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(6-Bromo-1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazine (Carbhydraz) Acts as a GPER Agonist in Breast Cancer Cells

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Abstract: Estrogens control a wide number of aspects of human physiology and play a key role in multiple diseases, including cancer. Estrogens act by binding to and activating the cognate receptor (ER), however numerous studies have revealed that the G protein-coupled receptor named GPR30/GPER mediates also estrogen signals. As ER and GPER share the ability to bind to same compounds, the use of GPER-selective ligands has allowed a better understanding of the biological responses mediated by GPER. In the present study, we designed and synthesized two novel carbazole derivatives and then investigated their ability to interact with and activate the GPER-mediated transduction pathway in breast cancer cells. Both compounds did not activate the classical ER in MCF7 cells, whereas one of the two compounds synthesized triggered through GPER the rapid ERK activation in ER-negative SkBr3 cells, demonstrating a good affinity for GPER in docking studies. The characterization of this novel selective GPER agonist could represent a potential useful tool to provide further insights into the physiopathological role exerted by GPER.

Keywords: Breast cancer, Carbazole derivatives, Docking simulations, Estrogen/estrogen receptors, GPR30/GPER, Heterocycles.

INTRODUCTION

Nature is source of molecules with a deep impact on human health. Numerous natural metabolites have multiple and distinct biological properties, making them important health products or structural templates for drug discovery [1]. The current literature provides a growing interest on plant derived heterocycles, widely used in medicine, agriculture and technology [2]. Among nitrogen heterocycles, indole derivatives such as carbazole alkaloids display a wide variety of activities, including antibacterial, anti-inflammatory, psychotropic and anti-histamine properties [3-7]. Moreover, carbazoles show significant antitumor activity in cells derived from leukemia, melanoma, colon adenocarcinoma, kidney, brain and breast tumors [8-15]. For instance, a series of simple benzo[a]carbazoles has been shown to bind to estrogen receptor (ER) and inhibit breast cancer cell proliferation as well as the growth of mammary tumors in rats [16]. ERα and ERβ are members of the superfamily of nuclear receptors that regulate multiple cellular functions in normal and cancer tissues acting as ligand-activated transcription factors [17]. In particular, ERα plays a key role in the development of breast cancer and is considered one of the principal biomarker for the endocrine treatment [17].

Although the biological responses to estrogens are mainly mediated by the classical ER, the G protein-coupled receptor GPR30/GPER has been recently shown to mediate estrogen signals in a variety of normal and cancer cell types [18]. In particular, GPER has been involved in rapid events induced by estrogens, including the transactivation of the epidermal growth factor receptor (EGFR), the activation of the mitogen activated protein kinase (MAPK) and phosphoinositide3-kinase (PI3K) transduction pathways, the stimulation of adenylcyclase and the mobilization of intracellular calcium [19]. Furthermore, GPER exhibits many of the biological characteristics of an estrogen receptor, including the capability to bind to estrogens, antiestrogens, phyto- and xenoestrogens [20-23]. Likewise, a recently synthesized compound, referred to as MIBE, displayed the property to bind to and inhibit both GPER- and ERα-mediated signaling in breast cancer cells, representing a promising pharmacol-

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ogical approach targeting breast carcinomas expressing one or both receptors [24]. Hence, the possibility to differentiate the pharmacology of GPER over that ER by targeting each receptor subtype in a selective manner still represents a central concern in dissecting estrogen signaling. In this context, the recent identification of novel compounds able to act exclusively through GPER has greatly advanced our understanding on the role elicited by GPER in diverse biological systems and cancer [25-27]. We have *in silico* designed, prepared and functionally characterized novel carbazole derivatives. One of the compounds synthesized showed a good affinity for the GPER-binding pocket and exhibited the capability to activate the GPER-mediated signaling in a selective manner in ER-negative breast cancer cells.

