and pharmacological tests suggested that compound **3a** could exert its antiproliferative activity acting as a GPR55 inverse agonist.

KEYWORDS: Triple-negative breast cancer, naphthoquinone, antiproliferative, GPR55, molecular docking

Breast cancer is the most commonly diagnosed malignancy among the female population, and it is one of the leading causes of cancer-related deaths in women all over the world.¹ In addition to surgery and radiotherapy, endocrine therapy usually offers a hope to breast cancer patients. The triple-negative breast cancer (TNBC) encompassed 15–20% of all diagnosed cases and, compared to the other types of breast cancer, it is characterized by a more aggressive nature and poorer prognosis. Furthermore, the risk of relapses is high, and metastases frequently occur with shorter overall survival.^{2,3} TNBC owes its name to the lacking expression of three markers, estrogen and progesterone receptors (ER and PR) and human epidermal growth factor receptor 2 (HER2), common targets for breast cancer therapies.⁴ The peculiar heterogeneity of TNBC has always hampered the development of specific targeted therapies.⁵ Although recently an intense understanding of the molecular mechanisms involved in the physiology of TNBC has opened the perspective for alternative therapeutic approaches, they are still under clinical trials and preclinical studies, and no targeted therapies for TNBC have been approved yet. Currently, systemic chemotherapy is the only available option for patients with TNBC.^{6,7} Therefore, the identification of therapeutically targeted molecules remains an attracting clinical challenge.

In the last years, the membrane-bound G protein-coupled receptor 55 (GPR55), initially deorphanized as a cannabinoid

receptor, has emerged as a potential target for treating cancer, including TNBC.⁸ In particular, GPR55 is highly expressed in TNBC cell lines, and its activation correlates with cancer proliferation and invasion.^{9–11} Despite increasing evidence, the paucity of specific ligands of GPR55 has limited its study as anticancer target. Most of them possess electronegative groups and pendant aromatic or heterocyclic rings that make critical hydrogen-bonding and aromatic interactions with specific residues in the receptor binding pocket.^{12–14} Based on these data, the design and the discovery of new potent ligands of GPR55 may be the key for TNBC targeted therapies.¹⁵

The 1,4-naphthoquinone scaffold is often found in many natural and synthetic compounds, and the presence of the two conjugated carbonyl groups on C1 and C4 is responsible for the biological relevance of this system.¹⁶ Moreover, the number of specific substituents and their position on the naphthoquinone core are responsible for a wide range of biological activities, including anticancer.^{17–21} In particular, the antiproliferative activity is favored by the presence of a hydroxyl group on C5.²² Although a number of strides are

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Scheme 1. Synthetic Steps for Compounds 1-6a and 1-4b^a

^aReagents and conditions: (i) 1. a: AlCl_3 , NaCl , $140\text{--}150\text{ }^\circ\text{C}$; b: $170\text{--}175\text{ }^\circ\text{C}$, 5 min; 2. H_2O , HCl , rt, overnight, 99%; (ii) amine, Na_2CO_3 , DCM, rt, overnight, 17–28%; (iii) CH_3I , Ag_2O , CHCl_3 , rt, 22 h, 56%.

being made to understand the molecular mechanism of action of 1,4-naphthoquinones, their therapeutic target still remains unknown.

Herein, we report the synthesis of novel 5,8-dihydroxy and 5,8-dimethoxy derivatives of 1,4-naphthoquinone, bearing pendant nonaromatic heterocycles or aliphatic amines on C2, with the main purpose to identify new antiproliferative agents of TNBC. The molecular mechanism of action of the new derivatives was investigated to ascertain their target. Molecular docking studies were also carried out to support the *in vitro* results. The synthetic steps to obtain compounds 1-6a and 1-3b are outlined in Scheme 1. According to the method of Huot and Brassard,²³ 1a was prepared as a tautomeric pair through Friedel–Crafts condensation of 1,4-dimethoxybenzene and 2,3-dichloromaleic anhydride, in a melted mixture of aluminum and sodium chlorides. It was easily purified by crystallization and isolated in high yield. Due to its high reactivity in nucleophilic substitutions, 2-6a were quickly obtained by reaction of 1a with various cyclic and aliphatic amines.

