Final report on the NKFIH K-120224 project entitled „Design, synthesis and chemical characterization of new rigid macrocyclic ligand based Magnetic Resonance Imaging probes: the safe way in diagnosis with manganese(II) complexes”

The main goal of the NKFIH K-120224 project was to design, synthesize and characterize manganese(II) (Mn(II)) complexes formed with rigidified ligands. Controlling the physicochemical (stability, inertness), relaxation parameters (R_1, and R_2 relaxation and water exchange rates etc.) and structure of the Mn(II) chelates is a key step in the design of safer (Mn(II)-based) alternatives to Gd(III) contrast agents (CA) in Magnetic Resonance Imaging (MRI). On the other hand, by improving the dissociation kinetic properties of the Mn(II) complexes we aimed at gaining surplus in terms inertness which may be traded in a part when smart/responsive (intelligent) MRI CAs (SCAs) are synthesized because the introduction of the “handle” that allows for the modulation of relaxation properties of the Mn(II) complexes (i.e. responsible for the responsivity of the probe) is known to affect negatively the dissociation kinetic properties of corresponding complexes.

1. Results of the studies involving open-chain ligands

Nearly a decade ago (in 2012) our group published a report summarizing on the dissociation kinetic studies of Mn(II) complexes formed with numerous open-chain and AAZTA ligands (*Inorg. Chem.* **2012,** *51(19),* 10065-10067). These results have confirmed that among the commercially available ligands only the rigid *trans*CTDA chelator forms a relatively inert Mn(II) complex. In order to extend our knowledge on how the nature/rigidity of linker connecting the two iminodiacetate moieties affects the coordination chemical properties of the complexes, we have synthesized the *cis*CDTA[1], the PhDTA[2] and tri- and tetraacetate derivatives of 2,6-bis(aminomethyl)-pyridine[3] (formulae of these ligands are shown in Figure 1). Equilibrium, kinetic (solvent exchange and dissociation kinetic studies) and relaxometric studies (^{1}H and ^{17}O NMR) have been performed with the [M(II)(L)] complexes (L = *cis*CDTA, PhDTA, BPTA, BP^{OMe}TA, MeBP3A while M(II)=Mn(II), Ca(II), Mg(II), Cu(II) and Zn(II)) were performed and the physicochemical data obtained were compared to those published for the isomeric complexes formed with *trans*CDTA ligand (these data are shown in Table 1).

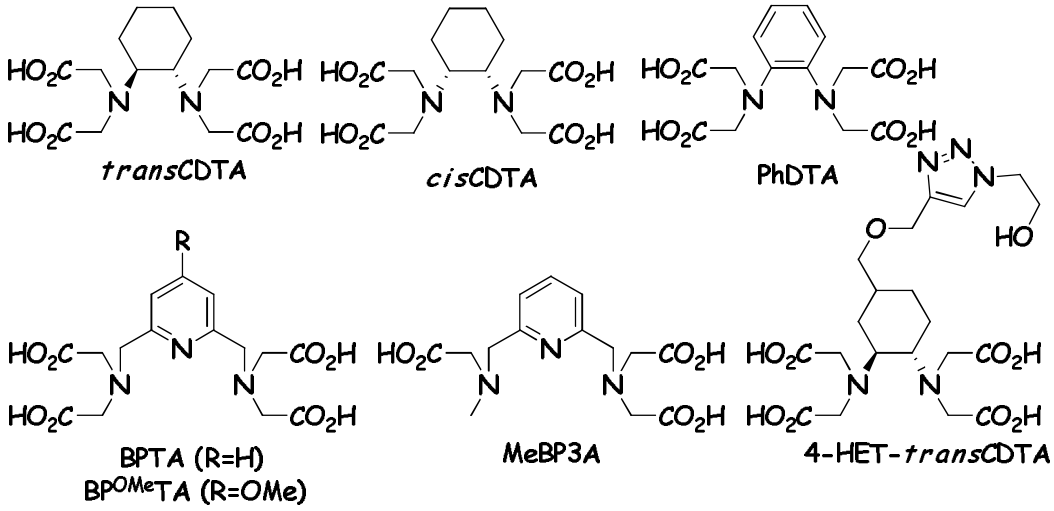

Figure 1. Formulae of open-chain ligands synthesized and studied.

Table 1. Comparision of the most relevant physico-chemical data for the Mn(II) complexes formed with rigid linear *trans*CDTA, *cis*CDTA, PhDTA BPTA, $BP^{OMe}TA$, and MeBP3A ligands (I = 0.15 M NaCl, T = 25 °C).

	trans-CDTA[a]	*cis*-CDTA[b]	PhDTA[c]	BPTA[d]	$BP^{OMe}TA$[d]	MeBP3A[d]	4-HET-*trans*CDTA[e]
log $K_{[Mn(L)]}$	14.32	14.19	11.79	14.13	13.89	11.97	13.80
pMn[f]	8.68	7.82	8.38	8.72	8.64	7.42	8.62
k_{ex}^{298} ($x10^7$ s^{-1})	14.0[g]	22.5	35.0[h]	–	–	280	17.6
r_{1p}^{298} ($mM^{-1}s^{-1}$)[i]	3.62	3.85	3.72	1.43	1.51	2.64	4.56
$t_{1/2}$ (h)[j]	12.2	0.47	19.1	6.7×10^{-6}	7.6×10^{-4}	1.5×10^{-2}	16.1

[a] *Inorg. Chem.* **2012,** *51(19),* 10065; [b] Ref. [1]; [c] Ref. [2]; [d] Ref. [3]; [e] Ref. [4]; [f] pMn values were calculated at pH=7.4 by using 0.01 mM Mn(II) and ligand concentration; [g] Ref. *Inorg. Chem.,* **2008,** *47,* 5702; [h] *Inorg. Chem.* **1977,** *16,* 2652; [i] at 25 °C and 20 MHz; [j] the half-lives (h) of dissociation were extrapolated to pH=7.4 by using 0.01 mM Cu(II) ion concentration.

As it can be seen form the data presented in Table 1. the thermodynamic data of the complexes are very alike with an exception for the complexes of PhDTA and MeBP3A ligands. This is likely explained by the basicity of the chelator in the case of PhDTA, while for the MeBP3A chelator the absence of the second binding moiety attached to the nitrogen atom lower the stability by offering not optimal coordination environment for the metal ion. However when comparing conditional data such as pMn values ($pMn=-\log[Mn(II)]_{free}$ in equilibrium at pH=7.4 in the presence 0.01 mM Mn(II) and ligand), the $[Mn(PhDTA)]^{2-}$ displays similar feature to that of parent $[Mn(trans CDTA)]^{2-}$ whereas the *cis*CDTA was

found to be a less attractive ligand for Mn(II) complexation (the pMn value of its Mn(II) complex near to physiological pH is equal to 7.82). Based on these data *trans*CDTA and PhDTA can be regarded as good chelators for Mn(II) complexation from a thermodynamic point of view. These are exactly those ligands which Mn(II) has the highest relaxation rate enhancement among the complexes studied. $[Mn(BPTA)]^{2-}$ and $[Mn(BP^{OMe}TA)]^{2-}$ complexes lack of metal bound water molecule required for the efficient transmission of paramagnetic effect, whereas $[Mn(MeBP3A)]^{2-}$ characterized by too fast water exchange preventing the efficient relaxation of the protons in the vicinity of paramagnetic center (the water exchange rate observed for $[Mn(MeBP3A)]^{2-}$ is among the highest values reported for a Mn(II) complexes in the literature).