MATERIALS AND METHODS

Molecular Modeling and Docking Simulations

We used the program GOLD v.5.0.1 (the Cambridge Crystallographic Data Center, UK) to perform docking simulations. GOLD is a program using a genetic algorithm that allows to investigate the full range of ligand conformational flexibility and a partial protein side chain flexibility. As protein target for our docking simulation, we used the three dimensional atomic coordinates of the GPER molecular model built by homology as described elsewhere [23]. We identified Phe 208 O atom, as the protein active site centre on the basis of our previous docking simulations [23], and we considered as the active site atoms, those located within 20 Å from this point. The default GOLD settings were used running the simulations. Residues Tyr123, Gln138, Phe206, Phe208, Glu275, Phe278 and His282 of GPER were defined with flexible side chains, allowing their free rotation. Ligand molecular structure was built and energy minimized with the programs InsightII and Discover3 (Biosym/MSI, San Diego, CA, USA). Figures were drawn with the program Chimera [28] and interaction diagram was built using the program Ligplot [29].

Cell Culture

SkBr3 human breast cancer cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS. MCF7 human breast cancer cells were maintained in DMEM with phenol red supplemented with 10% FBS. Cells were grown in a 37° C incubator with 5% CO_2. The day before experiments for immunoblots cells were switched to medium without serum, thereafter cells were treated as indicated.

Western Blot Analysis

SkBr3 was grown in 10-cm dishes and exposed to drugs for the appropriate time, then washed twice with ice-cold PBS and solubilized with 50 mM Hepes buffered solution, pH= 7.5, containing 150 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). Protein concentration in the supernatant was determined according to the Bradford method. Equal amounts (10-30 μg) of the whole cell lysate was electrophoresed through a reducing SDS/10% (w/v) polyacrylamide gel and electroblotted onto a nitrocellulose membrane which was probed with primary antibodies phosphorylated ERK1/2 (E-4), ERK2 (C-14), GPER (N-15) and β-actin (C-2) (all purchased from Santa Cruz Biotechnology, Milan, Italy). The levels of proteins and phosphoproteins were detected, after incubation with the horseradish peroxidase-linked secondary antibodies, by the ECL® (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy).

Transfections and Luciferase Assays

Plasmids and Luciferase Assays were previously described [30-31]. Cells were transferred into 24-well plates with 500 μL of regular growth medium/well the day before transfection. MCF7 cell medium was replaced with DMEM supplemented with 1% charcoal-stripped (CS) FBS lacking phenol red and serum on the day of transfection, which was performed using the X-tremeGENE 9 DNA transfection reagent, as recommended by the manufacturer (Roche Molecular Biochemicals, Milan, Italy) with a mixture containing 0.2 μg of reporter plasmid and 1 ng of pRL-CMV. After 5-6 h the medium was replaced again with serum-free DMEM lacking phenol red and supplemented with 1% CS-FBS, ligands were added at this point and cells were incubated for 16-18 h. Luciferase activity was then measured with the Dual Luciferase kit (Promega, Milan, Italy) according to the manufacturer recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase activity.

Gene Silencing Experiments

Cells were plated onto 10-cm dishes, maintained in serum-free medium for 24h and then transfected for additional 24h before treatments with a control vector or an independent shRNA sequence for each target gene using X-tremeGENE 9 (Roche Molecular Biochemicals, Milan, Italy). Short hairpin construct against human GPER (shGPER) was generated and used as previously described [32-33]. In brief, they were generated in lentiviral expression vector pLKO.1 purchased by Euroclone, Milan, Italy. The targeting strand generated from the GPER shRNA construct is 5'-CGCTCCCTGCAAGCAGTCTTT-3'.

EXPERIMENTAL SECTION

General

Commercial reagents were purchased from Aldrich, Acros Organics and Alfa Aesar and were used without additional purification. Melting points were determined on a Gallenkamp melting point apparatus. The IR spectra were recorded on a Fourier Transform Infrared Spectrometer FT/IR-4200 for KBr pellets. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Bruker 300 spectrometer. Chemical shifts are expressed in parts per million downfield from tetramethylsilane as an internal standard. Thin layer chromatography (TLC) was performed on silica gel 60F-264 (Merck). The 6-bromo-1,4-dimethyl-9*H*-carbazole-3-carbaldehyde **1** was prepared as described in the literature [34].

