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ARTICLE

New organometallics imines of Rhenium(I) as potential ligands of GSK-3β: Synthesis, characterization and biological studies

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Michelle Muñoz-Osses[a, c], Fernando Godoy[a,*], Angélica Fierro[b], Alejandra Gómez[a] and Nils Metzler-Nolte[c,*]

Substituted amino-piperazine derivatives have been synthesized and used as precursors in the preparation of a series of new organometallic Re(I) imine complexes of the general formula $[(\eta^5\text{-}C_5H_4CH{=}N\text{-}(CH_2)_5\text{-}Pz\text{-}R)Re(CO)_3]$ (Pz-R: -alkyl or aryl piperazine). The piperazine-based ligands were designed to be potential inhibitors of the GSK-3β kinase. All ligands and complexes were full characterized and evaluated in HT-29 and PT-45 cancer cell lines, where GSK-3β plays a crucial role. In this context, we carried out biological evaluation using the MTT colorimetric assay. In terms of structure activity relationship, our findings indicate improved biological activity when aromaticity is increased in the organic ligands (**3d**). In addition, the presence of the rhenium fragment in the imines (**5a-d**) leads to better activity with IC_{50} values in the range of 25-100 μM. Additionally, our experimental studies were complemented by computational studies, where the volume and the electrostatic surface of the organic ligands and the organometallic compounds as well as binding to the kinase protein were calculated.

Introduction

Bioorganometallic chemistry is the study of biologically active molecules that contain carbon directly bonded to metals or metalloids. The functionalization of metal fragment with organic ligands and/or conjugated biomolecules (amino acids, peptides, proteins or carbohydrates) has become a viable strategy to obtain new compounds that have high interest as targeted therapeutics.[1-14]

Although most of the work in this area has been focused on medium and late transition metal complexes, rhenium derivatives have been studied as potential anticancer agents.[15] With respect to cytotoxicity, some rhenium carbonyl complexes equal or even exceed that of the well-established anti-cancer drug cisplatin. There are a number of examples that include the Re(I) tricarbonyl core with ancillary ligands (N,N; alcoxido/hydroxido; N,O; P,P bidentate).[16,17] However, only a few half-sandwich compounds are known. Cyrhetrenyl complexes $[(Cp–R)Re(CO)_3]$ have been evaluated as potential biological candidates for binding to enzymes, proteins or receptors.[18-20] One of the best known examples is the "Re-Tamoxifen" compound from Jaouen's group, where a phenyl group was replaced by the cyclopentadienyl ring coordinated to the organometallic fragment, seeking to improve the activity of this new compound on the estrogen receptor (ER) and breast cancer.[8] Likewise, cyrhetrenyl sulfonamides have been shown to be excellent inhibitors of human carboanhydrase (hCa).[21,22]

One of the proteins that has received a lot of interest in recent years is glycogen synthase kinase 3 (GSK-3), a serine/ threonine kinase that is encoded by two known genes α and β. It has been described as a protein involved in glycogen metabolism.[23] However, it has also been shown to be involved in multiple biological processes by regulating different cellular functions in metabolic and signaling pathways, relating to the development of neurodegenerative and cancerous diseases.

GSK-3**β**, a cytosolic protein, is formed by 433 amino acids and has a mass of 47 kDa. It is distributed mainly in brain tissue, especially in neurons,[24] explaining and corroborating its role in neuronal signalling. Thus, the relationship with various central nervous system diseases such as Alzheimer's disease,[25] schizophrenia,[26] bipolar disorder,[27] as well as tissue inflammation[28] can be understood.

In addition, studies indicate that GSK-3**β** could act as a potential tumour suppressor due to its ability to phosphorylate pro-oncogenic molecules such as cyclin D17[29] and β-catenin.[30] On the other hand, recent studies have shown that GSK-3**β** plays a fundamental role as a positive regulator of the proliferation of cancer cells through different signalling

[a] Laboratory of Organometallic Chemistry, Faculty of Chemistry and Biology, Universidad de Santiago de Chile, Avda. Libertador Bernardo O´Higgins 3363, Estación Central, Santiago, Chile. Email: michelle.munoz@usach.cl; fernando.godoy@usach.cl; alejandra.gomezs@usach.cl
[b] Laboratory of Bioorganic and Molecular Simulation, Department of Organic Chemistry, Faculty of Chemistry, Pontificia Universidad Católica de Chile, Avda. Vicuña Mackenna 4860, Macul, Santiago, Chile. Email: afierroh@uc.cl
[c] Inorganic Chemistry I – Bioinorganic Chemistry, Ruhr University Bochum, Universitaetsstrasse 150, 44801 Bochum, Germany. Email: nils.metzler-nolte@rub.de

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pathways, [24,31] directly related to the development of various types of cancer such as renal,[32] pancreatic,[33] prostate,[34] ovarian,[35] colon,[36] leukemia,[37-38] lung,[39] thyroid,[40] mammary,[41] neuroblastoma,[42] and glioblastoma.[43] For this reason, GSK-3**β** has been proposed as a viable therapeutic target for the treatment of a broad spectrum of different types of cancer.
Several studies indicate that this protein is overexpressed in the cytoplasm and nucleus of colon and pancreas cancer cells[36, 44], suggesting a direct relationship between the activity of this kinase and the development of these types of cancer. Additionally, recent studies report that GSK-3β plays a crucial role in cell growth, regulating the activity of the nuclear growth factor through the transcription of genes. Thus, the inhibition of the protein attenuates the proliferation of colon and pancreas cancer cells and inducing cellular apoptosis[24, 29,31,33,36,45]. This information suggests GSK-3β as a potential new therapeutic target in human cancer.

The three-dimensional structure of GSK-3**β** was obtained by X-ray diffraction.[46-47] The amino acids that make up the binding site correspond to **Ile62, Val70, Ala83, Lys85, Glu97, Met101, Val110, Asp133, Tyr134, Val135, Pro136, Glu137, Thr138, Arg141, Lys183, Gln185, Asn186, Leu188, Cys199, Asp200,** which generate a cavity that is mainly polar. In general, the pharmaceutical industry has focused on the design of organic compounds as potential activators or inhibitors of kinases. For example, activity on GSK-3β and in cancer cell lines such as HT-29 was studied using piperazine derivatives, by a number of research groups.[48-52] These results indicate an important role for the piperazine fragment.

