Electronic Supplementary Information

A model radiopharmaceutical agent targeted to the Translocator Protein 18 kDa (TSPO)

Sara Piccinonna,[a] *Nicola Margiotta,*[*a] *Nunzio Denora,*[b] *Rosa Maria Iacobazzi,*[b] *Concetta Pacifico,*[a] *Giuseppe Trapani,*[b] *and Giovanni Natile*[*a]

[a]Dipartimento di Chimica, Università degli Studi di Bari. Via E. Orabona 4, 70125, Bari, Italy.

[b]Dipartimento di Farmacia-Scienze del Farmaco, Università degli Studi di Bari. Via E. Orabona 4, 70125, Bari, Italy.

[*]Corresponding Authors: NM, e-mail: nicola.margiotta@uniba.it; Tel. +39 080 5442759. GN, e-mail: giovanni.natile@uniba.it; Tel: +39 080 5442774

Chemicals

Commercial reagent grade chemicals, including $[ReBr(CO)_5]$, and solvents were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification. The TSPO ligand TZ6 was synthetized as previously reported.[1]

HAM'S F12, PBS, trypsin-EDTA, penicillin (10,000 U/mL), streptomycin (10 mg/mL), L-glutamine solution (100×), and foetal bovine serum (FBS) were purchased from Euroclone (Italy). Disposable culture flasks and Petri dishes were from Corning Glassworks (Corning, N.Y., USA). The radioligand [^{3}H]-PK11195 (85.7 Ci/mmol) was purchased from PerkinElmer Life Sciences, PK11195 was purchased from Sigma-Aldrich (Milan, Italy).

Instrumental measurements

Mass spectrometry: electrospray ionisation mass spectrometry (ESI-MS) was performed with an electrospray interface and an ion trap mass spectrometer (1100 Series LC/MSD Trap system Agilent, Palo Alto, CA).

^{1}H 1D, and 2D COSY and NOESY spectra were recorded on a Bruker Avance DPX 300 MHz instrument. Standard Bruker pulse sequences were used for the NMR experiments using gradient selected versions when necessary. Chemical shifts are given in ppm. ^{1}H chemical shifts were referenced to the residual protic peak of the solvent as internal reference (2.05 ppm for Acetone-d_6). Elemental analyses were carried out with an Eurovector EA 3000 CHN instrument.

Stability studies in water, phosphate buffered solutions, and dilute human serum

High performance liquid chromatography (HPLC) was used to evaluate the stability of the complex. HPLC was performed on a Waters 1515 instrument equipped with an isocratic HPLC pump and a Dual λ Absorbance Detector Waters 2487. The HPLC analysis utilized a reverse-phase column (Phenomenex® Inertsil5, C8, 5 μM, 3.20 x 150 mm). The mobile phase consisted of water/acetonitrile (40/60, v/v) with 0.1 % of trifluoroacetic acid and the flow rate was of 1 mL/min. The HPLC analysis was performed at ambient temperature using a double detection wavelength (λ_1 = 256 nm and λ_2 = 270 nm). The standard injection volume was 20 μL. A calibration curve of 1.25–50 μM was constructed from linear plot of peak area versus concentration. The calibration curve has been used as a quantitative reference for the complex-stability investigation.

The physiological stability of the complex was studied in diluted (1/1, v/v) human serum (Sigma-Aldrich cod. H4522) at 37 ± 0.2 °C in phosphate buffer (0.02 M, pH 7.4, ionic strength adjusted to 0.15 M with NaCl). The experiment was carried out by diluting the stock solution in DMF of the tested compound with a preheated (37 ± 0.2 °C) human serum/phosphate buffer solution (1/1, v/v)

to a final concentration of 10 μM. The percentage of the organic phase never exceeded 5 %. At determined intervals of time, aliquots of 50 μL were withdrawn and added to 200 μL of cold acetonitrile in order to precipitate the serum proteins. After mixing and centrifugation for 5 min at 13000 rpm, the clean supernatant was filtered and then analyzed by HPLC. All experiments were performed in triplicate.

