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VASORELAXATION EFFECT PROMOTED BY THE CALCIUM CHANNEL BLOCKER VERAPAMIL COORDINATED TO RUTHENIUM (II): CHEMICAL AND BIOLOGICAL PROPERTIES

Olivia Azevedo Nascimento [1], Elisangela Fabiana Boffo [1], Bruno Rodrigues Silva [2], Juliana Cristina Biazzotto [2], Lusiane Maria Bendhack [2], Roberto Santana da Silva[2] and Renata Galvão de Lima [*1, 3]

Departamento de Química [1], Universidade Federal da Bahia, Avenida Barão de Geremoabo number 147 , Ondina, Salvador Post Code 40170-115, BA, Brazil
Departamento de Física e Química [2], Faculdade de Ciências Farmacêuticas de Ribeirão Preto-Universidade de São Paulo, Avenida do Café s/n, Monte Alegre, Ribeirão Preto Post Code 14040-903, SP, Brazil
Faculdade de Ciências Integradas do Pontal-Universidade Federal de Uberlândia [3], Rua 20 number 1600, Tupã, Ituiutaba Post Code 38304-402, MG, Brazil

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Correspondence to Author:
Renata Galvão de Lima

Professor Adjunto in Chemistry Area, Universidade Federal de Uberlândia (UFU), Rua 20 number 1600, Tupã, Ituiutaba City, Minas Gerais State, Brazil.

E-mail: renatagalvao@pontal.ufu.br

ABSTRACT: This paper presents the synthesis and physical-chemical characterization of a new terpyridine (tpy) ruthenium complex coordinated to the L-type calcium blocker channels verapamil (vera) obtained from commercial pill. Elemental analysis (%C N H), infrared (IR) and ultraviolet-visible (UV-vis.) spectroscopy, mass spectrometry (MS), and ^{13}CNMR (nuclear magnetic resonance), and HPLC (high performance liquid chromatography) confirmed the pure and structure of the ligand verapamil and of the ruthenium complex structurally designed as $[Ru^{II}Cl_2(tpy)(vera)]$ Cyclic voltammetry aided investigation of the electrochemical behavior of [RuIICl2(tpy)(vera)] based in the metal ion redox potential. Verapamil ligand and [RuIICl2(tpy)(vera)] induced vascular relaxation in endothelium-intact and endothelium-denuded aortic rings. Verapamil drug and [RuIICl2(tpy)(vera)] complex coupled to verapamil elicited similar pharmacological results. The calcium ion chelating effects (Kd) promoted for verapamil ligand and [RuIICl2(tpy)(vera)] complex were found based in the fluorescence technic. The smooth muscle cell images studied to [RuIICl2(tpy)(vera)] complex acting as a luminescent agent important as biological probe.

INTRODUCTION: Voltage-sensitive calcium channel (L-type channels) blockers such as verapamil have found application in vasodilating therapy, being a great asset to treat hypertension and other cardiovascular diseases whose symptoms include abnormal arterial tone. Calcium entry through L-type channels in vascular smooth muscle cells (VSMC) contributes to vasoconstriction [1]. This contraction depends on increasing cytosolic calcium concentrations and subsequent activation of myosin-light chain kinase (MLCK) [2]. Clinicians have prescribed calcium-channel blockers to treat hypertension and angina and to manage cardiac arrhythmias [3]. Among these blockers is Verapamil (**Fig.1**), a drug belonging to the class of phenylalkylamines, which has been used in the therapy of some chronic conditions. This drug operates by selectively inhibiting Ca^{2+} influx through L-type calcium channels [4].

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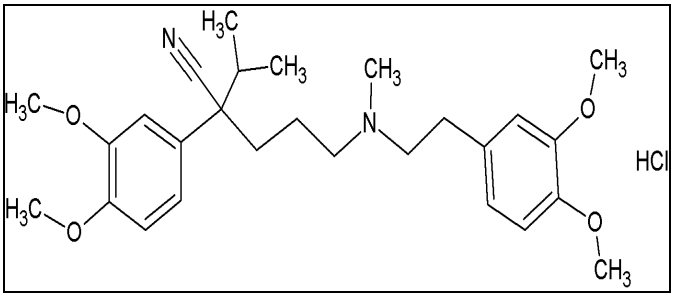


FIG. 1: CHEMICAL STRUCTURE OF VERAPAMIL.

Previous studies, based in molecular models, have suggested that, for verapamil to act, its relatively compact structure has to accommodate in the presence of calcium ion. Non-bonded interactions between the two dimethoxy aryl rings located on opposite ends of verapamil stabilize this drug in what is believed to be the most stable conformation of its isolated form [4-7]. Such conformational change may induce a different biological behavior and could be useful to describe the mechanism of action of this drug.

Although literature works have described that calcium and verapamil interact in aqueous medium [8], it is possible to observe calcium release in the presence of other metal ions. Therefore, describing the biological mechanism through which verapamil operates is complicated: several different reactions may occur between this drug and other metal centers. The use of a transition metal ion could help to overcome this difficulty and allow for better interpretation of verapamil conformational change during the ion calcium interaction, contributing with knowledge in the area of bioinorganic chemistry [9-12].

Therefore, the present study aimed to evaluate how Ca^{2+} interacts with the $[Ru^{II}Cl_2(tpy)(vera)]$ complex (where vera is verapamil, and tpy is 2,2′:6′,2″-terpyridine) (**Fig.2**). The highly stable interaction between ruthenium (II) and unsaturated ligands (terpyridine ligand) motivated this investigation[13-16] —this interaction restricts the spatial structure of verapamil when its coordinated to the metal ion center. This could be an important step toward a better description of the biological mechanism through which phenylalkylamine drugs function.

To evaluate the potential effects of $[Ru^{II}Cl_2(tpy)(vera)]$, we studied the rat aorta relaxation induced by the new complex and measured the cytosolic calcium concentration in VSMC as imaged by fluorescence microscopy.