Heterocyclic and diethylamino derivatives were obtained in high yield, while the lengthening of the alkyl amine chain resulted in a lower yield and formation of byproducts. The 5,8-dihydroxyl groups were replaced with methoxy substituents to evaluate their influence on the antiproliferative activity and to rule structure–activity relationship (SAR) considerations. Thus, 1b was obtained in a higher yield compared to its isomer through methylation of 1a with methyl iodide and silver oxide. Following the same reaction described before, 2-3b were obtained by nucleophilic substitutions of 1b with cyclic amines (see Supporting Information for reaction procedures).

To determine whether the new derivatives provide the desired TNBC antiproliferative activity, their EC_{50} was assessed against the MDA-MB-231 cell line, overexpressing GPR55, using the cell viability assay (Table 1). Although the small number of compounds, the *in vitro* results indicated the impact of the different substituents on the antiproliferative activity. The dihydroxyl derivative 1a, with two chlorine atoms on C2–3 positions, did not inhibit the cell proliferation. However, the presence of amino substituents on C2 showed some cytotoxic activity. Particularly, the presence of a six-membered ring, as in 2a and 3a, resulted in a potent cytotoxicity (Figure 1A,B). The analysis of dose–response

Table 1. Summary of the *in Vitro* Cytotoxic Activities of the New 1,4-Naphthoquinone Derivatives

compd	R	MDA-MB-231 ^a
1a		>20
2a	piperidin-1-yl	1.6
3a	morpholin-1-yl	2.7
4a	pyrrolidin-1-yl	>10
5a	$-\text{N}(\text{Et})_2$	>20
6a	$-\text{N}(\text{Bu})_2$	>20
1b		>20
2b	piperidin-1-yl	>10
3b	morpholin-1-yl	>10

^a EC_{50} values were determined by MTT assay after 24 h treatment and are expressed as micromolar concentrations. The values are averages of triplicate ($n = 3$) data and shown as the mean.

Figure 1. Dose–response curves of compound 2a (A) and 3a (B).

curve profiled compound 3a as ligand for GPR55. In any case, redox activity of compound 2a cannot be ruled out. The morpholino derivative 3a exhibited the best antiproliferative activity, probably due to hydrophobic contacts not found in 1a (see docking results). Substitutions with the 5-membered pyrrolidine ring or dialkylamino groups resulted in a significant loss of potency. Also in this case, reduction of the ring size and its opening into a dialkyl amine led to the lack of hydrophobic contacts with the binding site amino acids. Furthermore, dimethoxy derivatives 1-3b did not affect the proliferation of TNBC cells, even if six-membered rings are attached on C2, as in 2a and 3a. These results suggest that the presence of both six-membered rings on C2 and hydroxyl groups on C5 and C8 are crucial for the antiproliferative activity.

The most potent compounds **2a** and **3a** were then evaluated for their safety, testing their cytotoxicity against MCF10A, a primary human breast cell line. The EC₅₀ value of both **2a** and **3a** against MCF10A cells was higher, suggesting a slightly low antiproliferative activity than that found toward TNBC cell line (Table 2). Since most of chemotherapeutics are administered

Table 2. Comparison of the *in Vitro* Cytotoxic Activities of the Best Derivatives against TNBC and PBMC Cell lines

compd	MDA-MB-231 ^a	MCF10A ^a	PBMC ^b
2a	1.6	2.5	65%
3a	2.7	3.7	70%

^aEC₅₀ values were determined by MTT assay after 24 h treatment and are expressed as micromolar concentrations. The values are averages of triplicate (*n* = 3) data and shown as the mean. ^b100 μM test dose. Cell viability of PBMC was evaluated with trypan blue dye exclusion.

intravenously, we aimed to investigate the cytotoxicity of the selected compounds also against peripheral blood mononuclear cells (PBMC) to evaluate their safety. Compounds **2a** and **3a** did not affect the viability of PBMC either at 24 or 48 h of incubation (see Supporting Information), which was over 60% even at the highest test concentration (100 μM, Table 2). These data confirm the selective antiproliferative activity of the new compounds against TNBC and the potential application for the treatment of this deadly type of breast cancer in which combination therapies are emerging as strategy.³⁰