Curve fitting was performed by using SigmaPlot 9.0® as previously described.[2]

Cell cultures

Rat C6 glioma cells, from Interlab Cell Line Collection (ICLC) (Genova, Italy), were grown in HAM'S F12 with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine in a 5% CO_2 humidified atmosphere at 37 °C.

Membrane preparation

Membranes from the tumour cell line were prepared as described by Veenman et al.[3] with minor modifications. Briefly, rat C6 glioma were cultured to 80% confluence, the medium removed and the cells scraped in PBS (pH = 7.2). After detaching, the cells suspended in PBS were homogenized with a Brinkman Polytron (setting 5 for 3×15 s). The homogenate was centrifuged at 37,000*g* for 30 min at 4 °C and the supernatant was discarded. The final pellet was resuspended in ice-cold 10 mM PBS (pH 7.2) and stored at −80 °C until use.

Radioligand binding assay at TSPO

Binding of [^{3}H]-PK11195 to TSPO was performed according to Denora et al. with minor modifications.[4] In 0.5 mL of incubation buffer (PBS, pH 7.2) were suspended 100 μg of C6 membranes, 0.7 nM [^{3}H]-PK11195 and the compound under investigation or the reference compound (six to nine different concentrations). The samples were incubated for 90 min at 25 °C, then the incubation was stopped by rapid filtration on Whatman GF/C glass microfiber filters (pre-soaked in 0.3% polyethylenimine for 20 min) and the filters washed with 3 × 1 mL of ice-cold buffer (PBS, pH 7.2). Nonspecific binding was determined in the presence of 10 μM PK11195. Approximately 90% of specific binding was determined under these conditions.

Synthesis of fac-[ReBr(CO)$_3$(OH$_2$)$_2$]

[$ReBr(CO)_5$] (900 mg, 2.21 mmol) was suspended in 40 mL of H_2O in a 250 mL round-bottom flask and heated to reflux for 24 h under gentle magnetic stirring. Periodic rinsing of the reflux condenser allowed unreacted [$ReBr(CO)_5$] deposited on the condenser to be brought

back into the reaction solution. The crude mixture was cooled to room temperature and filtered through celite to remove small amounts of impurities. The colourless solution was dried under reduced pressure to give a white powder of the desired compound. With respect to the synthetic procedure adopted by Zubieta *et al.*,[5] who isolated the light green complex *fac*-[$Re(CO)_3(OH_2)_3$]Br by concentration of the mother liquor, we obtained a neutral white complex by complete evaporation of the mother liquor. Obtained 656 mg (76.9% yield). *Anal. Calculated* for [$ReBr(CO)_3(OH_2)_2$] ($C_3H_4O_5BrRe$): C, 9.33; H, 1.04 %. *Found*: C, 9.42; H, 1.10 %. *ESI-MS: calculated* for $[Re(CO)_3(OH_2)_3]^+$ ($C_3H_6O_6Re$) = 325. *Found*: *m/z* (% relative to the base peak) = 324.9 (100).

Synthesis of fac-[ReBr(CO)$_3$(TZ6)]

To a solution of TZ6 (14.3 mg, 0.03 mmol) in methanol (5 mL) was added, dropwise, a solution of *fac*-[$ReBr(CO)_3(OH_2)_2$] (13.5 mg, 0.03 mmol) in the same solvent (1 mL). The resulting solution was kept under magnetic stirring for 1 h at 40 °C meanwhile a yellow precipitate formed. The suspension was filtered and the separated yellow solid was washed with a small amount of cold methanol and dried under vacuum. Obtained 15.0 mg. A second crop (6.7 mg) of precipitate was obtained by concentration of the mother solution to ca. 3 mL. Obtained 21.7 mg (81.4% total yield). *Anal. Calculated* for [$ReBr(CO)_3$(TZ6)] ($C_{21}H_{20}BrCl_2N_4O_4ReS$): C, 33.12; H, 2.65; N, 7.36 %. *Found*: C, 33.14; H, 2.68; N, 7.38 %. *ESI-MS: calculated* for $[ReBr(CO)_3(TZ6) + Na]^+$ ($C_{21}H_{20}BrCl_2N_4NaO_4ReS$) = 784.5. *Found*: *m/z* (% relative to the base peak) = 784.8 (100) $[M + Na]^+$.