Although all the ligands synthesized and studied are rigid chelators, the dissociation kinetic parameters (as well as the half-life of dissociation) of their Mn(II) complexes vary over a wide time scale. A difference of more than six orders of magnitude was observed for the half-lives of dissociation of the Mn(II) complexes extrapolated to neutral pH. The best results were obtained for the ligand possessing a *ortho*-phenylenediamine linker connecting the two IMDA metal binding moieties ($t_{1/2}^{pH=7.4}$ ($[Mn(PhDTA)]^{2-}$) =19.1 h) while the parent $[Mn(transCDTA)]^{2-}$ complex had very similar dissociation kinetics parameters. Therefore *trans*-1,2-diaminocyclohexane and *ortho*-phenylenediamine structural motifs could be regarded as favorable "building blocks" for further ligand development aimed at finding ligands that form kinetically inert complexes with Mn(II). With this information in mind we designed and synthesized a *trans*CDTA ligand based bifunctional chelator (BFC) for bioconjugation purposes in which the "handle" (reactive group responsible for the conjugation) for synthetic modification was implemented on the cyclohexyl ring of the chelator.[4] The impact of this substituent on the parameters of the corresponding Mn(II) complex was assessed in solution through thermodynamic, kinetic, as well as NMR relaxometric studies performed with $[Mn(4\text{-}HET\text{-}CDTA)]^{2-}$ as a "model complex" (Table 1). By comparing the obtained results to those reported for the parent $[Mn(transCDTA)]^{2-}$ chelate we concluded that the BFC chelators designed and synthesized were promising precursors for the preparation of targeted, responsive, or high relaxivity Mn(II)-based PET/MR tracers (relying on $^{52/55}$Mn(II) isotopes).

2. Studies performed with macrocyclic chelators

Recently, a growing number of studies have shown that the formation of kinetically inert complexes can be predicted from macrocyclic ligands. Therefore we have turned our attention towards macrocyclic ligands with an aim to find the best cyclic platform for Mn(II) complexation. Our main focus was devoted to cyclen (1,4,7,10-teraazacyclodoecane) and related macrocycles, as these derivatives are expected to form the most inert Mn(II) chelates, based on our study performed some time ago (*Inorg. Chem.* **2014,** *53(10),* 5136–5149).

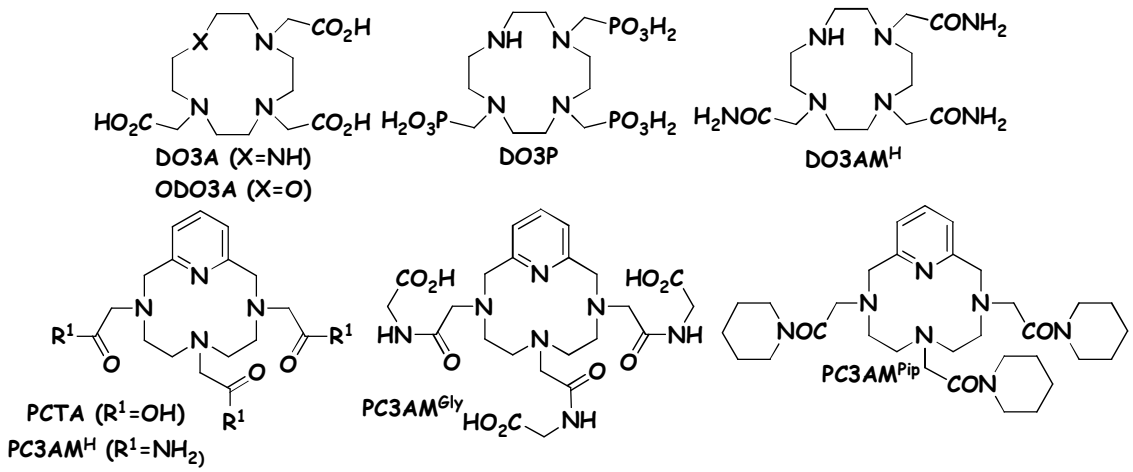

Figure 2. Formulae of 12-membered macrocyclic heptadentate ligands (DO3A, ODO3A, PCTA, DO3P, $DO3AM^H$, $PC3AM^H$, $PC3AM^{Gly}$ $PC3AM^{Pip}$) studied.

Since we had an access to several 12-membered macrocyclic heptadentate ligands (DO3A, ODO3A, PCTA, DO3P, $DO3AM^H$, $PC3AM^H$, $PC3AM^{Gly}$ $PC3AM^{Pip}$, Figure 2) from our previous studies performed with their Ln(III) complexes, we defined a project to study the stability and dissociation kinetic properties of the Mn(II) complexes formed with these chelators.[5] The structure of these chelators was expected to deliver information on how the rigidity (DO3A vs. PCTA, or for the corresponding primary amides $DO3AM^H$ vs. $PC3AM^H$), the nature of the donor atoms in the macrocycle (amine N, pyridine N, and etheric O atoms) and the nature of the pendant arms (i.e., replacement of acetate pendants by phosphonates (DO3A vs. DO3P) or amides (DO3A vs. $DO3AM^H$) including that of rigidified derivatives (PCTA vs. $PCTAM^H$ for) and the nature of amide pendants ($PC3AM^H$ vs. $PC3AM^{Gly}$ or $PC3AM^{Pip}$ representing the replacement of primary amides by secondary and tertiary amides, respectively) affect the physicochemical (stability and inertness) properties of the Mn(II) complexes. As expected, decreasing the denticity of DOTA (to afford DO3A) resulted in a drop in the stability and inertness of $[Mn(DO3A)]^-$ compared to $[Mn(DOTA)]^{2-}$. This decrease can be compensated partially by incorporating the fourth nitrogen atom (unsubstituted) in DO3A into a pyridine ring (e.g., PCTA) or by the replacement with an

etheric O atom (ODO3A). The substitution of primary amides for acetates resulted in a noticeable drop in the stability constant ($PC3AM^H$), but it increased as the primary amides ($PC3AM^H$) were replaced by secondary ($PC3AM^{Gly}$) or tertiary amide ($PC3AM^{Pip}$) pendants. The inertness of the Mn(II) complexes behaved alike as the rates of acid assisted dissociation increased going from DOTA (k_1 = 0.040 $M^{-1}s^{-1}$) to DO3A (k_1 = 0.45 $M^{-1}s^{-1}$). However, the rate constant decreased from 0.112 $M^{-1}s^{-1}$ observed for the anionic $[Mn(PCTA)]^-$ complex of to 0.0107 $M^{-1}s^{-1}$ and 0.00458 $M^{-1}s^{-1}$ for the cationic Mn(II) complexes of $PC3AM^H$ and $PC3AM^{Pip}$ ligands, respectively. These results clearly defined a set of structural elements of ligands (hexadentate, rigid macrocyclic platforms possessing acetate or tertiary amide pendants) which were than assembled to obtain suitable Mn(II) chelators. The new ligands based on pyclen, bispyclen as well as O-pyclen were designed, synthesized, properties of their Mn(II) complexes explored and patented[6-8].