Preparation of 1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazines (2a-c) and *N,N'*-bis-(6-bromo-1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazine (3)

Hydrazine hydrate, 98% (d= 1.029 g/mL; 5.97×10^{-3} mol; 0.29 mL) and 1,4-dimethyl-9*H*-carbazole-3-carbaldehyde

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1a-b ($1.48x10^{-3}$ mol) were dissolved in absolute ethanol (37.4 mL).

The resulting solution was heated under reflux for 3h. After cooling to room temperature, the reaction solution was evaporated under reduced pressure. The remaining residue was washed twice with Et_2O (20 mL). The filtrate was dried under reduced pression and the solid residue obtained was recrystallized from Et_2O to give **2a-b** as powder. The compound **3** has been isolated as cream powder for filtration of the reaction of **1a**.

(6-Bromo-1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazine (2a)

Orange powder; yield 60%; mp > 270 °C. IR spectrum, ν, cm^{-1}: 3398-3354 (NH_2); 3169 (NH); 1613 (CH=N); 1589; 1442; 857. ^{1}H NMR spectrum (DMSO-d_6), δ, ppm: 2.49 (s, 3H, C*H₃*); 2.78 (s, 3H, C*H₃*); 6.51 (s, 2H, N*H₂*); 7.45-7.49 (m, 2H, *Ar*); 7.61 (s, 1H, *Ar*); 8.22 (s, 1H, C*H*=N-NH_2); 8.26 (s, 1H, *Ar*); 11.47 (s, 1H, N*H*). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 15.12; 16.77; 110.73; 112.95; 117.92; 119.87; 124.32; 124.39; 125.30; 125.38; 127.04; 127.45; 138.48; 138.68; 139.11. Found, %: C 56.98; H 4.46; N 13.29. $C_{15}H_{14}BrN_3$. Calculated, %: C 56.95; H 4.50; N 13.31.

(6-Methoxy-1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazine (2b)

Green powder; yield 48%; mp > 270 °C. IR spectrum, ν, cm^{-1}: 3430-3379 (NH_2); 2924 (NH); 1612 (CH=N); 1464; 1210; 1130; 863. ^{1}H NMR spectrum (DMSO-d_6), δ, ppm: δ 2.79 (s, 3H, C*H₃*); 3.53 (s, 3H, C*H₃*); 3.87 (s, 3H, OC*H₃*); 6.51 (s, 2H, N*H₂*); 6.92-7.27 (m, 1H, *Ar*); 7.50-7.91 (m, 3H, 2 *Ar*, C*H*=N-NH_2); 8.30 (s, 1H, *Ar*); 11.29 (s, 1H, N*H*). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 15.32; 19.77; 55.87; 102.13; 105.95; 109.62; 112.19; 117.62; 121.39; 124.20; 125.05; 127.24; 134.67; 139.65; 143.06; 154.10. Found, %: C 71.89; H 6.41; N 15.72. $C_{16}H_{17}N_3O$. Calculated, %: C 71.92; H 6.39; N 15.69.

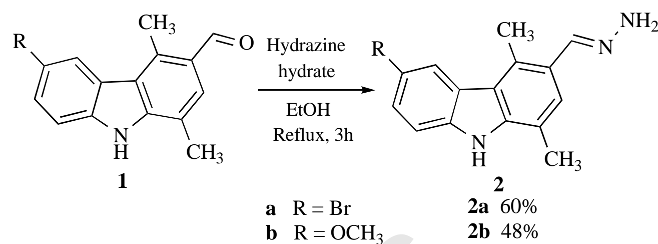
N,N'-bis-(6-bromo-1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazine (3)

Yield 15%; mp > 270 °C. IR spectrum, ν, cm^{-1}: 3419 (*NH*); 1614 (*CH=N*); 1589; 1438; 1247; 798. ^{1}H NMR spectrum (DMSO-d_6), δ, ppm: 2.58 (s, 6H, C*H₃*); 3.00 (s, 6H, C*H₃*); 7.55 (s, 4H, *Ar*); 8.01 (s, 2H, *Ar*); 8.34 (s, 2H, *Ar*); 9.18 (s, 2H, C*H*=N-); 11.52 (s, 2H, N*H*). ^{13}C NMR spectrum (DMSO-d_6), δ, ppm: 15.09; 16.72; 110.68; 112.94; 117.88; 119.86; 124.34; 124.38; 125.28; 125.37; 127.00; 127.41; 138.49; 138.68; 139.10. Found, %: C 60.02; H 4.03; N 9.33. $C_{30}H_{24}Br_2N_4$. Calculated, %: C 60.05; H 4.00; N 9.29.

