New poly(ADP-Ribose) Polymerase-1 Inhibitors with Antioxidant Activity Based on 4-Carboxamidobenzimidazole-2-yl-pyrroline and -tetrahydropyridine Nitroxides and their Precursors

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Abbrevations: DMF: Dimethylformamide; DTT: Dithiotreitol; EPR: Electron Paramagnetic Resonance; ip: intraperitoneally; MTT[+] : 3-(4,5-dimethylthiazol-2- yl]-2,5-diphenyltetrazolium bromide; PARP: poly(ADP-Ribose) Polymerase; PDB: Protein Data Bank; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen species

Abstract: 4-Carboxamidobenzimidazoles were previously described as PARP inhibitor compounds. Here we report upon 4-carboxamido-1*H*-benzimidazoles substituted in the 2-position with nitroxides or their amine or hydroxylamine precursors. Among the new molecules, a highly active PARP inhibitor **4h** (IC_{50} = 14 nM) was identified with antioxidant/radical scavenger activity. We concluded that in most cases sterically-hindered amines are better PARP inhibitors than their oxidized form and structural changes in the 2-substituted-4-carboxamido-1*H*-benzimidazoles, (such as *N*-substitution, or changing the position of the carboxamide group) was detrimental to PARP inhibition activity, but not on antioxidant activity. These results indicate the advantages in combining of an antioxidant nitroxide or nitroxide precursor with a PARP inhibitor molecule to decrease or eliminate the deleterious processes initiated by reactive oxygen and reactive nitrogen species (ROS and RNS). The radical scavenging capability of **4h** was demonstrated by EPR study of urine collected after drug administration.

Introduction Poly(ADP-ribose) polymerase-1 (PARP-1) (EC 2.4.2.30) is an abundant DNA-binding protein activated by damages to DNA structure that occur during inflammation, ischemia, neurodegeneration, hemorrhagic shock, exposure to genotoxic agents and other pathophysiological conditions affiliated with oxidative stress.[1,2] Activated PARP-1 consumes NAD^+ which is cleaved into nicotinamide and ADP-ribose, and polymerizes later onto nuclear acceptor proteins such as caspases, topoisomerases, histones and PARP itself. This process plays a key role in the genomic stability. Over-activation can lead depletion of NAD^+ and ATP which results in cell dysfunction and ultimately necrotic cell death.[3] A cellular suicide mechanism of necrosis and apoptosis by PARP activation has been implicated in the pathogenesis of brain injury and neurodegenerative disorders. This observation is confirmed also by the fact that PARP-1 knockout mice were found to be fertile and normally developing, but resistant to cerebral ischemia. These findings suggest that in the absence of DNA damage, PARP does not necessary for survival.[4] Therefore PARP-1 inhibition is a promising mechanistic target for drug development in the context of various forms of inflammation, ischemia and cancer therapy. The protective effect of PARP-1 inhibitors in a number of experimental models of human diseases, caused by oxidative or nitrosative stress and consequent PARP-1 activation, suggests that these compounds can offer therapeutic advances either to prevent or to slow down disease progression.[5,6] The majority of PARP inhibitors bind to the nicotinamide binding site, e. g. they are competitive inhibitors of NAD^+. It is well known that nicotinamide forms three hydrogen bonds in the catalytic region of PARP-1; one to the hydroxyl group of Ser904 and two to the backbone amides of Gly863. In addition,π-π interactions with Tyr 907 of the aromatic moiety is also important (Figure 1).[7] The proper interaction requires an anti conformation of the amide carbonyl group which is in equilibrium

with the syn-conformation. The first PARP-1 inhibitors were nicotinamide and 3-aminobenzamide as benchmark inhibitors[8] but because of their low potency, low aqueous solubility, and low specificity, new PARP inhibitors were needed. The low potency was attributed to the flexibility of the carboxamide group and therefore synthesis of conformationally-restricted PARP inhibitors, e.g. lactames or heterocyclic compounds resulted in more potent compounds by 2-4 orders of magnitude.[1,9] The carboxamide group rotation can also be restricted by intramolecular hydrogen bond as in case of benzoxazole-4-carboxamides and benzimidazole-4-carboxamides.[10]