Table 2. Comparision of the most relevant physico-chemical data for the Mn(II) complexes formed with 12-membered macrocyclic heptadentate ligands DO3A, ODO3A, PCTA, DO3P, $DO3AM^H$, $PC3AM^H$, $PC3AM^{Gly}$ $PC3AM^{Pip}$ (I = 0.15 M NaCl, T = 25 °C).

Ligand	$\Sigma \log K_{1,2}^H$	$\log K_{[MnL]}$	pMn	k_1 ($M^{-1}s^{-1}$)	$t_{1/2}$ (h)[a]	r_{1p} ($mM^{-1}s^{-1}$)
DO3A	19.00	16.55	8.66	0.45	1.1×10^4	1.18-1.31; 1.30
$DO3AM^H$	15.68	11.69	7.32	0.94	5.2×10^3	1.33
DO3P	23.92	17.45	6.43	2.4×10^5	3×10^{-3}	2.23
ODO3A	16.32	13.88	8.57	27	1.8×10^2	1.40
PCTA	16.70	16.83; 16.64	9.74	8.2×10^{-2}	5.9×10^4	1.50
$PC3AM^H$	12.86	11.94; 11.78	7.77	1.09×10^{-1}	4.5×10^5	1.27
$PC3AM^{Gly}$	13.40	13.20	8.37	1.07×10^{-2}	$3.\times10^2$ sb	1.44
$PC3AM^{Pip}$	14.51	14.05	8.86	1.64×10^{-2}	1.0×10^6	1.28

a at pH=7.4 in the presence of 0.01 mM Cu(II); [b] in 0.1 M HCl;

3. Result obtained with pyclen, O-pyclen and bispyclen diacetates and bis(amides)

The results outlined above performed with trisubstituted cyclododecane derivatives[5] have revealed greater conditional stability for $[Mn(PCTA)]^-$ than for $[Mn(DOTA)]^{2-}$ (near to physiological pH). The kinetic inertness of $[Mn(PCTA)]^-$ was found to be sufficiently high to

allow for the reduction of ligand denticity with an aim achieving the coordination of water co-ligand in its Mn(II) complex. Truncating the parent PCTA ligand however expected to return two regioisomeric ligands, the dissymmetric (3,6-disubstituted) and the symmetric (3,9-disubstituted) chelators (in parallel to those observed for the disubstituted 1,4,7,10-tetraazacyclododecanes 1,4-DO2A and 1,7-DO2A (*J. Inorg. Biochem.,* **2016,** *163,* 206-213)). Strait forward synthesis of regioisomeric ligands belonging to the pyclen family was not proposed in the literature therefore our first goal was to work out the details of synthetic protocols allowing to obtain these types of ligands off sufficient purity on large scales. The synthesis of symmetric (3,9-disubstituted) pyclen derivatives occurred by modifying published strategy which rely on the application of tert-butoxycarbonyl protecting group (Boc) on the nitrogen atom being opposite (*trans*) to the pyridine unit (the protecting group is built into the synthon before the macrocyclization step, while the cyclization was performed with the use of Richman-Attkins synthesis).[9] The dissymmetric (3,6-disubstituted) ligand was obtained by using a strategy which relies on the regioselective protection of one of the secondary amines of the pyclen macrocycle, in order to introduce the two metal binding arms (acetate and later picolinate moieties as well) on the remaining secondary amines. To that aim, pyclen-oxalate (Figure 3) was first synthesized following our own methodology published recently and used as a starting material.[9] Combination of these protocols allowed us to obtain ligands designed for Mn(II) complexation (3,6-PC2A, 3,9-PC2A)[10].

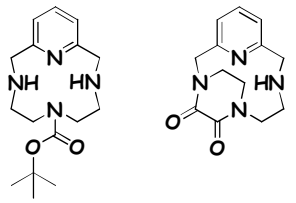

Figure 3. Formulae of the N(6)Boc-pyclen and pyclen-oxalate key synthetic intermediates.

Our parallel research projects performed with isomeric ligands returned very interesting results, as they pointed out that properties of Ln(III) complexes formed with such chelators (derivatives of cyclen macrocycle) might differ substantially [*Chem. Eur. J.* **2017,** *23(5),* 1110 and *Inorg. Chem.* **2020,** *59(12),* 8184]. At the same time, our results obtained for the Mn(II) complexes of isomeric DO2A ligands (1,4- and 1,7-) revealed less perceptible differences as far as the physicochemical properties of the Mn(II) complexes concerned.[11, 12] There was, however, one important difference between the isomeric Mn(II) complexes formed with isomeric DO2A derivatives, and that was the number of water molecules being directly coordinated to the metal ion (i.e. the parameter affecting the relaxation efficacy of the

complexes) which was slightly less than one in average for the 1,4-isomer, and it was 0 for the 1,7-disubstituted isomer (*Inorg. Chem*. **2013,** *52(6),* 3268). Therefore we have initiated a project aiming at the mapping of possible differences between the isomeric Mn(II) complexes of PC2A ligands (Figure 4). The relevant physico-chemical data (thermodynamic stability, dissociation and solvent exchange kinetics, and relaxivity values (at 25 °C)) are collected and compared in Table 4.

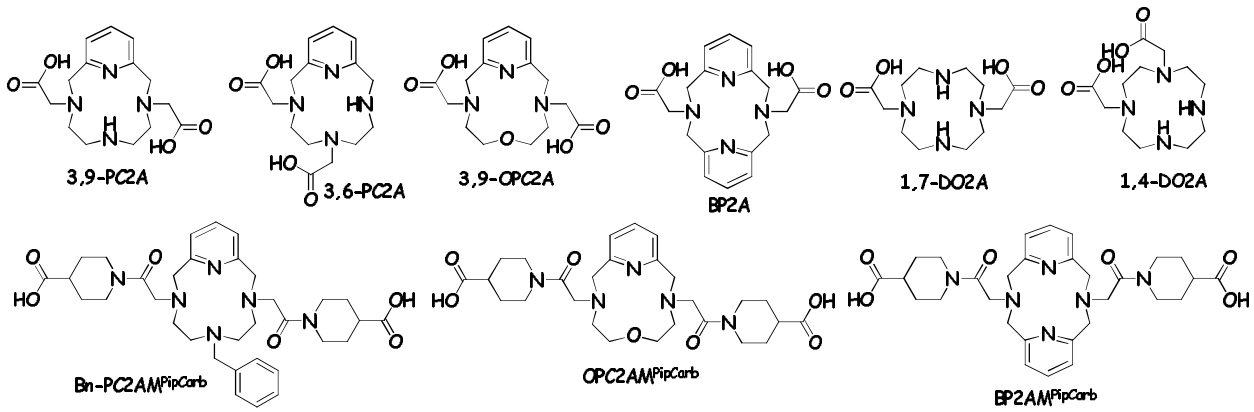

Figure 4. Formulae of pyclen, O-pyclen and bispyclen diacetates and bis(amides) studied.