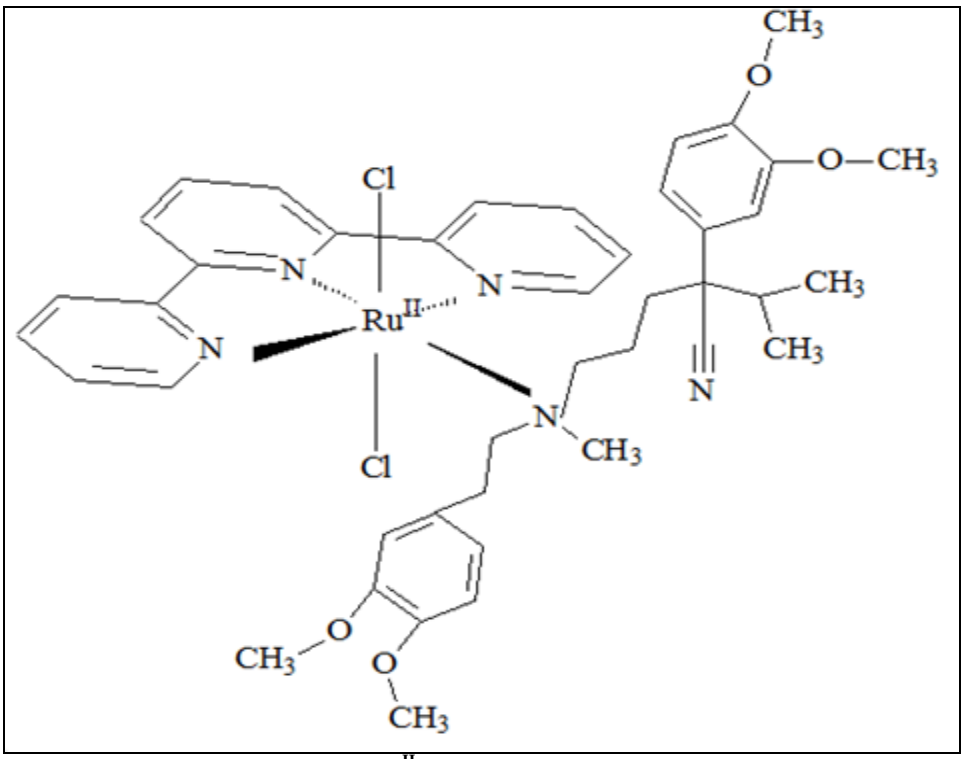


FIG. 2: CHEMICAL STRUCTURE FOR $[Ru^{II}Cl_2(tpy)(vera)]$ COMPLEX PROPOSED IN THIS WORK.

MATERIALS AND METHODS:

Apparatus: UV-vis. spectra were recorded on a Hitachi U-3501 spectrophotometer. Fluorescence spectra were acquired on Fluorolog-3 (JobinYvon-SPEX, Edison, NJ USA) spectrofluorometer equipped with an R928P Hamamatsu photomultiplier and a 450-W ozone-free Xe lamp, respectively. Infrared (IR) spectra were registered on a protegé 460 series FT-IR spectrometer, using solid samples pressed in KBr pellets. Cyclic voltammetry was conducted on an AUTOLAB®

model Drop Sense potentiostat/ galvanostat, employing a conventional three-electrode cell with a platinum disk working electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode. The chromatograms were obtained on a Shimadzu system using UV–vis. detection, and SPD-M20A detector, an LC-20AT pump with CBM-10A interface, a DGU-20A3 degasser, and Chemstation software; a CLC-ODS column (5 μm, 250 mm, 6 mm i.d.; Shimadzu) was employed. The samples were dissolved in the appropriate mobile phase; the injection volume was 20 μL. Mass spectrometry was accomplished on an ULTROTOFQ-ESI-TOF Mass Spectrometer (Bruker Daltonics, Billerica, USA) operating in the positive mode.

The sample was infused at a flow rate of 100 μL h^{-1}, using methanol/water (20:80, v/v) as solvent. The MS calibration was carried out with the aid of a Na–TFA (10 mg mL^{-1}) solution. All the NMR experiments were recorded at 27 °C, on a Varian Inova 500 spectrometer 11.7 Tesla (^{13}C at 125 MHz) equipped with a 5-mm direct detection probe from Cambridge Isotope Laboratories Inc. (USA), $CDCl_3$ (99.9%) was used as solvent. In vitro cell images were acquired under an Eclipse Ti fluorescent microscopy (Nikon). X-ray powder diffraction (XRD) experiment, a Shimadzu, model XRD-6000 diffractometer with cobalt tube (CoKα), operating voltage at 40.0 kV and 30.0 mA current was employed.

Dissociation constants (K_d) FLUO 3AM-Ca^{2+} (27 nmol L^{-1}) in the presence of verapamil (80 μmol L^{-1}) and of the [$Ru^{II}Cl_2$(tpy)(vera)] complex (80 μmol L^{-1}) were obtained by a linear regression fit of the fluorescence intensity $(I_0-I)/I_0$ vs the concentration of the added calcium ion ([Ca^{2+})], according to the following equation (1):

$$\frac{(I_0 - I)}{I_0} = Ca^{2+}.\frac{1}{K_d} \qquad (1)$$

$$\text{FLUO 3AM-}Ca^{2+} \rightarrow \text{FLUO 3AM} + Ca^{2+}$$

where I_0 and I_0-I are the emission intensity of FLUO 3AM at a selected wavelength, in the absence and in the presence of the compounds, respectively.

Chemicals and Reagents:

$RuCl_3.3H_2O$; 2,2′:6′,2”-terpyridine; and FLUO 3AM were purchased as high purity reagents from Sigma Aldrich Chemicals (Brazil) and were used as supplied. All the preparations and measurements were carried out under argon atmosphere, protected from light.

Methanol, ethanol, chloroform and dichloromethane were stored over molecular sieves. Other chemicals were of reagent grade and were used without further purification. Column chromatography was performed with basic aluminum oxide (Sigma Aldrich-Brazil).

Verapamil extraction methods:

Verapamil was obtained from a local market formulation containing 240 mg of verapamil hydrochloride as coated tablets from Sandoz® (Santana Pharmacy, Salvador-BA). First, the pill was ground in a mortar, and the resulting powder was sonicated in 10 mL of CHCl3, for 15 minute.After that, the suspension was centrifuged at 3000 rpm for 20 minute, to separate the liquid from the solid. The yellow chloroform extract was dried under vacuum, to give a solid.