Although several classes of PARP inhibitors move towards clinical development, new compounds are still needed.[11] In our previous studies we found that modification of cardiovascular drugs, such as mexiletine,[12] amiodarone[13] or trimetazidine[14] with pyrroline nitroxide precursors provided the parent compounds with an additional antioxidant and radical scavenging activity. For example alkylation of trimetazidine secondary amine with a 2,2,5,5-tetramethyl-2,5-dihydropyrrolin-3-ylmethyl group provided protection from ischemia-reperfusion-induced contractile dysfunction. This approach well suits the new stream of drug research, [15] e. g. incorporation of two drug pharmacophores in a single molecule with the intention to exert dual (cardiovascular and antioxidant) action. We considered the combination of nitroxides and their sterically-hindered amine precursors with PARP-inhibitors, realizing that most of deleterious processes resulting from PARP activation are initiated by harmful ROS and RNS. These types of compounds would inhibit not only poly-ADP-ribosylation, but simultaneously would suppress or decrease the harmful effect of initiator ROS and RNS as well.

Cyclic nitroxides can be regarded as synthetic, multifunctional antioxidant molecules, owing their unique features to either their reduced forms, hydroxylamines or their oxidized forms oxoammonium cations.[16] It has been shown a decade ago[17] that nitroxides form

superoxide dismutase mimetic oxoammonium cations, followed by reduction of the latter species with superoxide (eq. 1 and 2, Figure 2). Hydroxylamines can act as proton and electron donor molecules reducing any radical species (eq. 3.). Nitroxides ara also capable of inhibiting $^{\cdot}OH$ formation by oxidizing Fe^{2+} to Fe^{3+} and hence preventing its participitation in Fenton-reactions (eq. 4).[18] The fully reduced form of the nitroxide – a sterically hindered amine – is easily oxidized to nitroxide by various ROS (eq. 5). [19] This nitroxide is in an equilibrium with the hydroxylamine depending upon oxidative or reductive nature of its environment (eq. 6).[20]

Although 2-substituted-4(3*H*)-quinazolinone and 4-substituted-2*H*-phthalazin-1-one derivatives with nitroxide ring substituents were good antioxidants, these modifications resulted in reduced PARP inhibitory activity.[21] The fact that 1.) the biologoical activity of cyclic nitroxides is highly dependent on substituents borne upon the ring; 2.) in preliminary studies 2-(2,2,6,6-tetramethyl-1,2,3,6-tetrahydro-pyridin-4-yl)-1*H*-benzimidazole-4-carboxamide (**4h**) reduced the ADP-induced platelet aggregation[22]; 3.) the development of a similar PARP inhibitor 2-(1-propyl-4-piperidinyl)-1*H*-benzimidazol-4-carboxamide (ABT-472)[23] prompted us to synthesize and investigate the 4-carboxamidobenzimidazole series modified with nitroxides and their precursors. Here we report some structure-activity relationships of these compounds as PARP inhibitors.

Chemistry

Benzimidazoles were achieved by heating the mixture of paramagnetic alycyclic aldehyde or paramagnetic aromatic aldehyde (**2a-p**) with 2,3-diamino-benzamide (**1**)[10] in the presence of catalytic amount of *p*-toluenesulfonic acid acid in toluene, followed by oxidation of the crude condensed product with excess activated MnO_2 in $CHCl_3$ to give compounds **3a-n**.[24] This procedure however, suffers from the low solubility of 2,3-diaminobenzamide in toluene and