Table 4. Comparison of the most relevant physicochemical data of the Mn(II) complexes formed with 3,6-PC2A, 3,9-PC2A, 3,9-OCP2A, BP2A, 1,4- and 1,7-DO2A ligands (25 °C and 0.15 M NaCl).

	[Mn(**3,6-PC2A**)][a]	[Mn(**3,9-PC2A**)][a]	[Mn(**3,9-OPC2A**)][b]	[Mn(**BP2A**)][c]	[Mn(**1,4-DO2A**)][d]	[Mn(**1,7-DO2A**)][d]
log $K_{[Mn(L)]}$	15.53	17.09	13.13	14.67	15.68	14.64
pMn[e]	8.03	8.64	8.69	9.30	7.27	6.52
$t_{1/2}$ (h)[f]	63.2	21.0	21.9/1625[g]	1.80/4885[h]	48.3	56.8
k_{ex}^{298} ($\times 10^6$ s^{-1})	140	126	53	95	1130[i]	–
r_{1p}/r_{2p}^{298} ($mM^{-1}s^{-1}$)[j]	2.72/3.49	2.91/3.96	3.09/5.13	3.63/7.42	2.1/–[i]	1.5/–[i]

[a] Ref. [10]; [b] Ref. [13]; [c] manuscript in preparation; [d] Ref. [12]; [e] pMn values were calculated at pH=7.4 by using 0.01 mM Mn(II) and ligand concentration; [f] the half-lives (h) of dissociation were extrapolated to pH=7.4 by using 0.01 mM Cu(II) ion concentration; [g] the rate constant of spontaneous dissociation was neglected during the calculation; [h] calculated with the use of data obtained by ligand exchange reactions with PCTA; [i] *Inorg. Chem*. **2013,** *52(6),* 3268; [j] at 25 °C and 20 MHz.

As it can be seen form the data presented in Table 4, the Mn(II) complex formed with 3,9-PC2A is characterized by a higher thermodynamic stability as compared to the 3,6-isomeric chelate. Both complexes contain a water molecule coordinated to the metal ion displaying fast exchange kinetics which contributes to relatively high ^{1}H relaxivity (r_{1p} = 2.72 and 2.91 $mM^{-1}s^{-1}$ observed for the complexes of 3,6- and 3,9-PC2A, respectively at 25 ºC, 0.49 T) of the complexes. Mn(II) complexes are remarkably inert with respect to their dissociation, with half-lives of 63 and 21 h, respectively, at pH 7.4 in the presence of Cu(II) excess. In line with these results both complexes are stable in blood serum for at least over a period of 120 h as accessed by ^{1}H-relaxometry. In overall, both chelates are can be considered as "building blocks" when tailoring inert Mn(II) complexes for safe MRI applications, as well as for designing "smart"/responsive imaging probes. However the 3,9-PC2A isomer might require some fine tuning in order to enhance the inertness of its Mn(II) complex which is thought to be achieved by modifying the environment of the *trans* nitrogen atom in pyclen. With that aim we have synthesized the 3,9-OPC2A as well as the more rigid BP2A ligands. The data gathered for the Mn(II) complexes of these ligands confirm that the replacement of the N atom situated *trans* to the pyridine unit in pyclen for an etheric oxygen atom is followed by a notable decrease in the stability (log *K*[Mn(3,9-OPC2A)]=13.03 vs. log *K*[Mn(3,9-PC2A)]=17.09). However, near to physiological pH the conditional like constants (conditional stability or pMn values) indicate nearly equal stability of these complexes (as evidenced by the pMn values of the complexes pMn=8.69 vs. 8.64 respectively). What is more, the conditional stability increased further upon inclusion of the second pyridine ring in the macrocycle (pMn value is being equal to 9.30 for has been accessed by studying the rates of metal exchange reactions occurring with Cu(II) in the pH range of 3.6-5.0. The dissociation of the Mn(II) complexes formed with macrocyclic ligands usually occurs via acid catalyzed mechanism, which takes place nearly 80 times slower for the [Mn(3,9-OPC2A)] complex (k_1=2.81 $M^{-1}s^{-1}$) than the value corresponding to the [Mn(3,9-PC2A)] (k_1=221 $M^{-1}s^{-1}$) chelate, being in perfect agreement with the half-lives of dissociation calculated and compared at pH=7.4 in the presence of 0.01 mM Cu(II) ion ($t_{1/2}^{pH=7.4}$=1625 vs. 21.0 (h)). At the same time [Mn(BP2A)] complex possess a very unique dissociation kinetic behavior as the rates of its transmetalation reactions increase as the pH of the samples increase. Therefore calculation the half-life of dissociation in the presence of free Cu(II) ions returned low half-life value (1.80 h) albeit the rate constant characterizing the acid assisted dissociation is the smallest value (k_1=0.99 $M^{-1}s^{-1}$) evidence ever for a macrocyclic-diacetate derivative ligand. This rather unusual behavior can be explained by the effective attack of the $Cu(OH)^+$ species present at

relatively high concentration at pH above 4.40 on the [Mn(BP2A)] complex. The relaxivity of [Mn(3,9-OPC2A)] and [Mn(BP2A)] increased slightly (r_{1p}=3.36 and 3.63 $mM^{-1}s^{-1}$, respectively) when these values compared to that of [Mn(3,9-PC2A)] (r_{1p}=2.86 $mM^{-1}s^{-1}$) chelate as expected based on the changes observed in terms of water exchange rates or molecular mass of the chelates. Altogether the replacement of the N atom situated *trans* to the pyridine nitrogen in the macrocycle for etheric oxygen atom or another rigid "building block" affects positively the most important physicochemical data of their corresponding Mn(II) complexes by making the 3,9-OPC2A and BP2A platforms an attractive candidates for further ligand design (bifunctional ligands for Mn(II) chelation as well as scaffold for smart/intelligent contrast agents). It needs to be highlighted however that the unusual dissociation kinetic behavior of the [Mn(BP2A)] complex must be treated in order to restrain the dissociation path catalyzed by $Cu(OH)^{+}$ species. This can be achieved by various approaches and among these one possibility is to apply tertiary amide pendants (the same modification is expected not only shift the dissociation pathway, but improve the rates of dissociation as well). Therefore we have designed and synthesized bis(piperidine-4-carboxylate) amide derivatives of the PC2A, OPC2A and BP2A chelators (i.e. Bn-$PC2AM^{PipCarb}$, $OPC2AM^{PipCarb}$ and $BP2AM^{PipCarb}$). These ligands were found to form weaker complexes with the Mn(II) ion than the parent carboxylate based chelators do (log K_{MnL} are 13.99, 10.88 and 13.16 which translates to pMn of 7.33, 7.70 and 8.04, respectively). Despite the lower thermodynamic stability the kinetic inertness was improved as confirmed by the Zn(II) mediated dissociation of the complexes at pH=6.0 ($t_{1/2}$ at pH=6.0 are equal to 9.8, 638 and 3632 h, respectively). The r_{1p} (4.89, 5.15 and 5.04 $mM^{-1}s^{-1}$) and r_{2p} (10.14, 15.27 and 12.37 $mM^{-1}s^{-1}$) relaxivities of these complexes are greater than those of the Gd(III)-based CA available on the market, which allowed us to file patents for their Mn(II) complexes as possible alternatives to Gd(III)-based MRI CAs.[6-8] We have synthesized these ligands and their Mn(II) complexes in large scale (500-800 mg) and toxicity as well as biodistribution studies in mice were performed in collaboration with the researchers of Faculty of Medicine of the University of Debrecen. The results of these studies showed that the PC2AM derivative bis(amide) selected for the study (Bn-$PC2AM^{PypCarb}$) display significant toxicity even at the those being equal to 1/10th of the usual MRI dose (0.1 mmol/body weight kg), while the animals survived a twenty-five times higher dose when O-pyclen and bispyclen derivatives were applied. MRI studies performed in mice show that the complexes of $OPC2A2AM^{PypCarb}$ and $BP2AM^{PypCarb}$ chelators behave as expected from an extracellular MRI CA: their

excretion starts immediately after the injection through the kidneys and the majority of the complexes (99+%) excreted within the 1st h of administration.