NMR Characterization of fac-[ReBr(CO)$_3$(TZ6)]

The complex *fac*-[$ReBr(CO)_3$(TZ6)] was characterized by elemental analysis, mass spectroscopy, and ^{1}H 1D (Figure S1, top spectrum) and 2D NOESY and COSY NMR experiments in Acetone–d_6 (Figure S2). The two protons of the imidazopyridine moiety have been assigned on the basis of their coupling constant (1.80 Hz) which is typical of two protons in *meta* positions. In particular, the less shielded signal (8.85 ppm) was assigned to proton 5 which, unlike proton 7 (falling at 7.97 ppm), is flanked by an electronegative nitrogen atom. This assignment was also supported by a NOESY cross-peak (Figure S2, top spectrum) between proton 5 and the signal assigned to the methylene protons 1'' (4.78 ppm). The two protons of the thiazole ring have been assigned on the basis of their coupling constant (3.47 Hz), which is typical for two protons in *ortho* positions. Also in this case, the more downfield doublet (8.30 ppm) was assigned to proton

4', which is flanked by a nitrogen atom. Moreover, a 2D-COSY experiment (Figure S2, bottom spectrum) allowed the assignment of the remaining dipropylacetamidic chain. The two propyl substituents are not equivalent because of the restricted rotation about the C(2")-N(3") bond, which has a partial double-bond character. A NOESY cross-peak correlates the methylene protons H(1") to the signal at 3.63 ppm (not shown) assigned to protons H(4"). The signal ascribed to methylene 4" is correlated to the sextet falling at 1.89 ppm (assigned to methylene 5") and to the triplet falling at 1.06 ppm (assigned to methyl 9"). The signal at 3.35 ppm was assigned to methylene 7" on the basis of the absence of a correlation with methylene 1". The latter signal correlates with the sextet centred at 1.58 ppm which was assigned to methylene 8". This latter signal correlates with the triplet at 0.85 ppm which therefore was assigned to the methyl group 9".

Taken together (Table S1), the data indicate that there is a shift to lower field of all aromatic protons of the TZ6 ligand upon coordination to rhenium. The protons which undergo the highest deshielding are protons 7 ($\Delta\delta$ = 0.44 ppm), 5' ($\Delta\delta$ = 0.46 ppm), and 4' ($\Delta\delta$ = 0.36 ppm). The only protons shifted to higher field are those belonging to methylene 1", but the increase in shielding is rather small ($\Delta\delta$ = 0.07 ppm). Most remarkable is the switch of the latter two protons from a sharp singlet to a pseudoquartet upon coordination to rhenium. Rhenium coordinates to the imidazopyridine and to the thiazole nitrogens imposing to the thiazole and to the imidazopyridine moieties to be coplanar. Such a conformation not only hinders the free rotation about the 3–1" carbon–carbon bond but also renders the two protons of the 1" methylene group diastereotopic being the rhenium a center of asymmetry. Therefore, the two methylenic protons become chemically non equivalent and generate an AB spin system that appears as a pseudoquartet.