some aldehydes, therefore low yields were achieved (10-44 %). The paramagnetic benzimidazoles **3a-k** were reduced by Fe powder in AcOH to sterically-hindered amines **4a-k.**[25] A very convenient and alternative procedure for the above synthesis of diamagnetic benzimidazoles is the heating of **2a-p** aldehydes with 2,3-diamino-benzamide (**1**) in the presence of equivalent amount $Na_2S_2O_5$ in DMF to yield **3l-n** and **4a-m** 2-substituted benzimidazoles from moderate to good yield (55-81 %) (Scheme 1 and Table 1).[26] Aldehydes **2a,**[27] **2b,**[28] **2c,**[29] **2d,**[30] **2g,**[30] **2h,**[31] **2i,**[32] **2k,**[29] **2l,**[33] **2o**[34] were synthesized earlier, while aldehyde **2f, 2n** and **2p** were achieved by oxidation of alcohols **5,**[30] **6**[35] and **26**[36] with MnO_2. The Suzuki cross-coupling reaction[30] of **2c** vinyl bromide and 3-trifluoromethyl phenyl boronic acid in the presence of $PdCl_2(PPh_3)_2$ and $Ba(OH)_2$ in water/dioxane mixture afforded aldehyde **2e**. Acetylation of secondary nitrogen of compound **7**[33] with acetyl chloride gave aldehyde **2m,** while alkylation of **8** vaniline in the presence of K_2CO_3 in acetone yielded paramagnetic aldehyde **2j** (Scheme 2). The efficiency of 2-mercaptoquinazoline derivatives inspired us to synthesize a series of *S*-alkylated 2-mercapto-4-carboxamidobenzimidazoles as outlined in Scheme 3. The 2-mercapto-4-carboxamidobenzimidazole **10** was synthesized by treatment of compound **1** with CS_2 in THF in the presence of catalytic amount of NaOMe. Alkylation of compound **10** with allylic bromides **9** and **11** in methanol in the presence of KOH gave compounds **12a** and **12b.** Reduction of **12a,b** radicals with Fe powder in AcOH yielded **13a,b** sterically-hidered amines.

For further study the structure-activity realationship, additional structural modifications were made on the benzimidazole part. In this new compound series, *N*-alkylation on benzimidazole NH, removal of the carboxamide group, or alteration of its position was attempted. *N*-alkylation of compound **14**[10] with allylic bromide **9**[37] in acetonitrile in the presence of K_2CO_3 gave 1-4-disubstituted benzimidazole **15**. Reaction of *o*-phenylenediamine (**16**) and with aldehyde **2h** in the presence of equivalent amount $Na_2S_2O_5$ in DMF furnished 2-substituted-

benzimidazole **17** without a carboxamide group. The *N*-alkylated carboxamide was achieved by acylation of isopropyl amine with imidazolyde of **18** carboxylic acid[10] followed by reduction of the nitro group of compound **19** to give 2,3-diamino-*N*-isopropyl-benzamide (**20**). Reaction of compound **20** with **2a** aldehyde in the presence of *p*-toluenesulfonic acid in benzene, followed by oxidation with activated MnO_2 yielded compound **21**. Reduction of nitroxide **21** with powdered iron in glacial acetic acid furnished compound **22**. Compounds **24** and **25**, the isomers of compounds of **3h** and **4h,** respectively**,** were obtained by reaction of 3,4-diamino-benzamide (**23**)[38] and **2h** aldehyde analogously as described above (Scheme 4).

Results and discussion

Compounds in Table 1 exhibited IC_{50} for PARP inhibition in the range of 14 nM and 10 μM in the whole cell system assay, IC_{50} value ranged from 9 nM to 60 μM. In the *in vitro* antioxidant assay, all the compounds exhibited IC_{50} in the range of 0.5 nM-0.35 μM. During the structure-activity relationship monitoring we changed the size of the nitroxide ring (5-or 6-membered), the saturation of the ring, the substituents on the pyrroline ring, the spacer between the nitroxide ring and the benzimidazole ring, as well as the oxidation status of the ring nitrogen. The effect of substitution changes in the benzimidazole ring also has been tested.