Table 5. Comparison of the most relevant physicochemical data of the Mn(II) complexes formed with Bn-PC2A2AM^{PypCarb}, OPC2A2AM^{PypCarb} and BP2AM^{PypCarb} ligands (25 ^{o}C and 0.15 M NaCl).

	Bn-PC2AM^{PypCarb}	OPC2AM^{PypCarb}	BP2AM^{PypCarb}
$\Sigma \log K_2^H$	16.83	13.05	12.01
$\log K_{MnL}$	13.99(2)	10.88(5)	13.16(4)
$\log K_{MnHL}$	4.46(2)	4.11(4)	4.48(5)
$\log K_{MnH2L}$	3.86(1)	3.18(5)	3.77(1)
$\log K_{MnH-1L}$	12.28(4)	11.97(8)	12.33(6)
pMn[a]	7.33	7.70	8.04
$t_{1/2}$ (h) pH=6.0	9.78	638	3632
r_{1p}/r_{2p} ($mM^{-1}s^{-1}$)	4.89/10.14	5.15/15.27	5.04/12.37

[a] pMn values were calculated at pH=7.4 by using 0.01 mM Mn(II) and ligand concentration.

5. Results obtained with the smart/intelligent Mn(II)-based MRI probes

Bioresponsive (smart/intelligent) MRI probes (SCAs) capable of reporting about the tissue pH, partial oxygen pressure (pO_2), concentration of biological relevant metal ions (Zn(II), Ca(II) etc.) would allow to detect cancer at an earlier stage, or investigate the development of type II diabetes or study the mechanism of their drug action, development and progress of amyloid plaque formation (Alzheimer's disease) etc. Based on the results obtained for the isomeric PC2A complexes the given platform can be considered as an attractive candidate for designing SCA probes as it forms very stable, relatively inert, monaquated complex with Mn(II) ion. Their relaxivity is expected to increase with the decease in complex tumbling rates (rotational correlation time τ_r) as a results of non covalent or covalent interaction of the probes with biomacromolecules (HSA etc.). On the other hand, the attachment of the moiety displaying "on" and "off" coordination feature towards Mn(II) ion as a function of pH (or pO_2, in the presence essential metal ion (such as Zn(II)) etc.) is expected to create an environment when the transition between q=0 and q=1 in Mn(II) complexes occurs) followed by the activation of the agent (i.e. relaxivity response). With these aims in our minds we have designed series of Mn(II) complexes capable of human serum albumin (HSA) (angiographic MRI), or amyloid plaque binding (molecular imaging of

Alzheimer's disease), probes responding to hypoxic conditions as well as to the changes in pH or Zn(II) concentration based on 3,9-PC2A ligand platform (formulae of these ligands are shown in Figure 5. Among these, efficient HSA binding was achieved via the introduction of *para*-nitrobenzyl or mehylene-biphenyl moieties to the *trans* nitrogen atom (NO_2Bn-PC2A and PC2A-BP, respectively). pH-responsivity was probed by the introduction of ethyl-sulphonamide (PC2A-SA), ethylamine (PC2A-EA) and 4-nitrophenol moieties (PC2A-NP). Among these the PC2A-EA seems to offer the best pH-range (i.e. protonation of the complex) matching the biological window with its relaxivity change as a function of pH. Zn(II) responsive SCA was build by the attachment of a dipicolylamine (DPA) secondary metal binding moiety to the PC2A Mn(II) binding platform (PC2A-DPA) while candidates responsive to hypoxia were synthesized by applying the 2-nitroimidazol moiety as a "vector" (PC2A1M-^{C6}NIM and PC2ABn^{C6}NIM). Finally, for targeting the amyloid plaques Pittsburgh compound B (PIB) was attached through its OH group to the 3,9-PC2A platform (PC2A-PIB) via propylene linker.

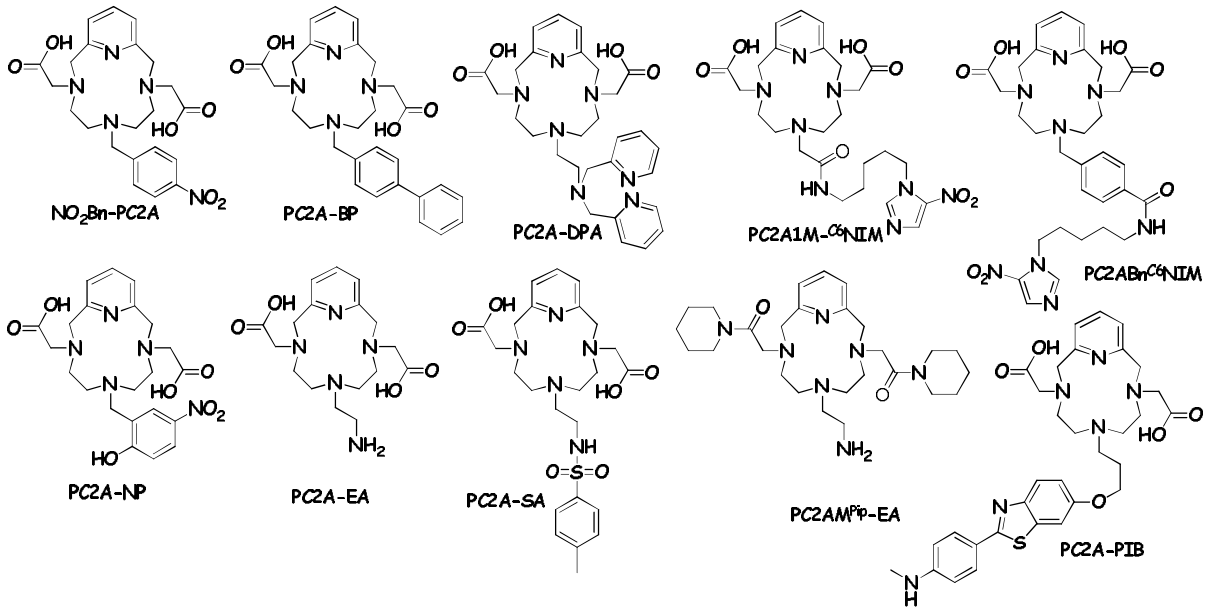

Figure 5. Formulae of the PC2A and PC2AM^{Pip} derivative SCAs designed, synthesized and studied.