Docking simulations were carried out to investigate the binding mode of **3a** on GPR55. No three-dimensional structure of GPR55 is currently available from experimental sources. The same docking protocol, already described by us for GPR40 agonists²⁴ and very similar to that applied by others to GPR55 simulations,^{25,13} has been used to evaluate the putative binding mode of the new 1,4-naphthoquinones on GPR55. The complementarity of the molecular surface of both **3a** and GPR55 binding site is illustrated in Figure 2A. Compound **3a**, in its best docking pose, showed the oxygen atoms in positions 4 and 5 in hydrogen bond contacts with the terminal ammonium group of Lys80 and the imidazole ring of His170 (Figure 2B). The importance of the 5-OH substituent for compound activity is in agreement with SAR on the naphthoquinones that shows a crucial role of the phenolic group for cytotoxic activity. In fact, docking simulations on methoxylated compounds **1b–3b** showed that they lacked the charge-reinforced hydrogen bond constituted by the imidazolium group of His170 and the phenate substituent at position 5 of the ligand.

The two hydrogen bonds of **3a** mimic those found between the phosphate group of the lysophosphatidylinositols (LPIs) and the same amino acids (Figure 2C). Moreover, this interaction pattern was also found in the complex between GPR55 and tetrahydromagnolol (THM),¹³ whose 2,2'-biphenyldiol moiety is reminiscent of the two 1,3-dioxygenated molecular pathways found in **3a** (namely, the 1-carbonyl/8-hydroxyl and 4-carbonyl/5-hydroxyl pairs).

In particular, a rough superposition between **3a** and THM clearly shows structural similarity (in size and shape) between compounds and match between oxygenated substituents (Figure 2D).

The morpholine ring of **3a** was accommodated within an aromatic cage comprising Tyr101, Phe102, Tyr106, Phe190, and Phe246 (Figure 2B), thus mimicking the pendant

Figure 2. (A) Complementarity between the molecular surface of **3a** (blue) and that of the GPR55 binding site (gray). (B,C) Graphical representation of the interactions between **3a** and LPI and the binding site of GPR55. The GPR55–**3a** complex (A) is stabilized by two hydrogen bonds (blue dashed lines) involving the ligand oxygens at 4 and 5 positions and by strong hydrophobic contacts (green dashed lines) mainly involving the morpholine ring at C2. Interestingly, LPI conserved the same hydrogen bonds (blue dashed lines, panel B), while its alkyl edge is superposed to the hydrophobic portion of morpholine of **3a**. (D) Rough superposition between **3a** (pink and atom type) and THM (green and atom type) shows a clear correspondence between the two phenolic hydroxyls of THM and the 4-carbonyl and 5-phenolic group of **3a**. Likewise, the overall size and shape of both molecules are comparable.

heteroaromatic ring found in already known GPR55 antagonists.¹³ This heterocyclic ring was also pointing toward Met105 that is one of the amino acids responsible for GPR55 activation. However, the overall size of **3a**, which is significantly smaller than other known GPR55 antagonists since a head region is not present (see below), allows such a compound to have a certain mobility within the binding site, as already hypothesized for other smaller GPR55 antagonists.¹³ As a consequence, molecular docking simulations cannot predict if **3a** is or is not able to prevent the amino acid conformational switch responsible for GPR55 activation. Given the uncertainty about the most appropriate form of GPR55 to be used for molecular modeling, docking simulations have been set starting from either the inactive or active GPR55 and resulted in very similar interaction patterns of **3a**. In fact, the key hydrogen bonds involving both Lys80 and His170 were maintained, further supported by hydrophobic interactions with the side chain of the aromatic cage above-described.