Regarding the PARP enzyme inhibition, the following compounds were found most effective: 4-carboxamidobenzimidazoles with 2-substituents as a five-membered nitroxide with a phenyl spacer (**3f**), six-membered-nitroxide without a spacer (**3h**), five-membered rings without a spacer and *N*-acetyl (**3m**), *N*-methyl (**3l**) and *N*-*O*-alkyl (**3n**) substituents and a six-membered ring with a secondary amine (NH) (**4h**). Compounds with substituents (Br, Ph, CO_2CH_3, dibenzofurane) on 4-position of pyrroline ring are less effective. In the PARP inhibition assay, longer spacers such as 4-phenoxymethylene (**3i, 3j**, **4i, 4j**) or a condensed

ring spacers (**3k, 4k, 4o, 4p**) are not well tolerated, the IC_{50} for these compounds is over 100 nM. The exception from the above tendencies are compounds **3a** and **4a** without any substituent on the nitroxide ring and without any spacer, featuring 721 nM and 345 nM IC_{50} values, respectively. Compounds **3h** and **4h** were found the best-performing PARP inhibitors. Compound **3h** was docked into the PARP-1 enzyme's active site and fits quite well. There are five H-bonds between the enzyme and substrate: the carboxamido groups forms H-bonds with Ser904, Gly863, water mediated H-bond between benzimidazole NH and Glu988, and nitroxide oxygen with Gln763 and π–π stacking interactions with Tyr907 (Figure 3). The docking was demonstrated with **3h** nitroxide form because it is the metabolite of compound **4h** (see bellow). Compounds with thiomethylene spacer and five-membered ring (**13a**, **13b)** or with six-membered ring (**14a, 14b**) had weaker inhibitory activity. Alkylation of benzimidazole NH or carboxamide nitrogen were detrimental to PARP inhibitory potency as we experienced for compounds **15, 21, 22.** Compounds **17, 24** and **25** were prepared to examine the influence of the carboxamide moiety on PARP inhibitory activity. As we presumed, removal of carboxamide as well as changing its position from 4 to 5 on the benzimidazole ring resulted in a loss of PARP inhibitory activity.

Cell death inhibition usually provides information on the cellular potency of the compound investigated. Surprisingly, there was a low correlation between cell death inhibition results and PARP inhibition results. The cell death inhibitory IC_{50} values are bellow 100 nM, the most effective ones being **3c** and **4c** with a bromine substituent on pyrroline ring, **3h** with a six-membered-ring, **3k** and **4k** with a thienyl ring spacer and **4j** with a 4-phenoxymethylene spacer. Compounds **13a, 13b, 14a, 14b** with -SCH_2-spacer did not exhibited antinecrotic activity as well as compounds without carboxamide function (**17**) or with dispositional carboxamide function (**24, 25**). The alkylation of carboxamide nitrogen with an isopropyl group did not decrease cell death inhibitory activity, as compared to compounds **3a** and **4a.**

From these results we conclude that the cell death inhibition, tested as protection of WRL-68 human liver cells from H_2O_2 induced cell death, does not exclusively reflect on PARP-inhibitory activity, but includes other protective mechanisms of tested compounds as well. This observation inspired us to study hydroxyl radical scavenging activity of the new PARP inhibitors in a Fenton-reaction. In this assay the water-soluble hydroxylamines were used and may reduce hydroxyl radicals to water while being oxidized to stable nitroxide free radicals. The formed nitroxides can also oxidize Fe^{2+} to Fe^{3+} and hence prevent its participation in the reaction (Figure 2, equations 3 and 4). Sterically hindered amines can be oxidized to nitroxides (Figure 2, equation 5.). These multiple ways of preventing $^{\cdot}OH$ formation in the Fenton reaction justify the very low IC_{50} values measured for studied compounds, which were mostly bellow 10 nM. This assumption is confirmed that compounds **3l, 3m, 3n** with higher IC_{50} value (over 70 nM) having blocked NH and NOH functions were unable to react with hydroxyl radicals or participate in electron transfers of Fenton reaction. Hydroxyl radical scavenging activities of compounds with $-SCH_2-$ spacer (**13a, 13b, 14a, 14b**) or with a condensed aromatic ring spacer (**3k, 4k, 4o, 4p**) were found to be limited.