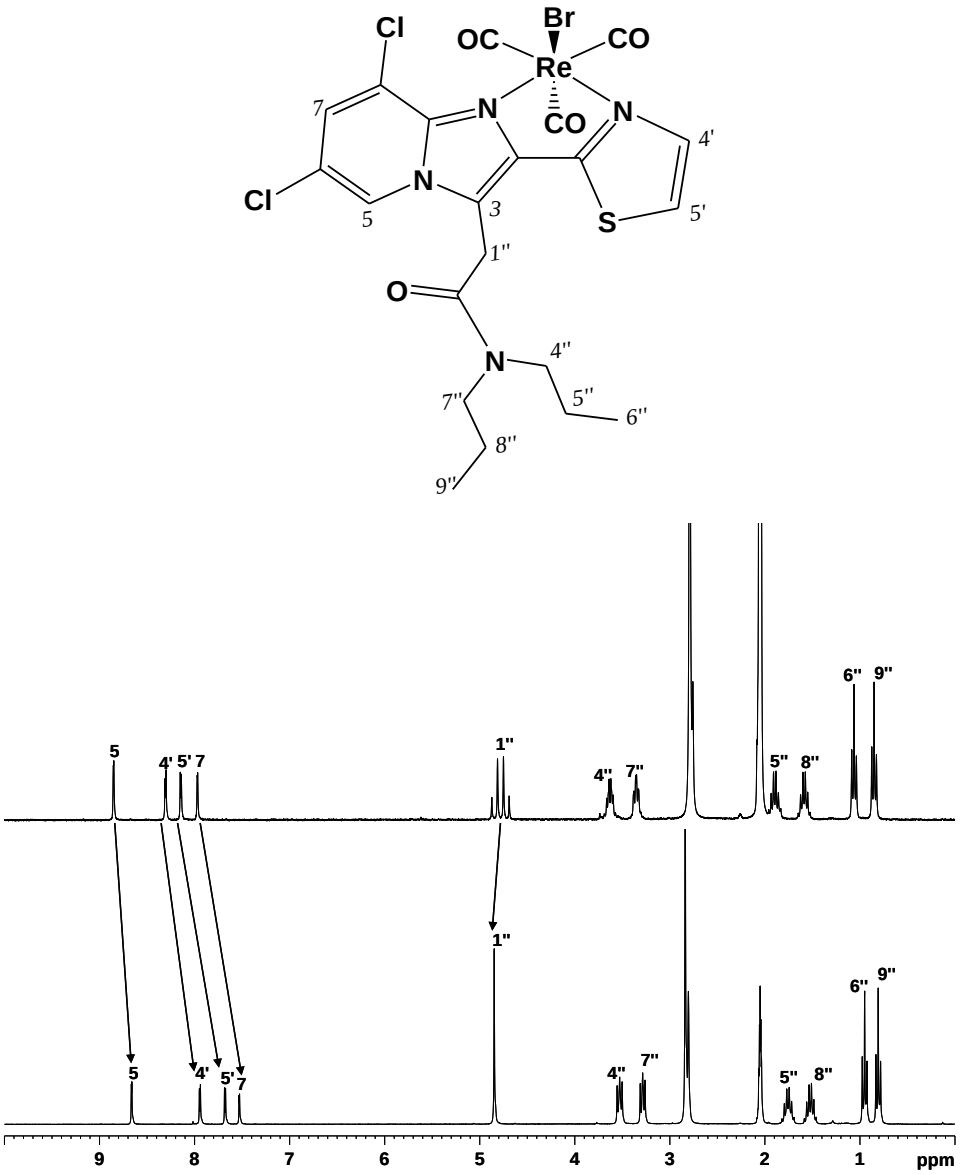

Figure S1: Comparison between the ^{1}H–NMR spectrum (^{1}H, 300 MHz) of *fac*-[ReBr(CO)$_3$(TZ6)] (top) and TZ6 (bottom) in Acetone–d_6.