Incorporating a metal or organometallic fragment has been an alternative strategy to design new compounds with biological activity. The metal centre may play a structural and electronic role associated with the distribution and spatial orientation of the organic ligands in the binding cavities of biological targets. With the right choice of metals, highly stable complexes are generated, usually with octahedral geometry. Half-sandwich Ruthenium(II) complexes have been evaluated as potential inhibitors of kinase proteins, some of those exhibiting good affinity for the GSK protein.[53-56] In the same way, cyrhetrene complexes functionalized with aminoquinoline, nitrofurane and glucosamine fragments, have been used as anticancer[57-58] and antimalarial[59-60] agents. The proven biological activity of rhenium complexes, together with the high stability of the Re(I) fragment and coupled with the high lipophilicity of cyrhetrene fragments, are some of the advantages of this metal fragment in the design of new compounds which exhibit a beneficial response to various biological targets.

Here, based on the structural GSK-3β characteristics[46-47] and the relevance in designing new ligands for kinase enzymes, a series of new cyrhetrene imine and amino piperazine derivatives were synthesized and then evaluated on cell lines where GSK-3β plays a crucial role.[24, 29-31, 61, 62]

Results and discussion

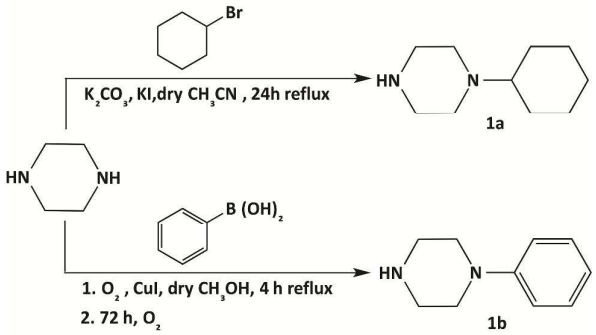
For the construction of new organometallic compounds, the organic and cyrhetrenyl fragments were first synthesized independently. It is important to mention that almost all of the compounds that are described below have not been reported in the literature until now. Generally, the synthesis of these new ligands gave pure compounds in acceptable yields.

Piperazine derivatives

Piperazines **1a** and **1b** were synthesized using a combination of methods described in literature[63-65] (Scheme 1). The ^{1}H NMR spectrum of **1a** shows the presence of two broad singlets at 2.88 and 2.53 ppm corresponding to the protons of the piperazine fragment, and at 2.19 ppm a multiplet that integrates for one proton indicates the presence of the mono-substituted ligand.

However, it was impossible to obtain **1b** using the same methodology as for **1a**, not even employing Cs_2CO_3 and CuI as a catalyst. In both cases, the ^{1}H NMR spectrum only shows the signals corresponding to the starting reagents. This can be attributed to the higher energy required to activate the C (sp^2)-Br bond present in the bromoarene; so a new synthesis methodology was applied using phenylboronic acid[56] instead of bromobenzene as the initial reagent (Scheme 1). In this case, **1b** was obtained as a pale yellow solid in 30% yield. The ^{1}H NMR spectrum shows two multiplets at 3.14 and 3.03 ppm corresponding to the protons of the piperazine fragment and a singlet centered at 1.78 ppm, which integrates for one proton corroborating the mono-substitution. Elemental analysis, ^{13}C NMR and ESI-MS are concordant with what was expected for **1a-b**. Besides, an HPLC of **1b** confirms that the ligand was obtained in analytically pure form.

Scheme 1. Synthesis of piperazine ligands: 1-cyclohexyl piperazine



(**1a**) and 1-phenyl piperazine (**1b**).

Cyano derivatives

The cyano ligands **2a-d** (Scheme 2) were obtained in high yields.[53-55] The IR spectra of **2** in CH_2Cl_2 show a frequency at 2230 cm^{-1}, which confirms the presence of the C≡N group. This frequency is consistent with related organic species (ν(CN): 2210-2360 cm^{-1}).

Also, the ^{1}H NMR spectra ratify the substitution reaction for the formation of ligands **2a-d**. In all cases, the chemical shift, multiplicity and coupling constants confirm the presence of the compounds. The presence of a cyano group is also confirmed by a signal at 119 ppm in the ^{13}C NMR spectra.

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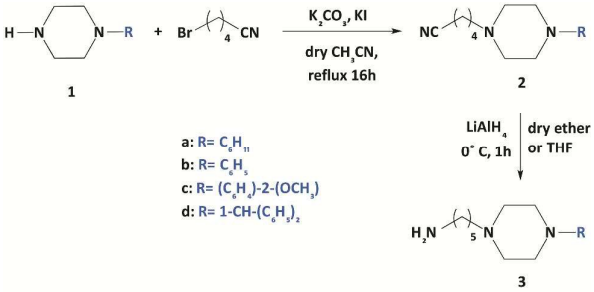
Amine derivatives

Subsequently, compounds **2** were reduced in the presence of $LiAlH_4$ using anhydrous ether or THF (Scheme 2). After work up the amine ligands **3a-d** were isolated as white, air-stable oily materials, which are soluble in polar solvents. In the case of **3c-d**, these were purified by column chromatography using neutral alumina as the stationary phase, while **3a** and **3b** were obtained in pure form without further purification.

Solution IR (CH_2Cl_2) spectra of **3** show the disappearance of the cyano band observed in the starting materials **2**, giving a tentative account of the reduction of this group. Furthermore, ^{1}H NMR ($CDCl_3$) spectra of **3** show the presence of a new triplet centered around 2.7 ppm, which integrates for two protons and can be attributed to the methylene group adjacent to the amino moiety. This confirms successful reduction of **2** to the ligands **3**.

Besides, for cyano and amino derivatives, the resonances assigned to the piperazine group are shifted to higher frequencies according to the acceptor character of the substituents on this fragment (**R**: Ph > Ph-OMe > $CH(Ph)_2$ > Cy). 2D NMR spectra (COSY, HMQC) were performed in order to correctly assign the protons present in the structures of cyano (**2**) and amino derivatives (**3**). Additionally, the mass spectra of the ligands **2** and **3** (ESI and EI) exhibit the presence of the molecular ion. It is important to mention that all chromatograms (HPLC) show the presence of a single peak, which confirms that all of them are analytically pure, just as shown by results from elemental analysis.



Scheme 2. Synthesis of new arylpiperazine cyano (**2a-d**) and amine (**3a-d**) ligands.

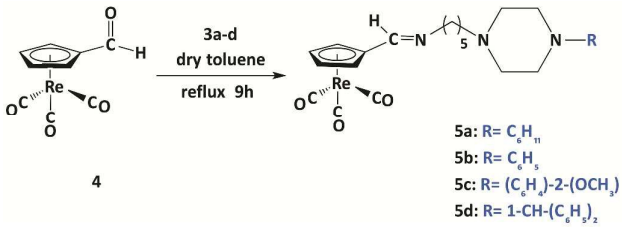
Organometallics compounds

The $[(\eta^5\text{-}C_5H_4CHO)Re(CO)_3]$ (**4**) complex was synthesized as reported in the literature[65] from the precursor $[(\eta^5\text{-}C_5H_5)Re(CO)_3]$[68]. The chromatogram (HPLC) showed the presence of a single peak at 22.68 minutes, indicating 98 % purity.