Final mass = 230 mg. Mass analysis: Calcd. for [$C_{27}H_{38}N_2O_4.1.3H_2O$] (MM. 478.02 g/mol): C, 67.78; H, 8.49; N, 5.85. Found: C, 67.28; H, 7.44; N, 6.05. ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ: 20.7 (CH_2), 34.9 (CH_2), 38.1 (CH_2), 40.2 (N-CH_3), 53.3 (N-CH_2), 55.4 (N-CH_2), 55.91 (O-CH_3), 55.96 (O-CH_3), 56.0 (O-CH_3), 56.3 (O-CH_3), 109.4 (CH), 111.2 (CH), 111.6 (CH), 112.0 (CH), 118.8 (CH), 120.2 (CH), 121.2 (CN), 129.7 (C), 148.3 (C-O), 148.7 (C-O), 149.4 (C-O), 149.4 (C-O). Its show X-ray powder diffractograms analyses characteristic of verapamil. HCl single enantiomer [17-18] demonstrated by high-intensity diffraction peaks detected at 2θ = 14.55°; 18.10°; 19.00° (**Fig. 3**).

The extracted sample was characterized by ^{13}C NMR spectroscopy (**Fig. 4**), and data were compared with published results[19]. FTIR: ν(C-O-C) 2949 cm^{-1} (methoxy groups), ν(CN) 2234 cm^{-1} (saturated alkyl nitrile), ν(C=N) 1608 1591 1518 cm^{-1} (benzene ring) [18].

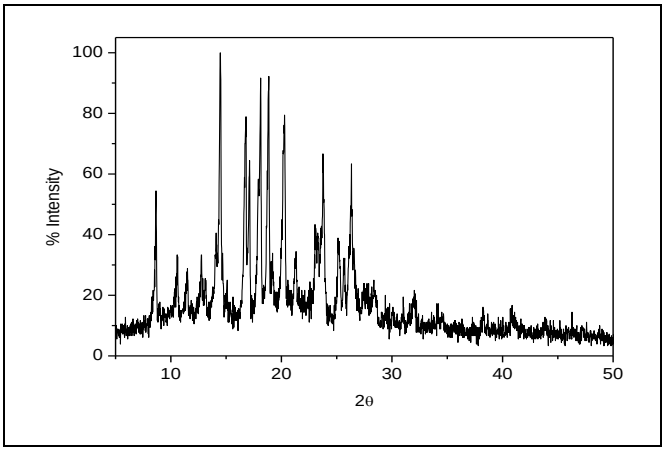


FIG. 3: XRD RESULTS OBTAINED VERAPAMIL HYDROCHLORIDE EXTRACTED SAMPLE.

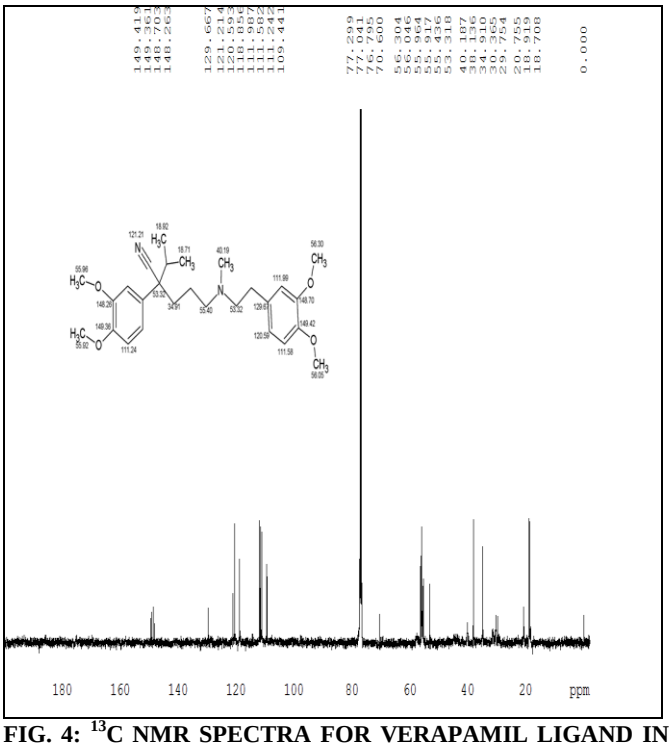


FIG. 4: ^{13}C NMR SPECTRA FOR VERAPAMIL LIGAND IN $CDCl_3$. IN SIDE: VERAPAMIL MOLECULAR STRUCTURE WITH CARBON NUMBERING SCHEME.

Synthesis of the ruthenium complexes:

$[Ru^{III}(tpy)Cl_3]$: The $[Ru^{III}(tpy)Cl_3]$ precursor complex was synthesized following a previously reported method[13].

$[Ru^{II}Cl_2(tpy)(vera)]$: The $[Ru^{II}Cl_2(tpy)(vera)]$ complex was synthesized using a method similar to that described by Suen and others[14,16] with some modifications. 247 mg (0.53 mmol) of the $[Ru^{III}(tpy)Cl_3]$ complex was added to 30 mL of ethanol/water (25:75 % v/v), followed by addition of 500 µL of triethylamine and 20 mg of LiCl. The mixture was heated at reflux for 15 minute, when the color changed from brown to purple. Next, 240 mg (0.53 mmol) of the extracted verapamil dissolved in ethanol (10 mL) was added to the purple solution. After 4 h under reflux at 80 ^{0}C, the purple mixture was cooled, and the volume was reduced to a minimum by rotary evaporation. The crude products were separated by classic chromatography on alumina oxide. The fraction eluted with methanol/dichloromethane (10:90% v/v) contained the target $[Ru^{II}Cl_2(tpy)(vera)]$ compound, isolated as the third fraction. Yield: 77 mg (32%). Mass analysis =. Calcd. for $[RuCl_2(C_{42}H_{49}N_5O_4)]$ (MM. 859.84 g/mol): C, 58.66; H, 5.74; N, 8.14. Found: C, 59.00; H, 5.64; N, 8.00.

Vascular Reactivity Studies Vessel preparation:

Male Wistar rats (400–450 g) were killed by decapitation in accordance with the Ethics Committee for Animal Research, Ribeirão Preto Campus, University of São Paulo, Brazil.