RESULTS AND DISCUSSION

Chemistry

The starting 6-bromo-1,4-dimethyl-9*H*-carbazole-3-carbaldehyde (**1**) was prepared by a published procedure [34]. The synthesis of (6-bromo-1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazine, referred to as Carbhydraz (**2a**), and its analogue **2b** was depicted in Scheme **1**.



Scheme 1. Synthesis of Carbhydraz (**2a**) and of **2b**.

This is a convenient modification of the Wolff-Kishner [35] reduction and requires the heating of the aldehydic compound **1** with hydrazine hydrate in absolute ethanol by one-pot reaction. The desired hydrazines **2** were obtained in good yield (48-60%). From reaction of **1a** was also isolated, as a byproduct, bis-carbazole **3** (Fig. **1**) (yield of 15%) with potential interest in medicinal chemistry [3].

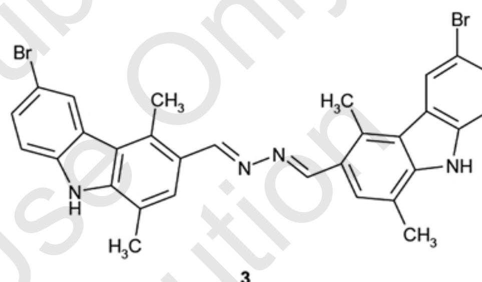


Fig. (1). *N,N'*-bis-(6-bromo-1,4-dimethyl-9*H*-carbazol-3-yl-methylene)-hydrazine (**3**).

RESULTS

Our previous studies [23-24, 27, 36-37] described the GPER binding pocket as a deep cleft in the protein core, surrounded by both hydrophobics and polar residues belonging to transmembrane helices TM III, TM V, TM VI and TM VII. Using a previously validated GPER molecular model as target [23], we performed docking simulations which confirmed a good affinity for the protein by the selective agonist G-1, as previously demonstrated both *in silico* and *in vitro* [25]. Next, we performed a docking simulation of the novel synthesized compounds Carbhydraz (**2a**) and **2b** for GPER using the same settings and parameters used for G-1. Both molecules were positioned within the GPER binding site (Fig. **2A-B**), similarly to G-1 (Fig. **2C-D**). Particularly, the bromine atom of Carbhydraz (**2a**) is positioned about 2.9Å from the nitrogen atom of H307, which is a residue belonging to helix TM VII. The primary amine of Carbhydraz (**2a**) forms a hydrogen bond with N118 (TM II), while the carbazole moiety forms hydrophobic interactions with V116, L119, M133, F206 and F208, which contribute to stabilize the complex.

A synthetic plot illustrating the interactions of Carbhydraz (**2a**) with GPER is shown in Fig. (**3**). The hydrazinic group of Carbhydraz (**2a**) is located in a favourable position to form hydrogen bonds with the carboxyl group of E115 and the hydroxyl group of C207. It should be noted that the functionalization of the carbazole nucleus in position 6 with a bromine (Br) present in Carbhydraz (**2a**) could be strategic for its affinity with GPER. In fact, the presence in compound **2b** of methoxy (OCH_3) rather than Br in the same position