New organometallics cyrhetrenyl imines

Through a condensation reaction between the precursor complex **4** $[(\eta^5\text{-}C_5H_4CHO)Re(CO)_3]$ and the amine arylpiperazine ligands **3a-d**, it was possible to obtain the organometallic imines **5a-d** respectively as air-stable oily solids, soluble in polar solvents (Scheme 3). The reaction was monitored by ^{1}H NMR through observing the disappearance of the aldehyde proton at 9.6 ppm and the appearance of a single new resonance at a lower frequency around 7.8 ppm, which is assigned to the imine proton. Likewise, only one set of triplets is observed which is related to the presence of the five-membered ring in the structures. In addition, the signal of the methylene protons adjacent to the imine moiety in **5a-d** is shifted to higher frequency with respect to that observed for the precursors **3a-d**. However, as expected, the proton resonances of the substituted piperazines in **5** are invariant to those observed for the amine ligands **3**.

These new organometallic Schiff bases were observed in a single isomeric form, which could be assigned to the E-isomer to avoid steric hindrance. This is also in line with related cyrhetrene imine complexes having a C=N bond, which have been fully characterized by conventional spectroscopic techniques and X-ray diffraction[69]. In all cases, a typical fragmentation pattern was observed for the rhenium complexes in high-resolution mass spectra of imines **5a-d**. Elemental analyses (see Experimental Section) also are in agreement with the proposed composition. Solutions of imines **5a-d** (DMSO), were stable for two months. Also, Imines were studied in solution pH 7.0 (0.5% DMSO in an aqueous solution) and were stable to hydrolysis. After this time, no resonances were detected that could be attributed to either amino-piperazine and cyrhetrene carboxaldehyde in the ^{1} H NMR spectra.



Scheme 3. Synthesis of new organometallics imines (**5a-d**).

Biological evaluation on HT-29 and PT45

The cytotoxicity of our new derivatives was studied on different cell lines by the MTT assay.[60] As mentioned above, the protein kinase GSK-3β (glycogen synthase kinase 3) is involved in the Wnt signaling pathways. Thus, this isoform of GSK has been related to the cancer development. The results are summarized in Table 1, and IC_{50} curves are shown in the Supporting Information.

Table 1: IC_{50} values of cyano (**2a-d**), amine (**3a-d**) and imine derivatives (**5a-d**) in HT-29 and PT-45 cancer cell lines as determined by the MTT assay with 72 h incubation time (triplicates).

Compounds	IC_{50} (μM) HT-29	IC_{50} (μM) PT-45
2a-d	n. a.	n. a.
3a-c	n. a.	n. a.
3d	18.11 ± 0.05	22.23 ± 0.04

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4	n. a.	n. a.
5a	97.27 ± 0.06	n. a.
5b	72.78 ± 0.04	n. a.
5c	76.38 ± 0.05	90.16 ± 0.05
5d	30.48 ± 0.03	25.82 ± 0.03
Cisplatin	32.6 ± 0.7	2.2 ± 0.3

n. a.: Not active up to a concentration of 250 μM.

Based on the values in Table 1, a difference in the activity related to different organic functions was observed after the change of C≡N to NH_2 group, and also with increasing the number of the aromatic ring. Importantly, when the metal was introduced in the molecule an increase in cytotoxic activity was observed, in addition to an interesting selectivity toward HT-29. Is important to mention that in comparison with the standard cisplatin, our compounds **3d** and **5a-b** displayed higher activity and improved selectivity (respectively) on colon cancer cells. This result suggests that rhenium derivatives offer a strong and attractive alternative to be explored in the design of new anticancer compound.

Considering the potential participation of GSK-3β on tumor suppression and as a positive regulator of proliferation, we rationalize our experimental results considering the physicochemical characteristic of the binding cavity of GSK-3β as a biological target of our new derivatives.

***In-silico* evaluation**

The proposal of new ligands for GSK-3β raised in this study contemplates arylpiperazine derivatives substituted with aromatic and aliphatic substituents and their modification with organometallic fragments, which could involve different types of molecular interactions with amino acids in the active site. Considering the description of the GSK-3β binding cavity, putative ligands should possess functional groups that generate van der Waals and coulombic interactions, and have a volume of less than 600 $Å^3$.

Both, electronic and stereochemical characteristics were evaluated using computational tools. Area, volume and electrostatic surface (Figure 1) were determined for the organic ligands arylpiperazine (**3a-d**) and the organometallic imines (**5a-d**).

This study was performed considering the biological conditions of the system, which is reflected in the protonation of the amines (**3a'-d'**). The volume of the organic ligands varies between 280 and 390 $Å^3$, while for organometallic complexes this ranges from ~ 470 to 560 $Å^3$. Thus, it is possible that all proposed systems could interact with the binding site of the protein.

On the other hand, a description of the electrostatic potential of the amino derivatives **3'** shows that in all cases there is a segment of high electron density determined by the presence of aromatic rings present in the structures (**3b´-d´**) and a localized electron deficiency at the other end, where the terminal ammonium group is appreciated. On the other hand, the presence of the organometallic fragment in tricarbonyl imine complexes (**5a-d**) increases the polarization of the electrostatic potential in the molecule, *i.e.* the concentration of electrons is located mainly on the arylpiperazine moiety (in red, Figure 1B) and as consequence a deficit of electron density results in the cyrhetrenyl fragment.