The thoracic aorta was quickly removed, dissected free, and cut into 4-mm-long rings. Next, the relaxation induced by these compounds in endothelium-intact (E^+) and endothelium-denuded (E^-) arteries was investigated. In the latter case, the endothelium was mechanically removed. The aortic rings were placed between two stainless-steel stirrups and connected to an isometric force transducer (Letica Scientific Instruments), to measure tension in the vessels. The rings were placed in a 10 mL organ chamber containing Krebs solution with the following composition (mol L^{-1} x 10^3): NaCl 130, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 14.9, glucose 5.5, and $CaCl_2$ 1.6. The solution was maintained at pH 7.4 and 37 ^{0}C, gassed with 95% O_2 and 5% CO_2.

The rings were initially stretched to a basal tension of 1.5 g (previously determined by length–tension relationship experiments), before allowing them to equilibrate for 60 minute in the bath, which was changed every 15–20 minute. Endothelial integrity was qualitatively assessed on the basis of the degree of relaxation elicited by acetylcholine (ACh, 1 x 10^{-6} mol L^{-1}) in the presence of contractile tone induced by phenylephrine (1 x 10^{-7} mol L^{-1})[20]. The efficacy (E_{max}) and potency (pD_2) of $[Ru^{II}Cl_2(tpy)(vera)]$ and verapamil in inducing vascular relaxation was examined.

Culture Cell:
VSMC (A7R5) was obtained from the Rio de Janeiro Cell Bank (BCRJ/UFRJ). Cells were seeded in a Petri dish with a 1 cm diameter laminated hole containing DMEM, 10% fetal bovine serum, and 5% penicillin. The cells were then cultured in a humidified incubator at 37 ^{o}C for 24 h, under 5% CO_2.

Fluorescence Microscopy:
Fluorescent cell imaging helped to confirm the results observed for FLUO 3AM using the fluorescence technique. To assess the transient cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), VSMC (A7R5) was incubated with Fluo 3AM (10 μmol L^{-1}) for 30 minute, at room temperature. Excess dye was removed by washing with phosphate-buffered saline solution (PBS); 30 minute were allowed for intracellular desterification of Fluo 3AM to Fluo 3. The cells were imaged in PBS buffer pH 7.4, followed by assessment under a fluorescence microscope. Fluo 3AM fluorescence was excited with the 488-nm line of a high-pressure mercury lamp using a filter for wavelength selection; the emitted fluorescence was measured at 510 nm. After acquisition of the cell/Fluo 3AM image, a DMSO solution (0.2 mmol L^{-1}) of the $[Ru^{II}Cl_2(tpy)(vera)]$ complex or verapamil was added to the different cell cultures, which were analyzed under visible and ultraviolet excitation.

RESULTS AND DISCUSSION: Verapamil hydrochloride is a chiral drug that is commercially available as a racemic mixture, usually termed racemate [18]. HPLC analysis confirmed the purity of extracted verapamil where the retention time was 2.94 minutes (**Fig. 5**, see experimental section for HPLC conditions), and the spectrum recorded for this peak presented the typical ultraviolet bands (at 225 and 277 nm) of this drug[17, 21].

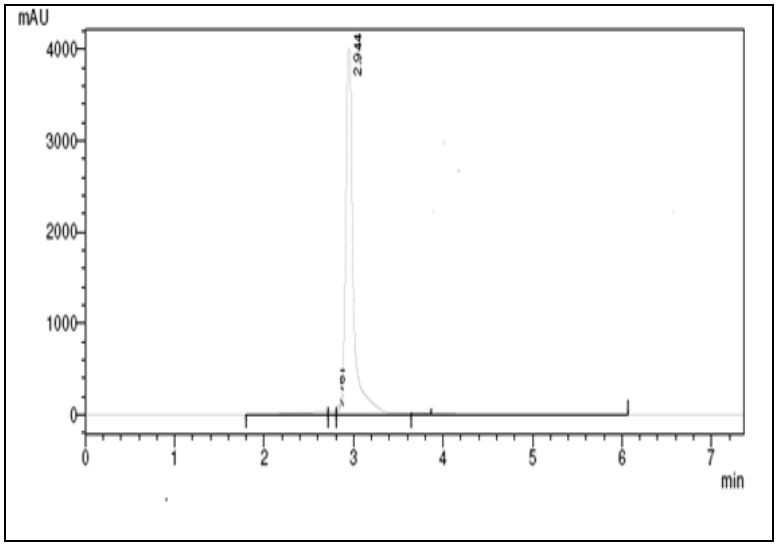


FIG.5: CHROMATOGRAM OF VERAPAMIL EXTRACTED. ISOCRATIC ELUTION WITH (%) 80:20 ACETONITRILE: WATER, WAS USED AT A CONSTANT FLOW-RATE OF 1.0 ml min^{-1}, UV DETECTION 270 nm AND 25 ± 0, 1 ^{0}C.

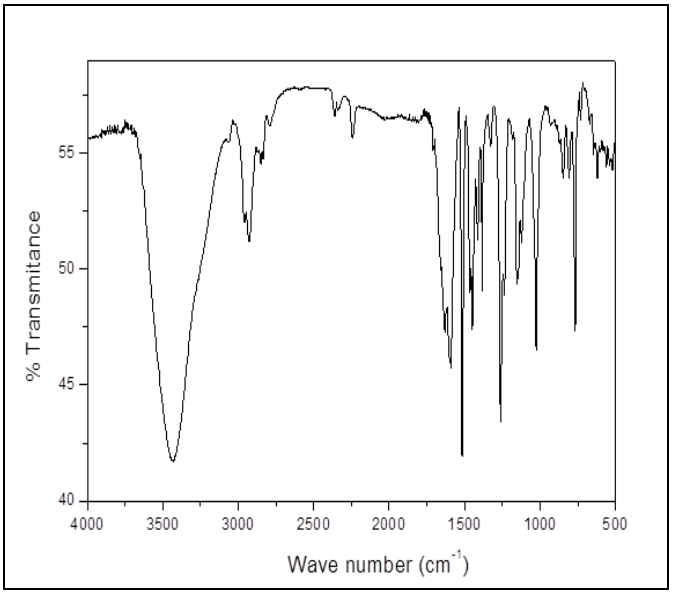
Next, we synthesized the $[Ru^{II}Cl_2(tpy)(vera)]$ complex from $[Ru^{III}(tpy)Cl_3]$, by substituting one chloride in the ruthenium coordination sphere with verapamil, followed by ruthenium(III) reduction with triethylamine. Substitution of a chloride of $[Ru^{III}(tpy)Cl_3]$ by an incoming ligand (along with ruthenium(III) reduction) resulted in formation of *trans*-$[Ru^{II}(tpy)LCl_2]$ complexes, like previously synthesized when L= CH_3CN, CH_3CH_2CN and pyridine [14]. In this proposed, we infer that the chloride ligands in the $[Ru^{II}Cl_2(tpy)(vera)]$ complex are in *trans* position.