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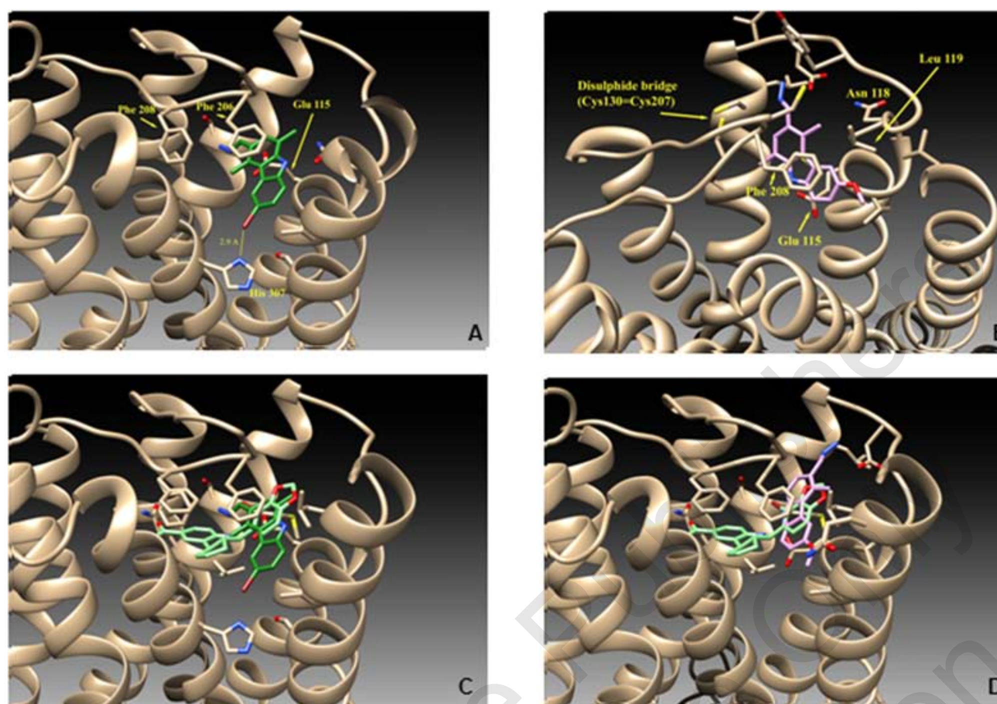

Fig. (2). Ribbon representation of GPER (drawn in tan) bound to different compounds. Panel A reports the binding mode of Carbhydraz (**2a**), drawn as dark green sticks. A yellow line connects the bromine to the histidine indolic nitrogen atom. In Panel B the compound **2b** is drawn in purple. The G-1 moiety is drawn in light green and superposed to Carbhydraz (**2a**) in Panel C and to **2b** in Panel D.

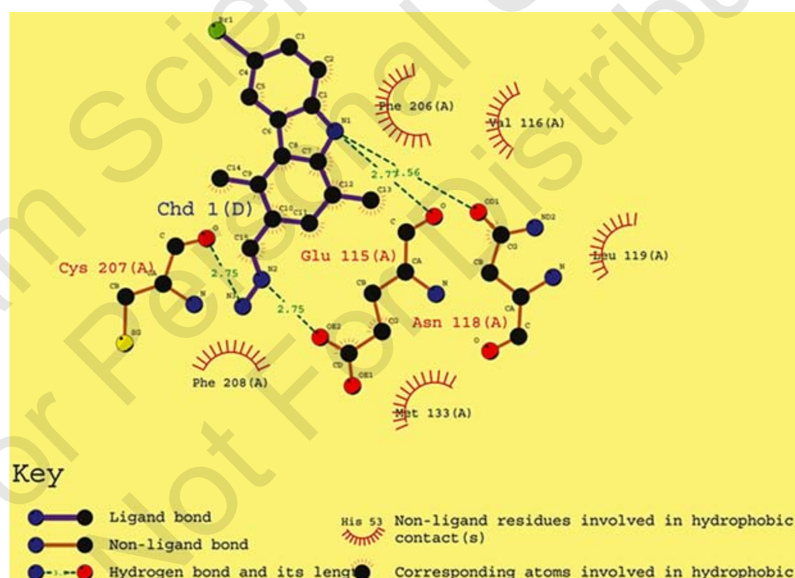

Fig. (3). Plot illustrating the interactions of Carbhydraz (**2a**) and the GPER binding site.

could determine a 180° rotation of the carbazole ring, then lowering the interaction between this compound and GPER helices TM I and TM VII.