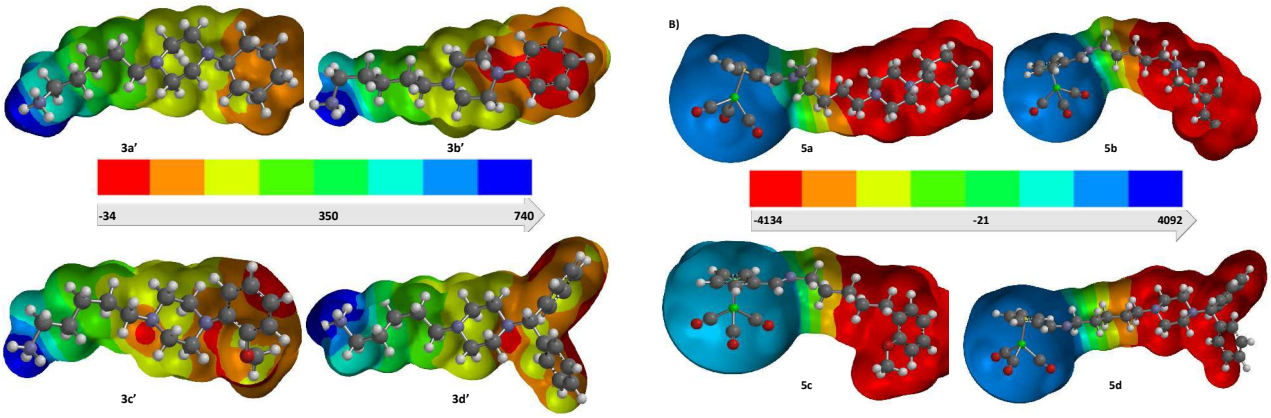

Figure 1. The electrostatic potential from ESP calculations obtained for **3a-d**, and **5a-d** in A) and B) respectively**.**

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However, unfortunately, the number of compounds with significant biological activity was not enough in this study to derive a quantitative relationship between electrostatic potential and cytotoxicity. Qualitatively, the compounds with higher dipolar moment (μ, negative charge density concentrated on the arylpiperazine segment) are more active on HT-29 and PT-45. A direct relation was observed for the organometallic compounds (**5a-5d**), which are displayed in Figure 2. Thus, is possible to assume that the electron-rich substituent in the nitrogen of piperazine is advantageous for the interactions with a biological target responsible of the cytotoxic effect.

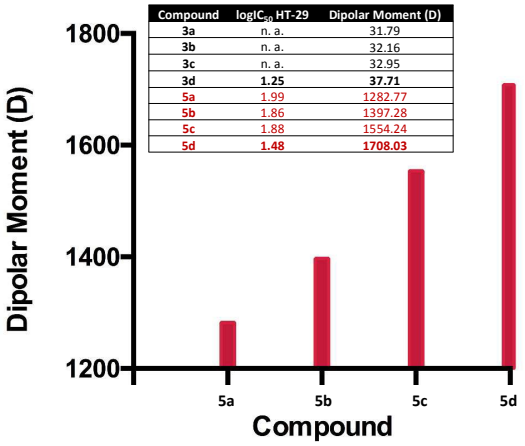

Figure 2. Dipolar moment of organometallic compounds (**5a** to **5d**). Inset shows specific values of dipolar moment and its relationship with experimental data.

Considering this electronic distribution, and that the union cavity has amino acids such as tyrosine, aspartate, glutamate, and arginine (among others), it is possible to indicate that the proposed compounds could generate coulombic type interactions with the amino acids present at the binding site of GSK-3β.

In order to describe specific interactions in the protein-ligand complexes, docking studies into the binding cavity were done. It is important to mention that specific parameters to describe the coordination of metal as rhenium-cyclopentadienyl (η^5) are not available into the force field. For that reason, the docking was carried out just with the organic compounds (**3a-d**). In all cases, the ligands were located in the same cavity and main interactions were conserved. The binding energies for the organic compounds were negative (from -4.41 to -6.98 kcal/mol; more detail in Electronic Supporting Information). The favorable interactions were observed to **3d,** which displayed the more negative binding energy (-6.98 kcal/mol). In Figure 3, a general representation of the complex **3d**-GSK-3β is shown.

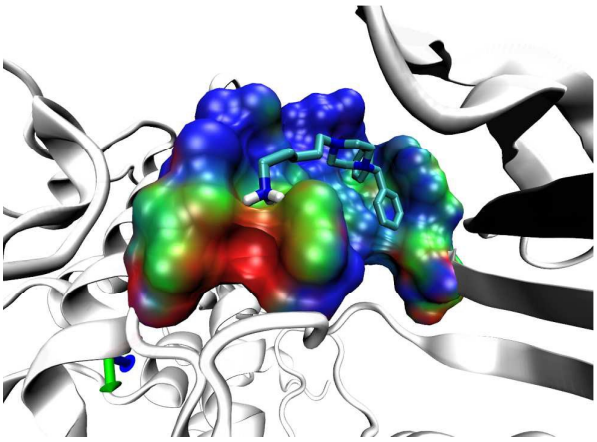

Figure 3. Compound **3d** complexed in GSK-3β and the electrostatic potential of the binding cavity is shown in surface (Blue: positive charged Green: non-charged surface Red: negative charged).

Our results for **3d** show that the protonated amino groups interact with a negatively charged segment of the cavity corresponding to the location of **Asp200**. Furthermore, the aromatic ring stacks with **Tyr134**, **Arg141** and **Asp133** (in the blue surface in Figure 2). Finally, our computational description supports the hypothesis that compounds with a high polarization of electrostatic potential could be ligands of GSK-3β.

Experimental

General Methods

All reactions were carried out using standard Schlenk technique under nitrogen atmosphere. All solvents were purified and dried by conventional methods and were distilled under nitrogen prior to use, except those used for thin layer and column chromatography, which were used without purification. $[(\eta^5\text{-}C_5H_5)Re(CO)_3]$[57], $[(\eta^5\text{-}C_5H_4CHO)Re(CO)_3]$[58] (**4**) and Tollens reagent were synthesized following literature procedures. Piperazine 1-(diphenylmethyl), 1-(2-methoxyphenyl), and 5-bromovaleronitrile were purchased from Aldrich and used without purification or treatment.

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Synthetic procedures and characterization

Synthesis of cyclohexyl piperazine $HNC_4H_8N(C_6H_{11})$ (1a)

1.0 g of piperazine (11.6 mmol) and 1.9 g of bromocyclohexane (11.6 mmol; d= 1.324 g/ml) in 30 mL of anhydrous CH_3CN were dissolved. 829.3 mg of K_2CO_3 (6.0 mmol) and 30.0 mg KI (0.2 mmol) were added and refluxed for 24 h. After this time, the reaction mixture was filtered and the resulting solution was evaporated to dryness. The reaction crude was purified by column chromatography, where from the fraction eluted with chloroform: methanol (1:1) it was possible to obtain a white solid attributed to compound 1a (yield 48%; 944.0 mg; 5.6 mmol). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.88 (t, 4H, J_{H-H}= 5.0 Hz, NC_4H_8N); 2.53 (t, 4H, $_{JH-H}$= 5.0 Hz, NC_4H_8N); 2.19 (m, 1H, C_6H_{11}); 1.91 (bs, 1H, C_6H_{11}); 1.85 (m, 2H, C_6H_{11}); 1.77 (m, 2H, C_6H_{11}); 1.61 (psd, 1H, NH); 1.20 (m, 4H, C_6H_{11}); 1.07 (m, 1H, C_6H_{11}) .^{13}C-NMR (400 MHz, $CDCl_3$) δ: 64.22 (s, CH); 50.44 (s, NC_4H_8N); 46.75 (s, NC_4H_8N); 28.96 (s, C_6H_{11}); 26.51 (s, C_6H_{11}); 26.10 (s, C_6H_{11}). ESI-MS (m/z) (CH_3OH): 168.92 [M^+]. Anal. Calc: C, 71.37%; H, 11.98%; N, 16.65%. Found: C, 70.71%; H, 11.95%; N, 16.69%.