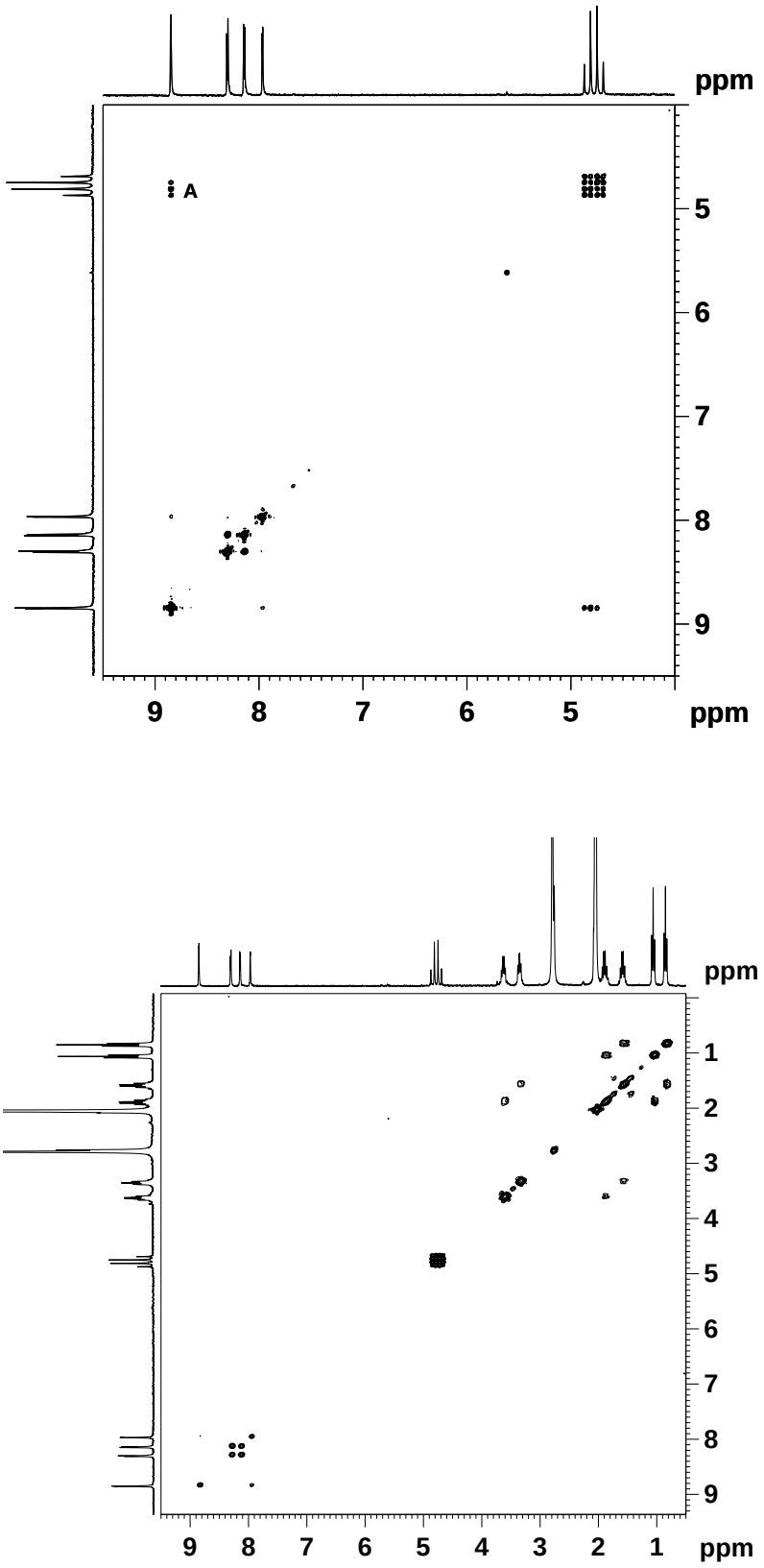

Figure S2: Portion of 2D NOESY (^{1}H, 300MHz) (top) and COSY (^{1}H, 300MHz) (bottom) spectra of *fac*-[$ReBr(CO)_3(TZ6)$] in Acetone–d_6.