Based on nitrogen oxidative status obvious structure-activity relationships can not be drawn yet, compound **4h** appears to be the best antioxidant and PARP inhibitor regarding the PARP enzyme inhibition, cell death inhibition and hydroxyl radical-scavenging results. The toxicity of **4h** was further assessed on 8 mice, and in comparison with a 5-membered analogue (**4a**). The LD_{50} for **4a** and **4h** were 620 mg/kg and 740mg/kg, respectively, both being higher than 500 mg/kg the acceptable therapy index.

Metabolism study of compounds 3h and 4h : During the metabolism studies HCl salts of both compounds (**3h, 4h**) were administered to rats intraperitoneally (ip.) in 8 mg/kg doses

and urine samples were collected before administration, for 4 hour after administration and after 4h till 18h. An isotropic triplet in the EPR spectra demonstrates the presence of nitroxide radicals and thus the oxidative metabolism of sterically hindered amine **4h,** i. e. it is metabolysed to **3h** *in vivo***.** The concentration of **3h** in the urine sample collected for 4 hour is 0.7 nM, referred to 10 nM solution of **2h,** however the EPR absorption increased almost 3-fold upon adding PbO_2 to urine samples indicating the presence of not only nitroxide but also of hydroxylamines oxidizable to nitroxides. The final concentration of radical species was 1.7 nM (Figure 4). Ip. administered hydroxylamine HCl salt of **3h** to rats and collecting their showed that hydroxylamine mostly (74%) oxidized to nitroxide after 4 hour (the concentration was 87nM, based on EPR measurements) and only 26% remained in hydroxylamine form. Further EPR active substances were formed upon addition of PbO_2 (Figure 5.) and final concentration of EPR active substance was 116 nM. Based on these observations we conclude that sterically hindered secondary amine moiety of PARP inhibitor **4h** is oxidized to nitroxide **3h,** while nitroxide **3h** and its hydroxylamine are in equilibrium in the rat model. Hydroxilamines are rapidly (0.2-1.2 $\times 10^4$ M^{-1}) oxidized by ROS ($O_2^{\cdot -}$ or peroxynitnitrite or their decomposition products), although autooxidation during sample collection can not be exluded either. More detailed studies demonstrated the oxidation of hydroxylamines to nitroxide of with rates influenced by ring size and substituents of the nitroxide.[39] Saito et al. demonstrated that both hydroxylamine and nitroxide forms are present, although hydroxylamine is more dominant from a freshly prepared kidney.[40] Urine collected between 4 hour to 22 hour after administration compounds **3h** or **4h** practically did not show triplett EPR signal (data not shown), presumably because the loss of the PARP-inhibitor metabolites.

Conclusions

In summary, we reported the synthesis and study of a series of 4-carboxamidobenzimidazole PARP-1 inhibitors carrying nitroxides and their precursors in the 2-position of benzimidazole ring[41] In structure-activity studies we found that five- or six-membered rings without substituents on the nitroxide ring and without spacer between benzimidazole and nitroxide moieties were the most efficient PARP-1 inhibitors. The results of PARP inhibition and antioxidant studies did not correlate, because the cell death inhibition is based not only on the PARP enzyme inhibition, but probably on the ROS scavenging activity also. Compound **4h** was found as a potent PARP inhibitor (IC_{50}=14 nM) with anti-apoptotic (IC_{50}=98 nM) activity, and an acceptable therapy index LD_{50}> (500mg/kg) . The structure of **4h** as a 6-membered, sterically-hindered amine connected directly to the 2-position of 4-carboxamidobenzimidazole and lacking substituents on the cyclic amino moiety. On a rat model we evaluated the metabolism of compound **4h** and found that during its oxidative metabolism a nitroxide is formed which is partially reduced to hydroxylamine. It is interesting to note that this structure is very similar to one, synthesized independently by an American research group in 4-carboxamidobenzimidazole series, namely 2(1-propynylpiperidin-4-yl)-1*H*-benzimidazole-4-carboxamide (ABT-472).[42] Further biological studies with compound **4h** are currently underway.