Carbhydraz (2a) Induces ERK 1/2 Phosphorylation through GPER in Breast Cancer Cells

In order to verify the results obtained by docking simulation regarding the potential of the two novel synthesized compounds to interact with GPER, we evaluated in ER-negative SkBr3 breast cancer cells the ERK1/2 phosphorylation, which is known to be a hallmark of ligand-activated GPER [23-24, 27, 36, 38-40]. As only the compound referred to as Carbhydraz (**2a**) was able to induce ERK1/2 phosphorylation (Fig. **4A-B**), we aimed to determine whether this effect occurs through GPER. Knocking-down GPER expression with a shGPER in SkBr3 cells, the ability of Carbhydraz (**2a**) to induce ERK1/2 activation was abrogated (Fig. **4C-D**). Taken together, these data suggest that Carbhydraz triggers ERK1/2 phosphorylation through GPER, confirming the findings obtained by docking simulations.

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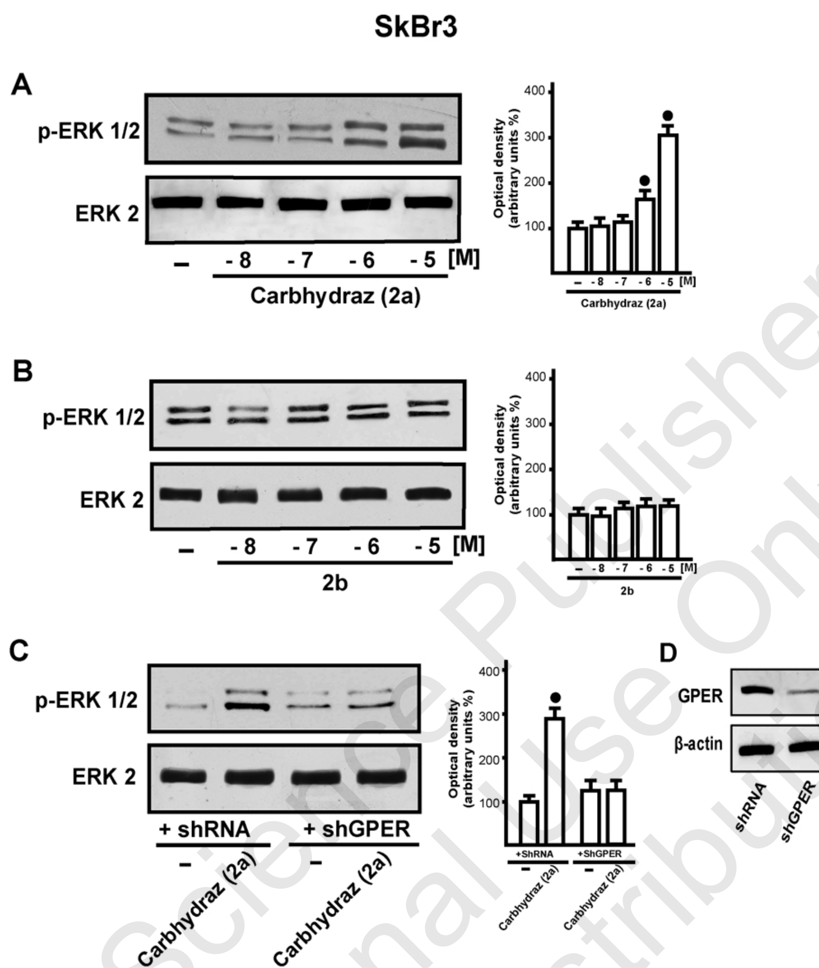

Fig. (4). Carbhydraz (2a) activates ERK1/2 in a GPER-dependent manner. (A-B) ERK1/2 activation in SkBr3 cells treated for 15 min with increasing concentrations of Carbhydraz (**2a**) or **2b**. (C) ERK1/2 activation in SkBr3 cells transfected with shRNA or shGPER and then treated for 15 min with vehicle (-) or 10 μM Carbhydraz (**2a**). Side panels show densitometric analysis of the immunoblots normalized to ERK2. (D) The efficacy of GPER silencing was ascertained by immunoblots. Each data point represents the mean ±SD of three independent experiments. (●) indicate $p<0.05$ for cells receiving vehicle (-) versus treatment.