Table S1. ^{1}H NMR Chemical Shifts (ppm) for TZ6 and *fac*-[ReBr(CO)$_3$(TZ6)] in Acetone–d_6.

Compound	5	7	4'	5'	1"	4"	7"	5"	8"	6"	9"
TZ6	8.66	7.53	7.94	7.68	4.85	3.53	3.29	1.74	1.52	0.95	0.81
	d	d	d	d	s	t	t	ss	ss	t	t
***fac*-[ReBr(CO)$_3$(TZ6)]**	8.85	7.97	8.30	8.14	4.78	3.63	3.35	1.89	1.58	1.06	0.85
	d	d	d	d	q	t	t	ss	ss	t	t

Numbering is as reported in Scheme 1 and Figure S1. s = singlet, d = doublet, t = triplet, q = quartet, ss = sextet.

X-ray crystallography.

Reflections were collected with Mo-*K*α radiation by using a Bruker AXS X8 APEX CCD System.

The numbers of independent reflections for [ReBr(CO)$_3$(TZ6)] were 49884 (θ_{max} = 26.39°), all reflections were indexed, integrated, and corrected for Lorentz, polarization, and absorption effects using the program SADABS.[6] Data collection, data reduction, and unit cell refinement were carried out with SAINT-IRIX package.[7]

The model was refined by full-matrix least-square methods. All non-hydrogen atoms were refined anisotropically. All hydrogen atoms were placed at calculated positions and refined given isotropic parameters equivalent to 1.5 (methyl groups) or 1.2 (other groups) times those of the atom to which they are attached

All calculations and molecular graphics were carried out using SIR2004,[8] SHELXL97,[9] PARST97,[10] WinGX,[11] and ORTEP-3 for Windows packages.[12] Details of the crystal data are listed in Table S2.

Table S2. Crystal data and structure refinement for [ReBr(CO)$_3$(TZ6)]

Chemical formula	$C_{21}H_{20}BrCl_2N_4O_4ReS$
Formula Mass	761.48
Crystal system	Monoclinic
a/Å	7.5111(4)
b/Å	27.2939(15)
c/Å	12.9838(7)
α/°	90.00
β/°	98.115(1)
γ/°	90.00
Unit cell volume/Å^3	2635.1(2)
Temperature/K	293(2)
Space group	*P*21/*c*
No. of formula units per unit cell, *Z*	4
Absorption coefficient, *μ*/mm^{-1}	6.444
No. of reflections measured	49884
No. of independent reflections	5403
R_{int}	0.0433
Final R_1 values ($I > 2\sigma(I)$)	0.0388
Final $wR(F^2)$ values ($I > 2\sigma(I)$)	0.0905
Final R_1 values (all data)	0.0492
Final $wR(F^2)$ values (all data)	0.0945
Goodness of fit on F^2	1.151

Additional informations on the X-ray structure. In the crystal the complex forms dimeric aggregates and the two molecules are related by an inversion centre. In particular, two weak halogen bonds, involving Br1 and S1A (Br1···S1A distance 3.475(2) Å), link the two subunits. (Figure S3). In the crystal packing are present other halogen bonds together with some weak hydrogen bonds. The halogen bond involves Cl6 and O2B (Cl6···O2B distance 3.103(5) Å) of adjacent molecules.
Several studies, on halogen bond as a potential tool for the rational design and construction of drugs that interact with DNA and other biological macromolecules are present in the literature.[13-15]

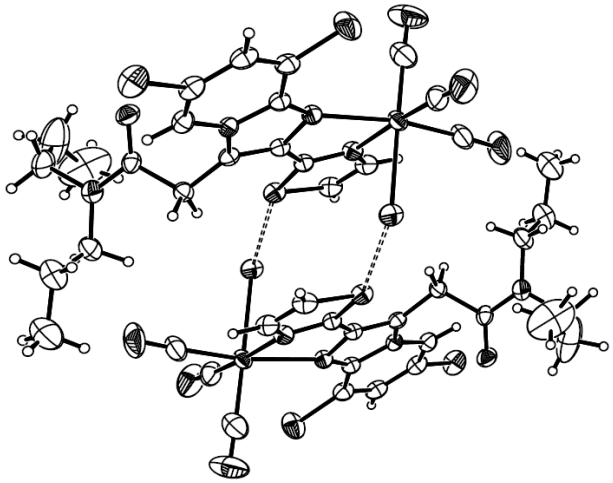

Figure S3. ORTEP drawing of two complexes linked by halogen bonds.