The side chains of Val242 and Leu270 provided profitable hydrophobic contacts that contributed to further stabilize the complex (Figure 2B). Docking simulations strongly suggested that such hydrophobic interactions played a fundamental role in defining compound activity. In fact, compounds bearing a small substituent (Cl or a dialkyl amine side chains, such as in **1a**, **5a**, and **6a**, respectively) or a five-membered heterocycle (such as in **4a**) instead of the six-membered heterocyclic rings of **2a** and **3a**, showed a significant reduction or a complete lack of hydrophobic interactions with the two hydrophobic amino acids and with the aromatic cage (not shown), which are

probably responsible for their loss of activity. Interestingly, the 8-OH substituent of **3a** was superposed to the alcoholic groups of the LPI sugar moiety, although clear electrostatic or hydrogen bond interactions with GPR55 were not evidenced (Figure 2C).

Although **3a** was able to maintain the hydrogen bond interaction with Lys80 that is the primary interaction site of the complexes between GPR55 and antagonist compounds,¹³ the overall structure of **3a** was not characterized by the presence of the three structural elements described for GPR55 antagonists (namely, a head edge, a central portion, and a pendant aromatic ring).¹³ This results in the inability to predict if **3a** could act as a GPR55 agonist or antagonist. In this view, we performed a pharmacological test using CID16020046 (thereafter referred to as CID160), a selective antagonist of GPR55 in order to characterize the pharmacodynamics profile of **3a**.

Clinical data identified LPIs as biomarkers of poor prognosis in cancer patients.²⁶ LPIs were found to be endogenous ligands of GPR55.¹⁰ Recently, the LPI/GPR55 axis was demonstrated to enhance human breast cancer cell migration independently of the TNBC phenotype.²⁹ Therefore, we tested **3a** on both TNBC and hormone-responsive breast cancer cell lines. The exposure of MDA-MB-231 and MDA-MB-468 (Figure 3A,C)

Figure 3. Activity of the GPR55 selective antagonist CID16020046 and **3a**, alone and in combination on two TNBC cell line MDA-MB-231 and MDA-MB-468 (A,C) and on two hormone-responsive cell lines MCF-7 and T47D (B,D). Values with the same letter are not significantly different, while values with different letters are significantly different (* $p < 0.05$ and ** $p < 0.001$ one-way ANOVA followed by Bonferroni's comparison test, as posthoc; each sample was run in triplicate ($n = 3$)).

as well as MCF-7 and T47D (Figure 3B,D) to **3a** led to a significant decrease of the proliferation rate compared to DMSO-treated cell. The evidence that CID160 caused a decrease of cell viability in 3 of the 4 tested cell lines could indicate the presence of endogenously produced ligand.²⁶ In this view, the exposure of MDA-MB-231, MDA-MB-468, and MCF-7 to CID160 for 30 min before treatment with **3a** was able to reverse its effect. This suggested that **3a** acts as inverse

agonist of GPR55. It is of note that the GPCRs exist in an active and an inactive form. In the physiological system, both forms are in equilibrium.^{27,28} Generally, agonists of GPCRs have higher affinity for the active form. In addition, based on this, **3a** is an inverse agonist since it is able to evoke an antiproliferative effect.

In conclusion, a new class of 1,4-naphthoquinones was designed and synthesized to identify potent antiproliferative agents. On the basis of the data obtained *in vitro*, we demonstrated that **3a** is a GPR55 inverse agonist. Moreover, molecular docking studies showed the interactions between **3a** and the binding site residues of GPR55. Further *in vivo* studies are needed to elucidate the pharmacokinetics profile of **3a** as antiproliferative agent in tumors harboring aberrant expression of GPR55, including TNBC.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00333.

General experimental information; full synthetic procedures and characterization data for all new compounds; NMR, GC-MS, and IR spectra; experimental procedure for PBMC isolation and cell viability assays; statistical analysis; computational details (PDF)

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Notes

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■ ABBREVIATIONS

DCM, dichloromethane; ER, estrogen receptor; GPR55, G protein-coupled receptor 55; HER2, human epidermal growth factor receptor 2; LPI, lysophosphatidylinositol; PBMC, peripheral blood mononuclear cells; PR, progesterone receptor; THM, tetrahydromagnolol; TNBC, triple-negative breast cancer

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