The IR spectrum of $[Ru^{II}Cl_2(tpy)(vera)]$ in KBr pellet displayed the band corresponding to nitrile stretching at 2241 cm^{-1} (**Fig. 6**), resembling the band relative to C≡N vibration in the IR spectrum of free verapamil (2235 cm^{-1}) [18].

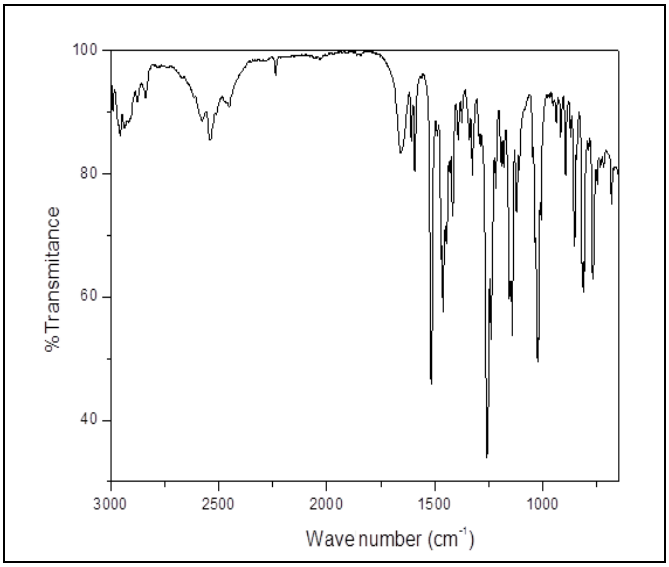
The structure of verapamil contains two different coordination points, nitrile and tertiary ammine, which can act as Lewis base. The IR analyses helped to infer that the ruthenium metal center coordinated to the tertiary ammine in the verapamil ligand, because the nitrile stretching in the

spectrum of [$Ru^{II}Cl_2$(tpy)(vera)] did not change significantly as compared with the spectrum of the free ligand, contrasting with observations made for other ruthenium complexes[22-23].

The mass spectrum of [$Ru^{II}Cl_2$(tpy)(vera)], acquired in the positive mode, (**Fig. 7**) displayed a signal at m/z 861.30, typical of protonated species of the complex like {[$Ru^{II}Cl_2$(tpy)(vera-H)] + H^+}. An intense peak emerged at m/z 455.28, probably referring to a protonated ligand product ([vera + H^+]) [24] derived from [$Ru^{II}Cl_2$(tpy)(vera)] fragmentation.



(A)



(B) POTENTIAL vc Ag/AgCl

FIG. 6: INFRARED SPECTRUM FOR [$Ru^{II}Cl_2$(tpy)(vera)] COMPLEX (A) AND VERAPAMIL EXTRACT (B) IN KBr PELLET.

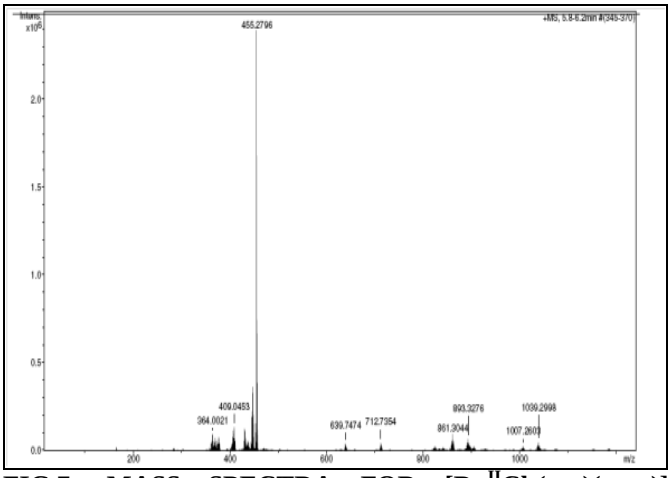


FIG.7: MASS SPECTRA FOR [$Ru^{II}Cl_2$(tpy)(vera)] COMPLEX IN METHANOL.

The spectroscopic behavior of ruthenium (II) terpyridine complexes containing a nitrile ligand is usually discussed in texts about spectroscopic transitions, including the ligand centered and metal-ligand charge transfer bands. The UV-vis. spectrum of [$Ru^{II}Cl_2$(tpy)(vera)] in dichloromethane (**Fig. 8)** displayed bands similar to those detected for *trans*-[$RuCl_2$(tpy)(CH_3CN)] and *trans*-[$RuCl_2$(tpy)(CH_3CH_2CN)][14].

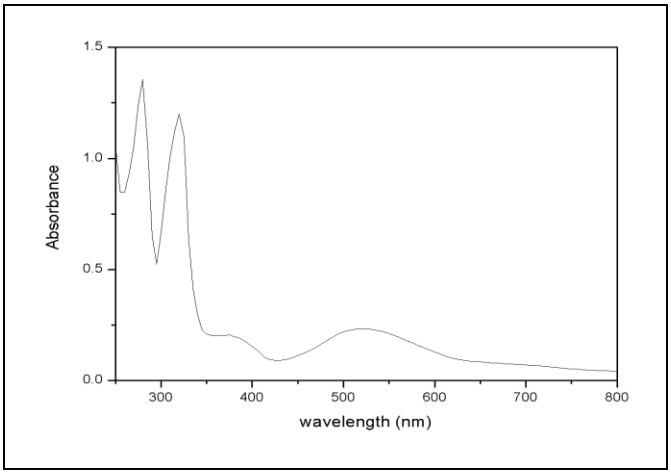


FIG. 8: ELECTRONIC SPECTRA IN UV-VIS. REGION OF [$Ru^{II}Cl_2$(tpy)(vera)] COMPLEX [3,70 x 10^{-5} mol L^{-1}] IN CH_2Cl_2.