Table 6. Relevant physico-chemical data for some of the Mn(II)-based SCA candidates designed, synthesized and studied.

Ligand	Function	logK_{MnL}	pMn	$t_{1/2}$ (h) at pH=6.0 and 37 °C	$^{a}r_{1p}$ of „off" and "on" forms	Ref.
NO_2Bn-PC2A	HSA bind.	14.72	8.49	14.7	4.64/7.64	[6]

PC2A-BP	HSA bind.	14.86	8.35	11.4 (25 °C)	5.08/23.5	[6, 14]
PC2A-SA	pH-sens.	17.96	9.76	40.2 (25 °C)	1.39/4.03	[6]
PC2A-EA	pH-sens.	19.01	9.27	321 (25 °C)	1.88/3.34	[6, 15]
PC2A-NP	pH-sens.	18.05	9.67	264 (25 °C)	1.58/3.03	[6]
$PC2AM^{Pip}$-EA	pH-sens.	14.73	8.23	1275 (25 °C) 181	1.37/4.18	[6]
PC2A-DPA	Zn(II)-sens.	15.87	7.79	64.5	4.03/20.0	[16]

a $mM^{-1}s^{-1}$ at 0.49 T.

The most relevant physicochemical data for some of these ligands and their Mn(II) complexes are shown in Table 6. The majority of Mn(II)-based SCA probes possess similar or considerably higher thermodynamic stability when the data compared to the parent 3,9-PC2A complex followed by a considerable improvement in their inertness. The relaxivity response in most of the cases are sufficiently high to detect these changes by using MR imaging on phantoms. With some of these systems we have launched in vivo animal studies using mice. The most cheering results obtained by MRI are summarized in Figures 7, 8 and 9. The former shows phantom MRI images taken with the use of [Mn(PC2A-EA)] present at 1.0 mM concentration in human blood serum near physiological pH (in the pH range of 6.67-7.50). The effect of pH on the contrast is apparent to the naked eye on the T_2-weighted images (right).[15]

As it is seen form the data presented in Table 6. the relaxivity response of the [Mn(PC2A-EA)] probe triggered by pH change is relatively low, therefore we have designed its bis(piperidine)amide derivative ($PC2AM^{Pip}$-EA) ligand to address the sensitivity question for which the relaxivity change observed as a function of pH (i.e. “off” and “on” forms) was doubled associated with a considerable improvement in its inertness. We have paid the price of this improvement by loosing in terms of complex stability as the stability constant characterizing the $[Mn(PC2AM^{Pip}\text{-EA})]^{2+}$ chelate dropped by slightly more than four log units (however the stability constant as well as the pMn value for the given system is being high enough allowing for its safe in vivo application).

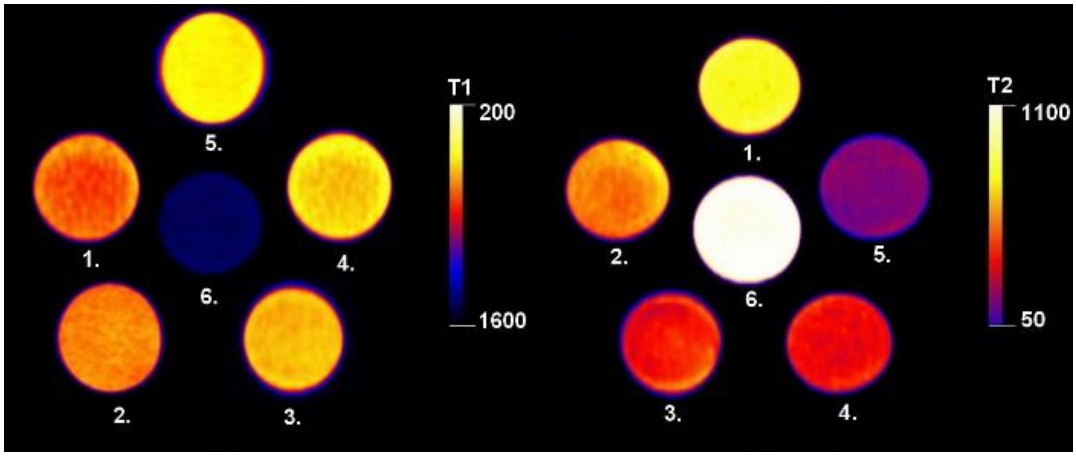

Figure 7. Representative T_1- (left) and T_2- weighted (right) MRI images (at 3 T) of the [Mn(PC2A-EA)] complex at a dose of 1mM in human serum (Seronorm) at different pH values (pH=7.50 (1), 7.25 (2), 7.08 (3), 6.81 (4) and 6.67 (5), sample (6) contained the serum solution only).

The performance of the angiographic probe [Mn(PC2A-BP)] was tested in vivo by MRI using Swiss mice. As it can be seen in Figure 8 the probe provided a substantial increase in blood signal that allowed the visualization of brain vascularization in more details (B) than without the agent (A). It highlighted micro-vascularization which was invisible without injecting the chelate as demonstrated in Figure 8 which represents pre- (A) and post-injection (B) angiographic MR images as well as the signal intensity difference between the two (C). The increase in blood signal intensity was retained even 30 minutes after the injection, and could be quantified as +26% at this time point with respect to the signal intensity prior to injection.[14]

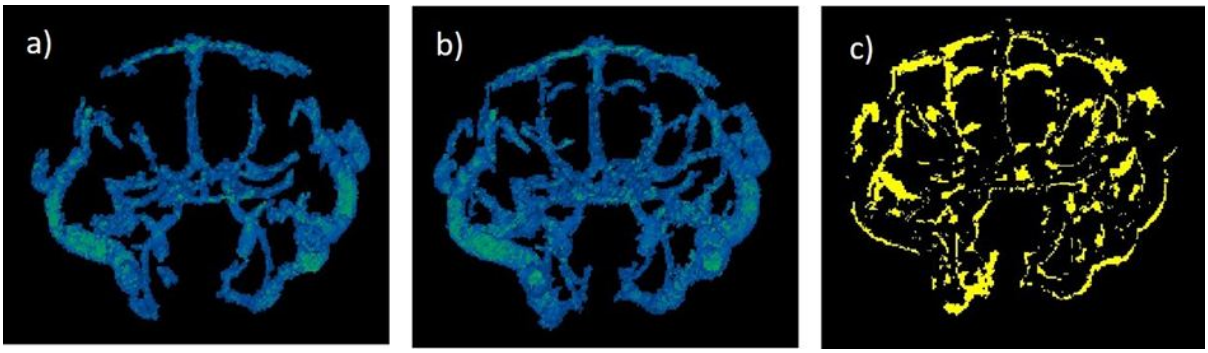

Figure 8. Angiographic MR images of a Swiss mouse brain acquired (a) before and (b) 30 min after i.v. administration of [Mn(PC2A-BP)] at a dose of 25 μmol/kg, using a FLASH sequence (FOV =1.83*1,83 cm, x 14 mm^2, matrix = 256 x 256, *TR*/*TE* = 30/5 ms, 51 axial slices 0.2 mm thickness, NA = 4, experimental time = 13 min). (c) Signal intensity increase observed at 30 min post-injection with respect to the pre-injection angiographic MR image.