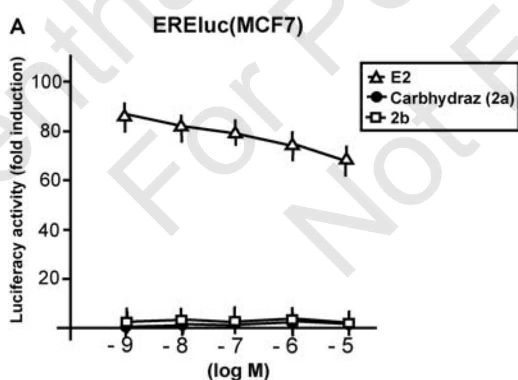

Fig. (5). Carbhydraz (2a) and 2b do not activate ERα. MCF7 cells were transfected with an ER luciferase reporter gene along with the internal transfection control Renilla Luciferase and then treated with increasing concentrations (logarithmic scale) of 17β-estradiol (E2), Carbhydraz (**2a**) and **2b**. The normalized luciferase activity values of cells treated with vehicle were set as 1-fold induction, upon which the activity induced by treatments was calculated. Each data point represents the mean ± SD of three experiments performed in triplicate.

To further evaluate whether the synthesized compounds might be able to activate ERα, we transiently transfected an ER reporter gene in MCF7 breast cancer cells. Only 17β-estradiol (E2) transactivated the endogenous ERα in MCF7 cells, demonstrating that Carbhydraz (**2a**) activates the GPER-mediated signaling in a selective manner (Fig. **5**).

DISCUSSION AND CONCLUSION

The seven-transmembrane G protein-coupled receptors (GPCRs), which belong to the largest superfamily of signal transduction proteins, play a crucial role in many physiological functions as well as in multiple diseases [41-42]. One member of this family, named GPR30/GPER, may mediate physiological responses in the reproductive, nervous, endocrine, immune and cardiovascular systems and contribute to the progression of several types of tumors [18]. In particular, the expression of GPER has been associated with aggressive features of breast, endometrial and ovarian tumors [43-45]. In line with these findings, numerous investigations demonstrated that GPER is involved in the progression of diverse tumor cells, including breast, endometrial, ovarian, thyroid, prostate and testicular germ cells [20, 39-40, 46-50]. Al-

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though several members of the GPCR family, including GPER, control key biological functions, existing drugs target only few members. Consequently, huge efforts are currently underway to develop new GPCR-based drugs, particularly toward new anti-cancer treatments. Considering that the crystallization of GPCRs is particularly difficult, computer based methods have been increasing successful in identifying their atomic structure on the basis of the primary structure [37]. In this context, the availability of a GPER 3D model allowed us to pursue a “protein-based” approach in order to characterize the potential interaction of different molecules with this receptor [37]. Moreover, following different strategies (“ligand-based” as well as mixed biomolecular and virtual screening), several GPER ligands have been identified by our and other groups [25, 47-48, 51-56]. Ligand binding studies validated the results obtained by molecular modeling and docking simulations. Additionally, functional assays allowed the characterization of the biological effects elicited by numerous compounds through GPER in multiple contexts. For instance, the two well-known ERα ligands and activators namely E2 and estriol showed an opposite action through GPER, as E2 activated and estriol inhibited the GPER-mediated pathway [23], whereas the ER antagonists tamoxifen and ICI 182,780 acted as GPER agonists [21, 33, 40]. In addition, a series of natural and synthetic compounds has been identified and characterized as GPER ligands with either agonist or antagonist properties [24-26, 57-58].

In the present study, we have designed and synthesized novel carbazole derivatives and we have performed docking simulations together with functional assays to assess their potential to activate the GPER-mediated signaling. In particular, the compound termed Carbhydraz (**2a**) displayed the potential ability to interact with GPER in docking simulations. Accordingly, in breast cancer cells Carbhydraz (**2a**) activated ERK phosphorylation, which is known to characterize the ligand-induced activation of GPER. Moreover, Carbhydraz (**2a**) did not exhibit any ERα activating property, suggesting a selective action through GPER. Diverse molecules bind to both ER and GPER making difficult the differential evaluation of the GPER/ER pharmacology. In this context, Carbhydraz (**2a**) would contribute to better dissect the distinct functions mediated by GPER.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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