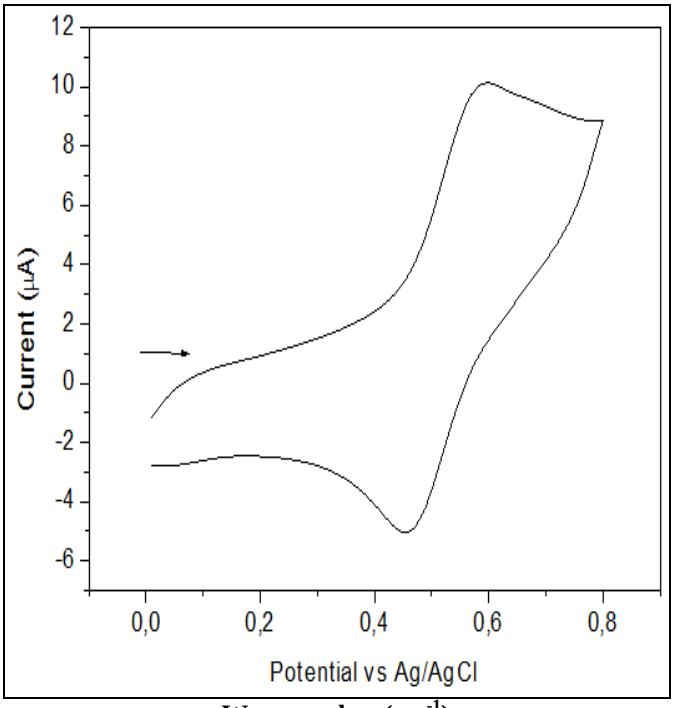
The bands in the UV region were attributed to the intraligand (IL) $\pi \rightarrow \pi^*$ transition centered mainly on the terpyridine and verapamil ligands. In the visible region, bands at 380 and 520 nm emerged, characterized as singlet metal-to-ligand charge transfer (^{1}MLCT) transitions from the ruthenium t_2g state to the π^* orbital on the terpyridine ligand (**Table 1**). Both verapamil and [$Ru^{II}Cl_2$(tpy)(vera)] exhibited fluorescence emission under UV excitation only (**Table 1**).

TABLE 1: ELECTRONIC DATE AND REDOX POTENTIAL FOR VERAPAMIL AND [$Ru^{II}Cl_2$(tpy)(vera)] COMPLEX IN CH_2Cl_2

Sample	λ_{MAX} (nm)/ log ε	Electronic transition	λ_{em} (nm)[b]	$E_{1/2}$ (V) vs Ag/AgCl
Verapamil	277/3.99[a] 225/3.07[a]	π→π*	310	-
[$Ru^{II}Cl_2$(tpy)(vera)]	280/4.69 320/4.59 380/3.84 520/3.79	π→π* (tpy, vera) π→π* (tpy) MLCT (dπRu^{II}→π*(tpy) MLCT (dπRu^{II}→π*(tpy, vera)	350	+ 0.52

a: ref.[21] , b: excitation wavelenght at 290 nm in DMSO

The Ru^{II}/Ru^{III} reversible process occurring at $E_{1/2}$= + 0.52 V vs Ag/AgCl (**Fig. 9**) was consistent with results obtained for similar ruthenium terpyridine complexes[14].



Wavenumber (cm^{-1})

FIG. 9: CYCLIC VOLTAMOGRAM FOR [$RuCl_2$(tpy)(vera)] COMPLEX [1.0 x 10^{-3}mol L^{-1}] in CH_2Cl_2. SCAN RATE 100 mV s^{-1}.

In addition to the physicochemical analyses, pharmacological experiments helped to determine how much the ruthenium metal ion influenced the pharmacological effect of verapamil.

To this end, we dissolved [$Ru^{II}Cl_2$(tpy)(vera)] in a 1% dimethylsulfoxide (DMSO)/Krebs buffer solution, due to the hydrophobic characteristics of the ruthenium complex. At 37 ^{0}C, no spectroscopic alterations in the spectrum of the [$Ru^{II}Cl_2$(tpy)(vera)] solution occurred along time (**Fig. 10)**, which indicated the chemical stability of the complex in this medium.

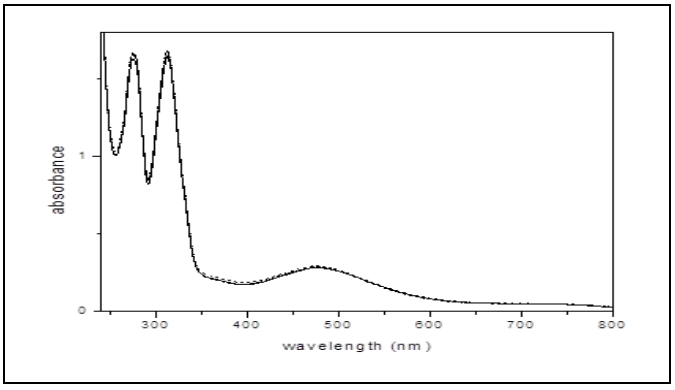


FIG.10: UV-VISIBLE SPECTRAL BEHAVIOR Of [$Ru^{II}Cl_2$(tpy)(vera)] COMPLEX IN KREBS BUFFER SOLUTION: 1% DMSO AND 37 ^{0}C (SOLID LINE) AFTER 30 MIN. (DASH LINE), 45 MIN. (DOT LINE) AND 1 H (DASH DOT LINE).

Fig.11 depicts the concentration-effect curves for the rat aorta relaxation induced by increased concentrations of verapamil and the [$Ru^{II}Cl_2$(tpy)(vera)] complex on endothelium-intact (E^+) and endothelium-denuded (E^-) aortic rings pre-contracted with phenylephrine. For E^+ aortic rings, both compounds induced relaxation in a concentration-dependent way. In both cases, the maximum effect was reached at the same concentration, with no differences in the potency calculated from the pD_2 values (**Table 2**).