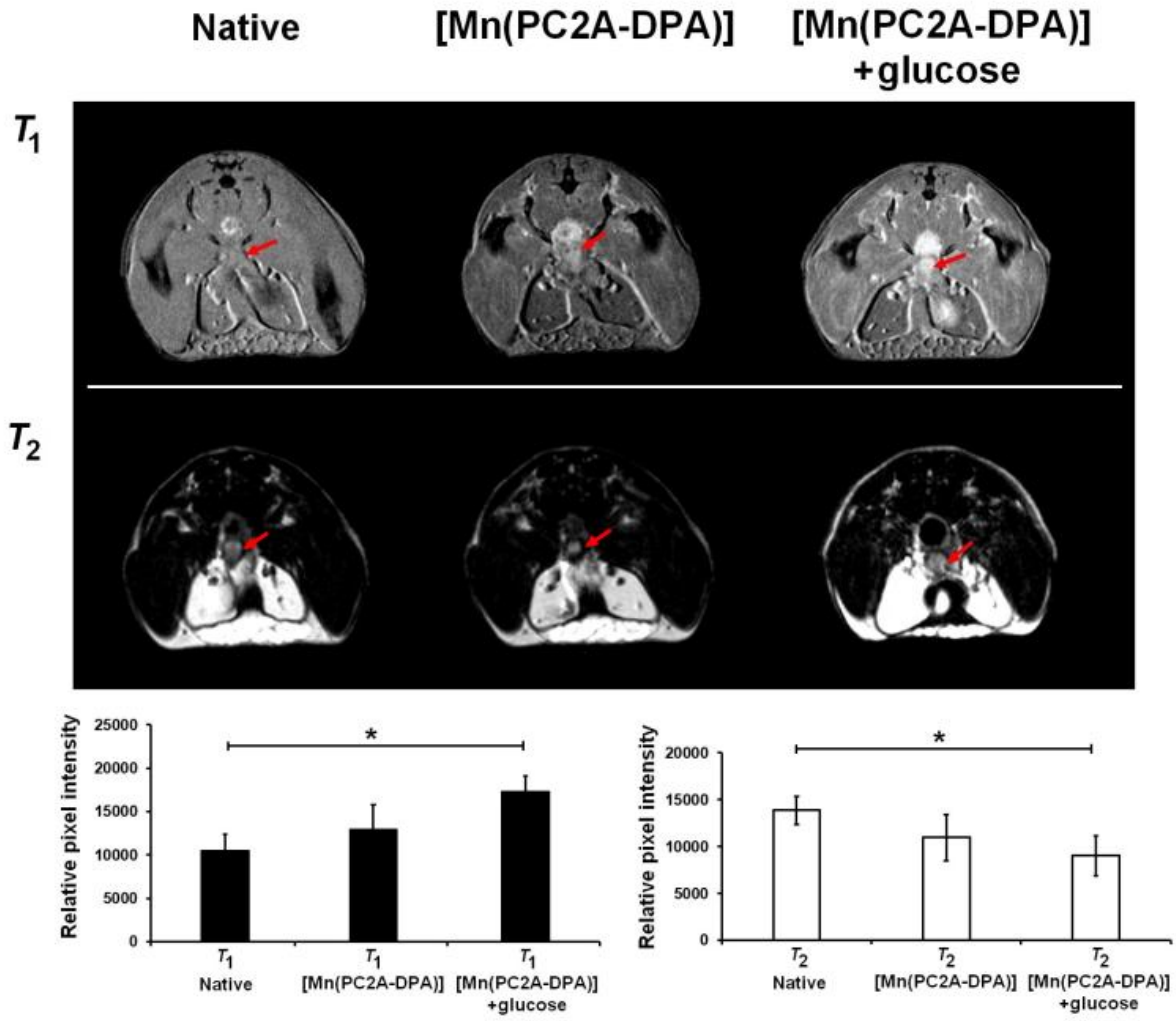

Figure 9. Representative transaxial T_1- (upper row) and T_2-weighted (middle row) MR images of the prostate of healthy male BALB/c mice imaged at 3 T magnetic field. Pre-injection (native) and post-injection of [Mn(PC2A-DPA)(H_2O)] without and with the i.p. co-injection of D-glucose (50 μL 20%). Quantitative image analysis (lower row) of the prostate (n=3/group). The prostate is marked with a red arrow. Values are presented as mean±SD. Significance level between the native and [Mn(PC2A-DPA)]+glucose values: $p \leq 0.05$ (*).

The Zn(II) responsive probe was also tested by preliminary in vivo MRI studies performed with healthy male BALB/c mice (imaged at 3 T). It is known that the healthy prostate tissue contain large concentration of Zn(II) ions. Increase in blood glucose concentrations triggers its release via the glucose stimulated zinc secretion (GSZS) mechanism. As it can be seen in Figure 9 co-injection of the Mn(PC2A-DPA)] SCA candidate with glucose resulted in a significant contrast enhancement (approx. 60% higher as compared to the native images) as observed on both T_1- or T_2-weighted images (Figure 9).[16] Prof-of-concept studies involving the hypoxia sensitive (PC2A1M-NIM and PC2ABn^{NIM}) and

amyloid-beta binder (PC2A-PIB) complexes are in progress with the use of PET (^{52}Mn isotope) and MRI imaging (natural ^{55}Mn isotope) techniques.

6. Mn(II) complexes of extremely rigid 14- and 15-mebered macrocyclic ligands

Members of rigid macrocyclic ligands based on 14- and 15-membered macrocycles were also synthesized because literature evidences published for Cu(II) and other metal ions indicate an exceptional inertness for the complexes of the given ligand class (Figure 10). Out of 15-anePyN$_5$Pip, 15-anePyN$_3$O$_2^{Bn}$ and 15-anePyN$_5^{Bn}$ chelators designed the 15-anePyN$_3$O$_2^{Bn}$ chelator was prepared and its complexation properties studied in detail (it was impossible to obtain the 15-anePyN$_5^{Bn}$ ligand as one of its synthons appeared to be unstable and in the possession of the less attractive physicochemical data corresponding to the complexes of 15-anePyN$_3$O$_2^{Bn}$ we gave up further efforts to synthesize the given chelator).[17] On the other hand, we observed the formation of a dimeric congener of 15-anePyN$_5$Pip while attempting to synthesize the 15-anePyN$_5$Pip ligand as during the macrocyclization process 2:2 macrocyclization occurred delivering a symmetric 30-membered decaazamacrocylic (30-anePy$_2$N$_{10}$Pip$_2$) ligand (which is a unique ligand, yet it was found to be a poor Mn(II) binding chelator).

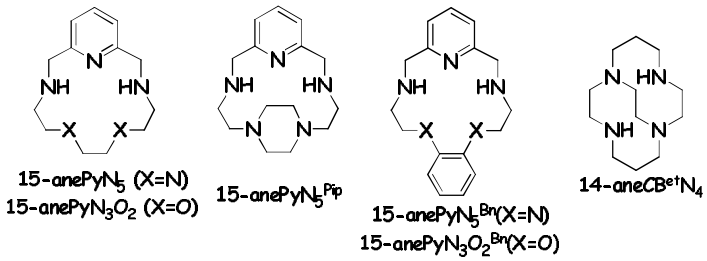

Figure 10. Formulae of 15-anePyN$_5$, 15-anePyN$_3$O$_2$, 15-anePyN$_5$Pip, 15-anePyN$_3$O$_2^{Bn}$,15-anePyN$_5^{Bn}$ and 14-aneCBetN$_4$ ligands.