Synthesis of phenyl piperazine HNC_4H_8N (C_6H_5) (1b)

500.0 mg of piperazine (5.8 mmol) and 800.0 mg of phenylboronic acid (6.6 mmol) were dissolved in 20 mL of anhydrous methanol in the presence of CuI as catalyst (44.0 mg, 0.2 mmol). The reaction mixture was bubbled with O_2 and refluxed for 4 hours. After this time, 10 ml of anhydrous methanol was added and the mixture was allowed to reflux at room temperature with constant stirring for 48 hours. Subsequently, the solution is evaporated to dryness. The reaction crude is washed with ethyl acetate to give a white solid which corresponds to the unreacted free piperazine. The solution is evaporated to dryness to give a pale yellow solid attributed to ligand **1b** (yield 30%; 278.0 mg; 1.7 mmol). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.27 (m, 2H, C_6H_5); 6.93 (d, 2H, J_{H-H}= 8.2 Hz, C_6H_5); 6.86 (t, 1H, J_{H-H}= 7.2 Hz, C_6H_5); 3.14 (m, 4H, NC_4H_8N); 3.03 (m, 4H, NC_4H_8N); 1.78 (s, 1H, NH). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 152.01 (s, C_6H_5); 129.18 (s, C_6H_5); 119.77 (s, C_6H_5); 116.14 (s, C_6H_5); 50.51 (s, NC_4H_8N); 46.31 (s, NC_4H_8N ESI-MS (m/z) (CH_3OH): 162.94 [M^+]. Anal. Calc: C, 74.03%; H, 8.70%; N: 17.27%. Found: C, 68.89%; H, 7.76%; N, 15.40%. HPLC (H_2O: CH_3CN): retention time: 3.78 minutes, 98% purity.

Synthesis of cyano derivatives: $_2HN(CH_2)_5$-NC_4H_8N-(C_6H_{11}) (2a); $_2HN(CH_2)_5$-NC_4H_8N-(C_6H_5) (2b); $_2HN(CH_2)_5$-NC_4H_8N-(C_6H_4)-2-(OCH_3) (2c); $_2HN(CH_2)_5$-NC_4H_8N-($CH(C_6H_5)_2$) (2d)

General experimental procedure

1.0 g of corresponding piperazine (**1a**: cyclohexyl piperazine (5.9 mmol); 1b: phenyl piperazine (6.2 mmol); 1c: 2-methoxyphenyl piperazine (5.2 mmol); **1d**: diphenylmethyl piperazine (4.0 mmol) and equimolar 5-bromovaleronitrilo (d=1.388 g/ml) were dissolved in 35 ml of anhydrous acetonitrile. 829.3 mg of K_2CO_3 (6.0 mmol) and 30.0 mg KI (0.2 mmol) were added and the solution was refluxed for 16-20 h. After this time, the reaction mixture is filtered to remove excess potassium salts, and the resulting solution is evaporated to dryness.

2a: crude was purified by column chromatography on neutral alumina, where from the fraction eluted with acetone it was possible to obtain a white oily solid corresponding to **2a** (yield 80%; 1.2 g, 4.7 mmol). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.53 (bs, 4H, NC_4H_8N); 2.40 (br s, 4H, NC_4H_8N); 2.31 (m, 4H, CH_2); 2.16 (m, 1H, C_6H_{11}); 1.82 (psd, 2H, CH_2); 1.73 (psd, 2H, CH_2); 1.60 (m, 5H, C_6H_{11}); 1.14 (m, 5H, C_6H_{11}). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 119.61 (s, CN); 63.44 (s, C_6H_{11}); 57.60 (s, CH_2); 53.63 (s, NC_4H_8N); 48.88 (s, NC_4H_8N); 29.03 (s, C_6H_{11}); 26.33 (s, CH_2); 25.89 (s, C_6H_{11}); 25.79 (s, C_6H_{11}); 23.59 (s, CH_2); 17.10 (s, CH_2). ESI-MS (m/z) (CH_3 OH): 249.98 [M^+]; 168.12 [M^+ - NC_4H_8N-1- (C_6H_{11})]. Anal. Calc: C, 72.24%; H, 10.91%; N, 16.85%. Found: C, 70.05%; H, 10.47%; N, 17.13%.

2b: crude was purified by column chromatography using neutral alumina as the stationary phase. From the fraction eluted with ethyl acetate, it was possible to obtain compound **2b** as an orange solid (yield 93%; 1.4 g, 5.8 mmol). ^{1}H-NMR (400 MHz, methanol-d_4) δ: 7.23 (t, 2H, J_{H-H} = 8.0 Hz, C_6H_5); 6.96 (d, 2H, J_{H-H} = 8.3 Hz, C_6H_5); 6.84 (m, 1H, C_6H_5); 3.18 (t, 4H, J_{H-H}= 4.9 Hz, NC_4H_8N); 2.63 (t, 4H, J_{H-H}= 4.9 Hz, NC_4H_8N); 2.46 (m, 4H, CH_2); 1.68 (m, 4H, CH_2). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.26 (t, 2H, J_{H-H}= 8.0 Hz, C_6H_5); 6.93 (d, 2H, J_{H-H}= 8.3 Hz, C_6H_5); 6.85 (m, 1H, C_6H_5); 3.20 (t, 4H, J_{H-H}= 4.9 Hz, NC_4H_8N); 2.60 (t, 4H, J_{H-H}= 4.9 Hz, NC_4H_8N); 2.42 (m, 4H, CH_2); 1.72 (m, 4H, CH_2) ^{13}C-NMR (400 MHz, methanol-d_4) δ: 152.70 (s, C_6H_5); 130.09 (s, C_6H_5); 121.18 (s, C_6H_5); 119.61 (s, CN); 58.55 (s, CH_2); 54.22 (s, NC_4H_8N); 50.22 (s, NC_4H_8N); 26.51 (s, CH_2); 24.59 (s, CH_2); 17.23 (s, CH_2). ESI-MS (m/z) (CH_3OH): 244.29 [M^++ H]. Anal. Calc: C, 74.03%; H, 8.70%; N: 17.27%. Found: C, 72.50%; H, 8.54%; N, 16.84%. HPLC (H_2O: CH_3 CN): retention time: 16.78 minutes, 99% purity.