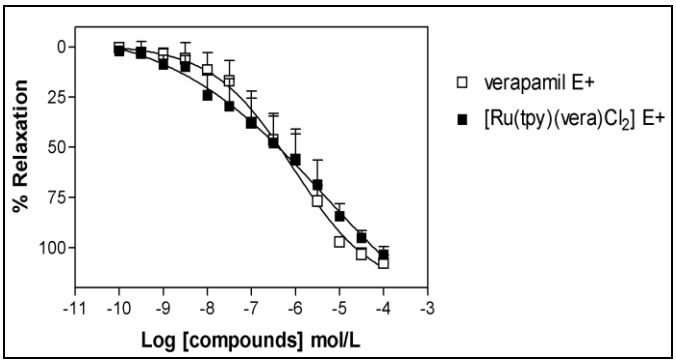


FIG. 11: VASCULAR RELAXATION INDUCED BY VERAPAMIL AND [$Ru^{II}Cl_2$(tpy)(vera)] ON RAT INTACT (E+) AORTIC RINGS. RESPONSES ARE REPRESENTED AS THE PERCENTAGE OF THE RELAXATION INDUCED BY COMPOUNDS ON THE PRE CONTRACTION WITH PHENYLEPHRINE. DATA ARE SHOWN AS MEAN ± SEM.

TABLE 2: E_{max} AND pD_2 VALUES TO PERCENTAGE (%) OF VASCULAR RELAXATION INDUCED BY VERAPAMIL AND [$RuCl_2$(tpy)(vera)] COMPLEX IN INTACT (E^+) OR DENUDED (E^-) AORTA FROM RAT.

(%) Relaxation						
	Verapamil			[$RuCl_2$(tpy)(vera)]		
Aorta	E_{max} (%)	pD_2	*n*	E_{max} (%)	pD_2	*n*
E^+	107.8±4.2	6.27±0.35	6	103.6±4.2	6.18±0.54	7
E^-	105.9±4.9	5.78±0.49	6	99.4±1.7	5.85±0.57	7

Data are shown as mean ± SEM of "n" independent experiments

Phenylalkylamines like verapamil are important in hypertension therapy. They act by blocking Ca^{2+} inflow into the cells, across the plasma membrane[5]. Previous studies have suggested that verapamil stabilizes as a relatively compact structure where non-bonded interactions between the two dimethoxy aryl rings hold Ca^{2+} [5-6].

We decided to investigate whether the similar pharmacological effects observed for verapamil and [$Ru^{II}Cl_2$(tpy)(vera)] originated from the capacity of the methoxy and alkyl nitrile functional groups on verapamil to hold Ca^{2+} via electrostatic interactions [5-6]. FLUO 3-AM, a chelating fluorescence calcium probe with maximum emission at 550 nm under visible excitation, aided us in this investigation.

Addition of the calcium ion solution to FLUO 3-AM in DMSO solution prompted a progressive, [Ca^{2+}]-dependent increase in the fluorescence intensity of the emission spectrum (**Fig. 12A**) (Calcium Signaling Protocols 1999). Plotting of $(I_0 - I)/I_0$ versus [Ca^{2+}] afforded a satisfactory straight line (Fig. 12B). The dissociation constant (K_d) of FLUO 3-AM in the presence of rising ion Ca^{2+} concentration was 1.15 μmol L^{-1}.This value was close to that reported for this same fluorescence probe in buffer solution (pH 7.2), at 22 ^{0}C is equal 3.90 μmol L^{-1} [25].

In the presence of verapamil and [$Ru^{II}Cl_2$(tpy)(vera)], the K_d of FLUO 3AM increased to 2.62 and 4.10 μmol L^{-1}, respectively. On the basis of the dissociation equation (1), the higher K_d values indicated a competitive chelating effect between the tested compounds and FLUO 3-AM for the calcium ion.

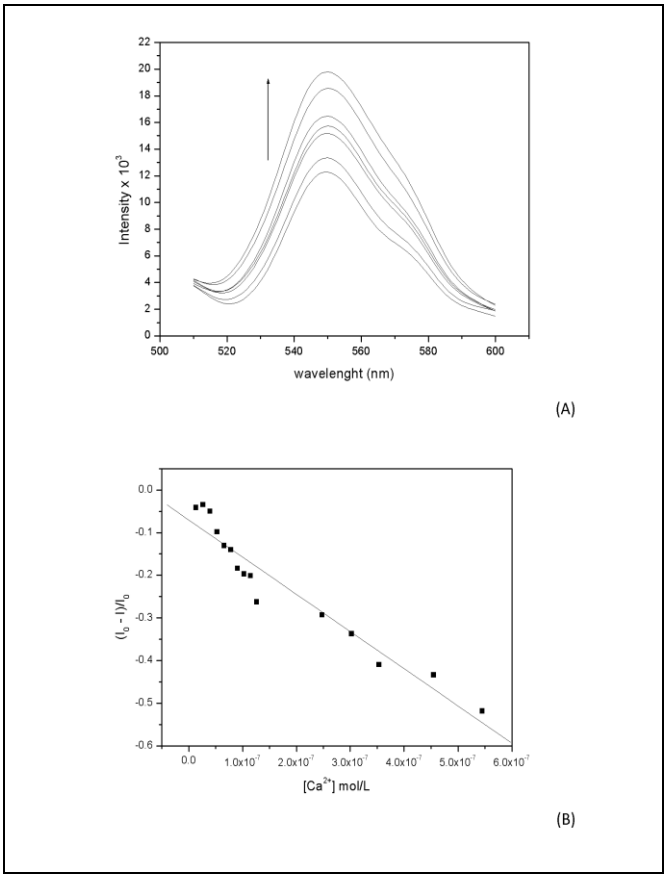


FIG. 12: FLUORESCENCE SPECTRA OF FLUO 3AM (27 nmol L^{-1}) WITH ADDED [CA^{2+}] (0 – 0.6 μmol L^{-1}) IN DMSO AT ROOM TEMPERATURE (≈ 25 ^{0}C) (A) AND PLOTTING OF $(I_0 - I)/I_0$ VERSUS [Ca^{2+}] GAVE A SATISFACTORY STRAIGHT LINE (B).