The results of the studies obtained for the 15-anePyN$_3$O$_2^{Bn}$ ligand can be summarized as follows: inclusion of the catechol (phenylene-1,2-diol) unit into the parent ligand backbone resulted in very similar ligand basicity, yet the stability of the complexes formed with the given derivative were 1-1,5 log unit lower (e.g. log K_{MnL}=5.44 (15-anePyN$_3$O$_2^{Bn}$) vs. log K_{MnL}=7.18 (15-anePyN$_3$O$_2$)) than those of the complexes formed with parent macrocycle. The relaxivity of the [Mn(15-anePyN$_3$O$_2^{Bn}$)] complex is 4.72 mM^{-1}s^{-1}, which on one hand confirms our assumption about the number of bound water molecule in the complex being equal to 2 (q=2) confirmed later by X-ray crystallography. On the other hand, the given value is similar to those of low molecular mass extracellular Gd(III)-based MRI CAs (i.e.

[Gd(DTPA)]$^{2-}$ or [Gd(DOTA)]$^{-}$). However, despite of the rigidification of the ligands backbone in the [Mn(15-anePyN$_3$O$_2$^{Bn})] chelate the rate of decomplexation remains relatively fast as it occurs with similar rates than that observed by É. Tóth and coworkers for the parent [Mn(15-anePyN$_3$O$_2$)] complex. These results indicate that the replacement of fluxional ethane-1,2-diol bridging unit by the rigid phenylene-1,2-diol moiety in the backbone of the 15-anePyN$_3$O$_2$ ligand resulted in very similar (or even a little worst) physicochemical parameters for the resulting complexes. Unfortunately, it has to be noted that as an adverse effect of rigidification, the Mn(II) chelate is unsuitable for further investigations as a contrast agent due to its labile nature under even mildly acidic conditions.[17]

Some representatives of rigid (cross and side bridged) 1,4,8,11-tetraazacyclotetradecane derivatives bearing benzyl, acetate and amide moieties were also obtained and tested for Mn(II) complexation. With the given class of ligand we experienced problems when synthesizing their Mn(II) complexes in aqueous solution. The only way we could obtain Mn(II) complexes of these extraordinary rigid ligands that may behave like proton sponges was reacting the [Mn(C$_6$H$_5$N)$_2$Cl$_2$] precursor obtained by following literature protocols. The relaxivity of the [Mn(14-aneCB^{et}N$_4$)]$^{2+}$ chelate formed confirms (r_{1p}=4,43 mM-1s-1) the bishydration of the complex (q=2) as expected based on the number of donor atoms present in the ligand. The dissociation of the [Mn(14-aneCBetN$_4$)]$^{2+}$ chelate was studied in the acid concentration range of 0,1 M-1,0 M. Fitting the pseudo-firs-order rate constants retuned the rate constant characteristic for the acid assisted dissociation being equal to k_1=3.56×10^{-3} M^{-1}s^{-1}, which is one order of magnitude lower than the value corresponding to [Mn(DOTA)]$^{2-}$ referred to as „gold standard” in the literature (k_1=4.0×10^{-2} M^{-1}s^{-1}). Although these results are very promising, yet the sensitivity of these Mn(II) complexes to O$_2$ (and sometimes even to H$_2$O) make these ligands not an ideal platforms for Mn(II) complexation. Nevertheless, we are continuously investigating the avenues to facilitate the synthesis of chelates and improve their redox stability as they might can be considered as valuable platforms during the design of SCA candidates.

7. Possible utilization of the results

Three patents filed and granted for the utilization of pyclen, O-pyclen and bispyclen bis(amides) and SCA probes based on PC2A platform maintain the interest of pharmaceutical companies (negotiations/joint research are ongoing with Bracco Imaging S.p.A. and General Electric companies).[6-8] The synthetic protocol proposed for the synthesis of 3,6- and 3,9-disubstituted pyclen derivatives supplemented with the application of different metal binding

moieties (acetates, picolinates and both moieties simultaneously) allowed us to obtain large number of octa- and nona-dentate chelators designed for Gd(III) complexation with an aim of finding Gd(III) chelates offering better physicochemical properties than the traditional ligand platforms currently suggested and applied widely for Gd(III) complexation (these ligands include 3,6-PC2APA, 3,9-PC2APA, 3,6-PC2PA and 3,9-PC2PA, Figure 11)[18, 19], chelation of luminescent lanthanides (Nd(III), Eu(III), Tb(III) or Yb(III) ions)[20], as well as fast and efficient binding of diagnostic or therapeutic radionuclides of rare earth metal ions (3,6-PCA2PA and 3,9-PCA2PA chelators).[21] Screening the synthesized ligands returned two candidates, the 3,6-PC2APA and the 3,9-PC2PA chelators, which form Gd(III) complexes of higher conditional stability, display slightly better relaxation properties, possess similar acid assisted dissociation rate constants yet form considerably faster than $[Gd(DOTA)]^-$ referred to as a "gold standard".[18, 19] Similar trends in terms of physico-chemical data were obtained for the Y(III) complexes, by making these new ligands classes very promising platforms for the complexation of lanthanide(III) ions (including the radiolanthanide isotopes of short half-life).[20, 21] What is more, our own and recent literature references show that rigid pyclen derivatives might be considered for the chelation of isotopes of more exotic elements such as antimony or zirconium (*Inorg. Chem.,* **2021,** *60(18),* 14253 and *Inorg. Chem.,* **2020,** *59(23),* 17473). Finally, synthetic protocol proposed by us is now used widely to obtain mono- and disubstituted (picolinate, furinate, 8-oxychinolinate etc. derivative) chelators for the complexation of Mn(II) and Fe(III) within the frame of NKFIH K-134694 proposal (thereby confirming the robustness of the proposed synthetic scheme).

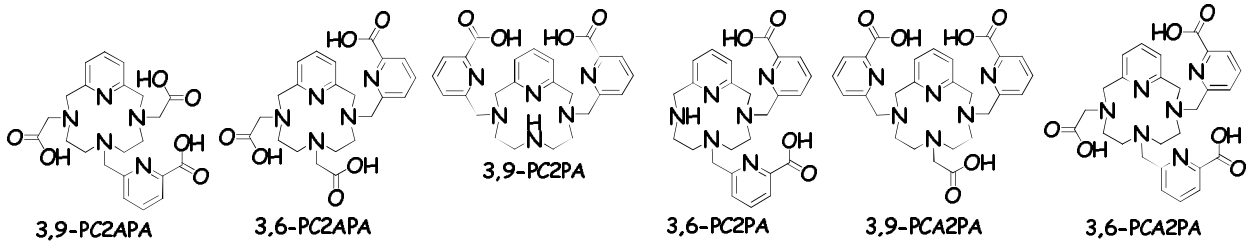

Figure 11. Formulae of pyclen derivative octa- and nonadentate acetates and picolinates synthesized and explored within the frame of the project.

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