2c: Ligand **2c** was purified by column chromatography and recovered in the fraction eluted with CH_2Cl_2 in 86% yield (1.3 g, 4.5 mmol). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.94 (m, 4H, C_6H_4); 3.87 (s, 3H, OCH_3); 3.09 (bs, 4H, NC_4H_8N); 2.65 (bs, 4H, NC_4H_8N); 2.42 (t, 4H, J_{H-H} = 6.3 Hz, CH_2); 1.71 (m, 4H, CH_2). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 152.47 (s, C_6H_4); 141.30 (s, C_6H_4); 123.07 (s, C_6H_4); 121.26 (s, C_6H_4); 119.88 (s, CN); 118.54 (s, C_6H_4); 111.42 (s, C_6H_4); 57.74 (s, CH_2); 55.48 (s, OCH_3); 53.54 (s, NC_4H_8N); 50.79 (s, NC_4H_8N); 25.73 (s, CH_2); 23.79 (s, CH_2); 17.24 (s, CH_2). ESI-MS (m/z) (CH_3OH): 295.92 [M^++ Na^+], 273.96 [M^+], 205.99 [M^+ - $(CH_2)_3$-CN], 190.18 [M+ - $(CH_2)_4$-CN]. EI (m/z): 273.1 [M^+]. Anal. Calc: C, 70.30%; H, 8.48%; N: 15.37%. Found: C, 69.73%; H, 8.31%; N, 15.46%. HPLC (H_2O: CH_3CN): retention time: 16.73 minutes.

2d: the crude was purified by column chromatography, where from the fraction eluted with ethyl acetate it was possible to obtain a white solid corresponding to compound **2d** (yield 88%, 1.20 g, 3.52 mmol). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.41 (d, 4H, J_{H-H}= 7.5 Hz, C_6H_5); 7.26 (t, 4H, J_{H-H}= 7.5 Hz, C_6H_5); 7.17 (t, 2H, J_{H-H}= 7.4 Hz, C_6H_5); 4.22 (s, 1H, CH); 2.45 (bs, 8H, NC_4H_8N); 2.36 (t, 4H, J_{H-H}= 6.7 Hz, CH_2); 1.65 (m, 4H, CH_2). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 142.90 (s, C_6H_5); 128.61 (s, C_6H_5); 128.10 (s, C_6H_5); 127.04 (s, C_6H_5); 119.77 (s, CN); 76.41 (s, CH); 57.49 (s, CH_2); 53.65 (s, NC_4H_8N); 52.02 (s, NC_4H_8N); 25.89 (s, CH_2); 23.68 (s, CH 2); 17.24 (s, CH_2). ESI-MS (m/z) (CH_3OH):

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337.28 [M^+]. EI (m/z): 333.1 [M^+]. Anal. Calc: C, 79.24%; H, 8.16%; N: 12.60%. Found: C, 78.33%; H, 8.03%; N, 12.52%. HPLC (H_2O: CH_3CN): retention time: 20.55 minutes.

Synthesis of amino derivatives: $_2HN(CH_2)_5$-NC_4H_8N-(C_6H_{11}) (3a); $_2HN(CH_2)_5$-NC_4H_8N-(C_6H_5) (3b); $_2HN(CH_2)_5$-NC_4H_8N-(C_6H_4)-2-(OCH_3) (3c); $_2HN(CH_2)_5$-NC_4H_8N-($CH(C_6H_5)_2$) (3d)

General experimental procedure

200.0 mg of ligand **2** (**2a**: 0.80 mmol, **2b**: 0.82 mmol, **2c**: 0.73 mmol, **2d**: 0.60 mmol) were dissolved in a Schlenk in 10 mL of dry ether (anhydrous THF was used for ligands **2a** and **2b**) and 1.5 ml (4.4 mmol) of a solution of $LiAlH_4$ (2M in THF) was added dropwise. The solutions were allowed to react with constant stirring for 1 h at 0 ° C under a nitrogen atmosphere. At the end of this time, 0.5 ml of methanol and an aqueous solution of KOH (10 ml, 10%) were added to the reaction mixture. The aqueous phase was extracted with CH_2Cl_2 (3 x 10 mL). The latter were dried over Na_2SO_4. The organic phases were concentrated to dryness to give the **3** (**a-d**) ligands.

3a: 192.6 mg of white solid (yield 95%, 0.76 mmol) was obtained. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 2.66 (t, 2H, J_{H-H} = 7.0 Hz, CH_2); 2.58 (bs, 4H, NC_4H_8N); 2.45 (bs, 4H, NC_4H_8N); 2.30 (t, 2H, J_{H-H} = 7.8 Hz, CH_2); 2.19 (m, 1H, C_6H_{11}); 1.86 (psd, 2H, C_6H_{11}); 1.77 (psd, 2H, CH_2); 1.60 (m, 1H, C_6H_{11}); 1.46 (m, 4H, CH_2); 1.19 (m, 8H, C_6H_{11}). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 63.61 (s, C_6H_{11}); 58.96 (s, CH_2); 53.91 (s, NC_4H_8N); 49.06 (s, NC_4H_8N); 42.22 (s, CH_2); 33.82 (s, CH_2); 29.10 (s, C_6H_{11}); 26.91 (s, CH_2); 26.51 (s, CH_2); 26.02 (s, C_6H_{11}); 25.06 (s, C_6H_{11}). ESI-MS (m/z) (CH_3OH): 254.07 [M^+]. EI (m/z): 254.3 [M^+]. Anal. Calc: C, 71.09%; H, 12.33%; N, 16.58%. Found: C, 66.10%; H, 11.13%; N, 14.18%.

3b: 195.4 mg of white solid (yield 96%, 0.79 mmol) was obtained. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.26 (m, 2H, C_6H_5); 6.93 (d, 2H, J_{H-H} = 8.2 Hz, C_6H_5); 6.85 (t, 1H, J_{H-H} = 7.3 Hz, C_6H_5); 3.21 (t, 4H, J_{H-H} = 4.9 Hz, NC_4H_8N); 2.70 (t, 2H, J_{H-H} = 6.9 Hz, CH_2); 2.60 (t, 4H, JH-H = 5.0 Hz, NC_4H_8N); 2.39 (t, 2H, J_{H-H} = 7.5 Hz, CH_2); 1.45 (m, 8H, CH_2). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 151.59 (s, C_6H_5); 129.34 (s, C_6H_5); 119.81 (s, C_6H_5); 59.08 (s, CH_2); 53.58 (s, NC_4H_8N); 49.08 (s, NC_4H_8N); 42.32 (s, CH_2); 34.05 (s, CH $_2$); 27.09 (s, CH_2); 25.05 (s, CH_2). ESI-MS (m/z) (CH_3OH): 248.00 [M^+]. Anal. Calc: C, 72.83%; H, 10.19%; N: 16.99%. Found: C, 68.33%; H, 9.31%; N, 14.67%.