Table S3. Selected bond lengths [Å] and angles [°] for *fac*-[ReBr(CO)$_3$(TZ6)]**.**

Re1−N1	2.230(4)	N1−Re1−C12	97.3(3)
Re1−N3a	2.171(4)	N3a−Re1−Br1	85.4 (1)
Re1−Br1	2.634(1)	N3a−Re1−C11	94.5(3)
Re1−C10	1.905(7)	N3a−Re1−C12	95.7(3)
Re1−C11	1.897(7)	C10−Re1−Br1	89.2(3)
Re1−C12	1.883(10)	C10−Re1−C12	89.7(4)
N1−Re1−N3a	74.3(2)	C10−Re1−C11	87.1(3)
N1−Re1−Br1	83.3(1)	C11−Re1−Br1	90.7(3)
N1−Re1−C10	103.5(2)	C11−Re1−C12	89.0(4)

References

[1] N. Margiotta, R. Ostuni, R. Ranaldo, N. Denora, V. Laquintana, G. Trapani, G. Liso, G. Natile, *J. Med. Chem.*, 2007, **50**, 1019;

[2] N. Denora, B.C.M. Potts, V. J. Stella, *J. Pharm. Sci.*, 2007, **96**, 2037;

[3] L. Veenman, E. Levin, G. Weisinger, S. Leschiner, I. Spanier, S. H. Snyder, A. Weizman, M. Gavish, *Biochem. Pharmacol.*, 2004, **68**, 689.

[4] N. Denora, V. Laquintana, M. G. Pisu, R. Dore, L. Murru, A. Latrofa, G. Trapani, E. Sanna. *J. Med. Chem.*, 2008, **51 (21)**, 6876.

[5] N. Lazarova, S. James, J. Babich, J. Zubieta, *Inorg. Chem. Comm.*, 2004, **7**, 1023.

[6] G. M. Sheldrick, SADABS, Program for Empirical Absorption Correction of Area Detector Data, University of Göttingen, Germany, 1996.

[7] Bruker: SAINT-IRIX. Bruker AXS Inc., Madison, Wisconsin, USA, 2003.

[8] M. C. Burla, R. Caliandro, M. Camalli, B. Carrozzini, G. L. Cascarano, L. De Caro, C. Giacovazzo, G. Polidori, R. Spagna, *J. Appl. Crystallogr*. 2005, **38**, 381.

[9] G. M. Sheldrick, *Acta Crystallogr. Sect. A* 2008, **64**, 112.

[10] (a) M. Nardelli, *Comput. Chem.* 1983; **7**, 95; (b) M. Nardelli, *J. Appl. Crystallogr*. 1995, **28**, 659.

[11] L. J. Farrugia, *J. Appl. Crystallogr*. 1999, **32**, 837.

[12] L. J. Farrugia, *J. Appl. Crystallogr*. 1997, **30**, 565.

[13] Y. Lu, T. Shi, Y. Wang, H. Yang, X. Yan, X. Luo, H. Jiang, W. Zhu *J. Med. Chem.* 2009, **5**2, 2854.

[14] A. R.Voth, F. A. Hays, P. S. Ho, *Proc. Natl. Acad. Sci. U.S.A.* 2007, **104**, 6188.

[15] P. Metrangolo, F. Meyer, T. Pilati, G. Resnati, G. Terraneo *Angew. Chem. Int. E*d. 2008, **47**, 6114.