Next, we assessed the calcium chelating activity of free verapamil and [$Ru^{II}Cl_2$(tpy)(vera)] in vitro, using VSMC A7R5 and FLUO 3-AM. Under visible light excitation, it was possible to observe the fluorescence emission of FLUO 3-AM chelated with Ca^{2+} in the VSMC (**Fig. 13B**). Incubation with verapamil suppressed this fluorescence (**Fig. 13D**). These results evidenced competitive Ca^{2+} chelation between FLUO 3-AM and verapamil, corroborating the results in. Incubation with [$Ru^{II}Cl_2$(tpy)(vera)] **Fig. 12** provided a similar outcome (**Fig. 13F**).

Moreover, VSMC incubated with [$Ru^{II}Cl_2$(tpy)(vera)] displayed fluorescence emission under ultraviolet light excitation (Fig. 13G), which emerged from the ruthenium complex located in the area of the cell nucleus. Verapamil did not prompt this effect in the cell (results not shown). Taken together, these results reflected efficient internalization of the ruthenium complex by the cell as compared with verapamil [26].

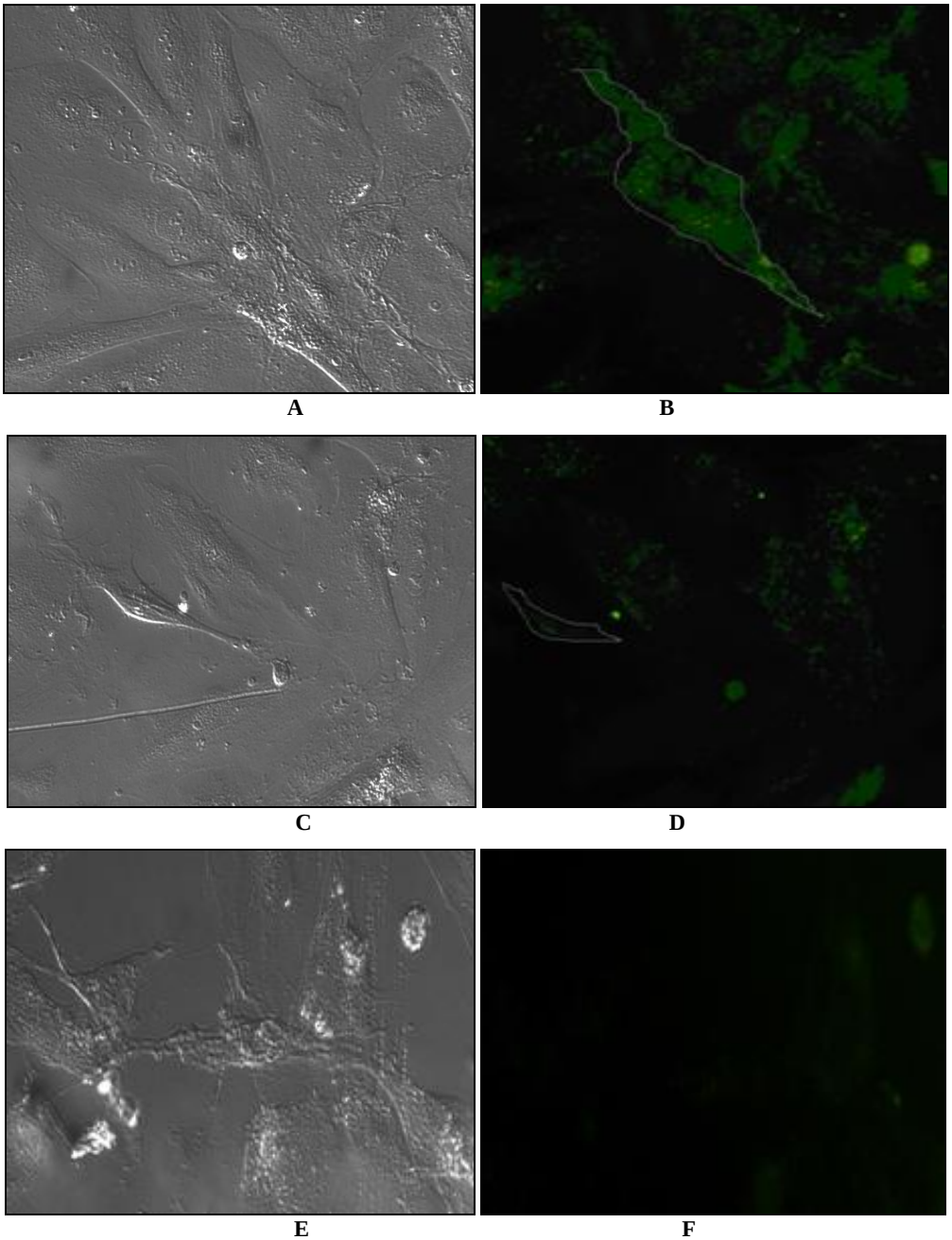


FIG.13: FLUORESCENCE MICROSCOPY ANALYSES FOR SMOOTH MUSCLE CELL A7R5 IN PRESENCE OF FLUO 3AM (10 μmol L^{-1}) (A), UNDER VISIBLE LIGHT EXCITATION (B), AFTER FREE VERAPAMIL (0.20 mmol L^{-1}) (C), UNDER VISIBLE LIGHT EXCITATION (D). MUSCLE CELL IN PRESENCE OF FLUO 3AM (10 μmol/L) AND [$Ru^{II}Cl_2$(tpy)(vera)] COMPLEX (0.20 mmol L^{-1}) (E), UNDER VISIBLE (F) LIGHT EXCITATION.

CONCLUSION: The mechanisms through which the [$Ru^{II}Cl_2$(tpy)(vera)] complex induces muscular relaxation remain unknown. However, the present study has shown that ruthenium verapamil compounds interact with calcium ions via the same electrostatic interaction involving the methoxy and alkyl nitrile substituents present in verapamil. The preliminary pharmacological results evidenced that [$Ru^{II}Cl_2$(tpy)(vera)] induced vascular smooth muscle relaxation, indicating the potential therapeutic action of ruthenium complexes as calcium blockers. Compared with verapamil, [$Ru^{II}Cl_2$(tpy)(vera)] offers the advantage of acting as a luminescent biological probe.

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