3c: the reaction crude was purified through column chromatography on neutral alumina, where from the fraction eluted with acetonitrile it was possible to recover the precursor ligand **2c** that did not react. Finally of the fraction eluted with methanol, ligand **3c** was obtained as a white solid, stable in air and soluble in polar solvents (yield 78%, 158.3 mg, 0.57 mmol). ^{1}H-NMR ($CDCl_3$) δ: 6.92 (m, 4H, C_6H_4); 3.85 (s, 3H, OCH_3); 3.09 (bs, 4H, NC_4H_8N); 2.69 (t, 2H, J_{H-H} = 6.8 Hz, CH_2); 2.63 (bs, 4H, NC_4H_8N); 2.40 (t, 2H, J_{H-H} = 7.4 Hz, CH 2); 1.54 (m, 2H, CH_2); 1.46 (m, 2H, CH_2); 1.36 (m, 2H, CH_2). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 152.42 (s, C_6H_4); 141.65 (s, C_6H_4); 122.95 (s, C_6H_4); 121.10 (s, C_6H_4); 118.30 (s, C_6H_4); 111.38 (s, C_6H_4); 58.96 (s, CH_2); 55.47 (s, OCH_3); 53.66 (s, NC_4H_8N); 50.78 (s, NC_4H_8N); 42.29 (s, CH_2); 33.96 (s, CH_2); 26.92 (s, CH_2); 25.02 (s, CH_2). ESI-MS (m/z) (CH_3OH): 278.02 [M^++ H], 261.30 [M^+ - NH 2], EI (m/z): 278.2 [M^+]. Anal. Calc: C, 69.27%; H, 9.81%; N: 15.15%. Found: C, 64.68%; H, 9.15%; N, 13.78%.

3d: 182.0 mg of white solid (yield 90%, 0.54 mmol) was obtained. 1H-NMR (CDCl3) δ: 7.41 (d, 4H, J_{H-H} = 7.5 Hz, C_6H_5); 7.26 (t, 4H, J_{H-H} = 7.5 Hz, C_6H_5); 7.17 (t, 2H, J_{H-H}= 7.3 Hz, C_6H_5); 4.22 (s, 1H, CH); 2.69 (t, 2H, J_{H-H} = 7.0 Hz, CH_2); 2.45 (bs, 8H, NC_4H_8N); 2.33 (t, 2H, J_{H-H} = 7.7 Hz, CH_2); 1.66 (broad s, 2H, NH_2); 1.47 (m, 4H, CH_2); 1.32 (m, 2H, CH_2). ^{13}C-NMR (400 MHz, $CDCl_3$) δ: 142.98 (s, C_6H_5); 128.54 (s, C_6H_5); 128.13 (s, C_6H_5); 126.99 (s, C_6H_5); 76.43 (s, CH); 58.81 (s, CH_2); 53.69 (s, NC_4H_8N); 52.06 (s, NC_4H_8N); 42.20 (s, CH_2); 33.65 (s, CH_2); 26.86 (s, CH_2); 25.03 (s, CH_2). ESI-MS (m / z) (CH_3OH): 338.12 [M^+]. EI (m/z): 338.2 [M^+]. Anal. Calc: C, 78.29%; H, 9.26%; N: 12.45%. Found: C, 78.21%; H, 9.31%; N, 12.12%.

Synthesis of [(η^5-C_5H_4CHO)$Re(CO)_3$] (4)

The aldehyde cyrhetrene was synthesized using a method described in literature[57]. The **4** compound was obtained as a yellow solid (CH_2Cl_2, (υCO) cm^{-1}]: 2033 (s), 1940 (s), S), 1694 (w) .^{1}H-NMR ($CDCl_3$) δ: 9.61 (s, 1H, CHO); 6.03 (t, 2H, J_{H-H} = 2.3 Hz, C_5H_4); 5.48 (t, 2H, J_{H-H} = 2.3 Hz, C_5H_4). HPLC (CH_3CN: H_2O) retention time: 22.68 minutes. Purity: 98%.

Synthesis of organometallic imines [(η^5-C_5H_4CHNH-$(CH_2)_5$-NC_4H_8N-1-(C_6H_{11}))$Re(CO)_3$] (5a);[(η^5-$C_5H_4CHNH(CH_2)_5$-NC_4H_8N-1(C_6H_5))$Re(CO)_3$] (5b); [(η^5-C_5H_4CHNH-$(CH_2)_5$-NC_4H_8N-1-(C_6H_4)-2-(OCH_3))$Re(CO)_3$] (5c); [(η^5-C_5H_4CHNH-$(CH_2)_5$-NC_4H_8N-1-($CH(C_6H_5)_2$)$Re(CO)_3$] (5d).

General experimental procedure:

100.0 mg of aldehyde precursor complex **4** (0.3 mmol) and 0.3 mmol of amine ligand **3a-d** (**3a**: 87.1 mg; **3b**: 85.0 mg; **3c**: 95,4 mg; **3d**: 116,0 mg) were dissolved in 20 ml of anhydrous toluene. Molecular sieve (4Å) was added to the solution, which was refluxed for 9 h under nitrogen atmosphere with constant stirring. After this time, the reaction mixture was filtered and the solvent evaporated under reduced pressure to give a yellow oily solid corresponding to the organometallic imines **5a-d**.

5a: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.87 (s, 1H, HC = N); 5.82 (t, 2H, J_{H-H}= 2.2 Hz, C_5H_4); 5.35 (t, 2H, J_{H-H}= 2.2 Hz, C_5H_4); 3.47 (t, 2H, J_{H-H} = 6.4 Hz, CH_2); 2.60 (bs, 4H, NC_4H_8N); 2.47 (bs, 4H, NC_4H_8N); 2.31 (t, 2H, JH-H = 7.8 Hz, CH_2); 1.82 (m, 1H, C_6H_{11}); 1.58 (m, 5H, C_6H_{11}); 1.24 (m, 11H, C_6H_{11} and CH_2). ESI-MS (m/z) (CH_3OH): 599.73 [M + H $^+$]. Anal. Calc: C, 48.14%; H, 5.72%; N, 7.02%. Found: C, 48.14%; H, 5.72%; N, 6.92%.

5b: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.88 (s, 1H, HC = N); 7.27 (m, 1H, C_6H_5); 6.88 (m, 4H, C_6H_5); 5.82 (t, 2H, J_{H-H} = 2.2 Hz, C_5H_4); 5.36 (t, 2H, J_{H-H} = 2.2 Hz, C_5H_4); 3.49 (t, 2H, J_{H-H} = 6.5 Hz, CH_2); 3.21 (pst, 4H, J_{H-H} = 4.8 Hz, NC_4H_8N); 2.60 (pst, 4H, J_{H-H} = 5.0 Hz, NC_4H_8N); 2.40 (t, 2H,

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J_{H-H} = 6.3 Hz, CH_2); 1.60 (m, 4H, -CH_2); 1.35 (m, 2H, -CH_2) ESI-MS (m/z) (CH_3OH): 593.98 [M + H^+]. Anal. Calc: C, 48.63%; H, 4.76%; N, 7.09%. Found: C, 48.31%; H, 4.80%; N, 7.05%.

5c: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.88 (s, 1H, HC = N); 6.91 (m, 4H, C_6H_4); 5.82 (t, 2H, JH-H = 2.2 Hz, C_5H_4); 5.36 (t, 2H, J_{H-H} = 2.2 Hz, C_5H_4); 3.86 (s, 3H, OCH_3); 3.50 (t, 2H, J_{H-H} = 6.6 Hz, CH_2); 3.11 (t, 4H, J_{H-H} = 4.9 Hz, NC_4H_8N); 2.64 (t, 4H, J_{H-H} = 4.27 Hz, NC_4H_8N); 2.40 (pst, 2H, CH_2); 1.60 (m, 6H, CH_2). ESI-MS (m/z) (CH_3OH): 623.82 [M + H^+]. Anal. Calc: C, 48.22%; H, 4.86%; N, 6.75%. Found: C, 48.46%; H, 4.98%; N, 6.57%.

5d: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.86 (s, 1H, HC = N); 7.41 (m, 4H, C_6H_5); 7.26 (m, 4H, C_6H_5); 7.17 (m, 2H, C_6H_5); 5.80 (t, 2H, J_{H-H} = 2.2 Hz, C_5H_4); 5.33 (t, 2H, J_{H-H} = 2.2 Hz, C_5H_4); 4.22 (s, 1H, CH); 3.47 (t, 2H, J_{H-H} = 6.6 Hz, CH_2); 2.46 (t, 8H, NC_4H_8N); 2.35 (t, 2H, J_{H-H} = 7.4 Hz, CH_2); 1.56 (m, 6H, CH_2). ESI-MS (m/z) (CH_3OH): 683.05 [M + H^+]. Anal. Calc: C, 54.53%; H, 5.02%; N, 6.15%. Found: C, 52.85%; H, 5.09%; N, 5.92%.

Cell Culture and Cytotoxicity Assays

For the growth of the cells was used Dulbecco's Modified Eagle's Medium (DMEM). It contains 10% FCS, 1% penicillin and streptomycin. HT-29 and PT-45 cells were detached from the wells with trypsin and EDTA, harvested by centrifugation and resuspended in cell culture medium.

The assays were carried out on 96 well plates with 6000 cells per well for both cell lines. After 24 h of incubation at 37 °C and 10% CO_2, the cells were treated with the compounds (**2a-d**, **3a-d**, **4** and **5a-d)** with DMSO concentrations of 0.5%, and a final volumen of 200 µL per well. For this, we used standar solution of each compound of 20 mM. For a negative control, one series of cells was left untreated. The cells were incubated for 48 h followed by adding 50 µL MTT (2.5 mg·mL^{-1}). After an incubation time of 2 h, the medium was removed and 200 µL DMSO were added. The formazan crystals were dissolved and the absorption was measured at 550 nm, using a reference wavelength of 620 nm. Each test was repeated in triplicates in at least three independent experiments for each cell line.[70]

In silico studies

Optimization Geometry and Electrostatic Potential for 3a-d and 5a-d

The compounds were calculated using Gaussian03[71] and the partial charges were corrected using ESP methodology. For organic ligands Hartree-Fock 6-31G* was used and for organometallic compounds Semiempiric PM3 was employed. The electrostatic potential surface was displayed in GaussView.

Docking Studies

In order to obtain information regarding the principal Protein-Ligand interactions the molecular docking of 3a-d with GSK-3β was done using AutoDock 4.0[72] suite. In general, the grid maps were calculated using the autogrid4 option and were located on the center of the receptor cavity. The volumes for the grid maps were 85x 85 x 85 points (a grid-point spacing of 0.375 Å). The autotors option was used to define the rotating bonds in the ligand. In the Lamarckian genetic algorithm (LGA) dockings, an initial population of 1500 random individuals with a population size of 100 individuals, a maximum number of 2.5 x 106 energy evaluations, a maximum number of 27,000 generations, a mutation rate of 0.02 and a cross-over rate of 0.80 were employed. The docked compound complexes were built using the lowest docked-energy binding positions.

Conclusions

Four different asymmetric alkyl and aryl cyano-piperazine derivatives have been synthesized and full characterized. The reduction of the cyano group with $LiAlH_4$ afforded amino-piperazines **3**, in quantitative yield. Subsequently, through a condensation reaction between cyrhetrenyl aldehyde (**4**) and the amino derivatives (**3a-d**), new organometallic piperazine-derived imines of rhenium (**5a-d**) were obtained in analytically pure form. All compounds were evaluated in two cancer cell lines, namely HT-29 and PT-45. With the exception of the amino-piperazine **3d**, only the organometallic compounds showed appreciable cytotoxic activity. Again, the **3d**-derived complex **5d** showed best activity in the range of 30 µM against both cell lines. In terms of structure-actvity relationship (SAR), our results indicate that by increasing the aromaticity in the organic ligands (**3d**), and incorporating an organometallic fragment into the structure of the compounds (**5a-d**), significant biological activity can be achieved on both cell lines. In addition, the general physicochemical characteristics of our compounds (**3a-d** and **5a-d**) and the binding site of the kinase protein could be related indirectly to the biological function. A computational study showed that **3d** interacts with amino acids like as **Asp200**, **Tyr134**, **Arg141**, **Asp133** in the active site of GSK-3β. This may present a hint for the highest IC_{50} value of this compound, and may provide a clue towards further optimization of this compound class as organometallic GSK-3β inhibitors.

Conflicts of interest

There are no conflicts to declare.

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Amino-piperazines and organometallic imines were synthetized and evaluated in HT-29 and PT-45 cancer cell lines. Computational studies were developed.