Experimental Procedures

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental analyses (C, H, N, S) were performed on Carlo Erba EA 1110 CHNS elemental analyzer. Mass spectra were recorded on an Automass Multi instrument in the EI mode (70 eV, direct inlet) or on a VG TRIO-2 instrument with thermospray technique. ESR spectra were obtained from 10^{-5} molar solutions ($CHCl_3$), using a Magnettech MS200

spectrometer and all monoradicals gave triplett line a_N = 14.7-16.5 G.. Preparative flash column chromatography was performed using a Merck Kieselgel 60 (0.040-0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 x 20 x 0.02 cm) coated with Merck Kieselgel GF_{254}. ^{1}H NMR spectra of diamagnetic compounds were recorded with a Varian Unity Inova 400 WB spectrometer; chemical shifts were referenced to TMS. Compounds **1**,[10] **2a,**[27] **2b,**[28] **2c,**[29] **2d,**[30] **2g,**[30] **2h,**[31] **2i,**[32] **2k,**[29] **2l,**[33] **2o**[34]**, 5,**[30] **6,**[41] **7,**[27] **9,**[31] **11,**[38] **14,**[10] **18,**[10] **23**[38] were prepared as described earlier and all other reagents and compounds were purchased from Aldrich or Fluka. All the compounds purchased or synthesized exhibited ≥95% purity by combustion analysis.

Biological activity studies

For biological studies the compounds were converted to water-soluble hydrochloride salts. Compounds **3a-k, 13a, 14a, 16, 18** and **23** were refluxed for 15 min. in ethanol, saturated previously with HCl gas. After evaporation of the solvent the hydroxylamine salt was crystallized from acetone or ether. Compounds **3l-n, 4a-p, 13b, 14b 19, 31, 24** were dissolved in ethanol, saturated previously with HCl gas. After evaporation of the solvent the salt was crystallized from acetone or ether.

Assay to test inhibitory effects of benzimidazole derivatives on PARP enzyme in vitro:

Poly-ADP-ribose polymerase was isolated from rat liver based on a known method.[43] The potential inhibitory effect of benzimidazole derivatives were tested using this assay system. The PARP activity was determined in 130 μL reaction mixture containing 100 mM Tris-HCI buffer, (pH 8.0), 10 mM $MgCl_2$, 10% glycerol, 1.5mM DTT, 1 mM[Adenine-2,8-^{3}H] NAD^+(4.500 cpm/nmol), 10μg activated DNA and 10μg histones. The incubation time was l5 minutes, and the reaction was stopped by addition of trichloro acetic acid (8%). After addition

of 0.5 mg albumine , precipitation was allowed to proceed for at least 20 minutes on ice, and the insoluble material was collected on glass filters, washed with 5% perchloric acid. The protein-bound radioactivity was determined using a LS-200 Beckman scincillation counter.

Protecting effect of compounds against H_2O_2 induced cell death, determined in WRL68-human liver cell line (Inhibitory Cell Death): *Cell culture*: WRL-68 human liver cells were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). Cell lines were grown in a humidified 5% CO_2 atmosphere at 37 ºC and maintained in culture as adherent monolayers using Dulbecco's Modified Eagle's Medium DMEM) containing 1% antibiotic-antimycotic solution (Sigma, St. Louis, Mo.) and 10% fetal calf serum. Cells were passaged at intervals of 3 days.

Detection of cell survival: Cells were seeded into 96-well plates at a starting density of 2.5×10^4 cells/well and cultured overnight in a humidified 5% CO_2 atmosphere at 37 ºC. The following day 0.3 mM H_2O_2 was added to the medium either alone or in the presence of 10, 2, 1, 0.5, 0.1 and 0.02 μM of the protecting agents (benzimidazole derivatives). Three hours later, the medium was removed and 0.5% of the water soluble mitochondrial dye (3-(4,5-dimethylthiazol-2- yl]-2,5-diphenyltetrazolium bromide (MTT^+) was added. Incubation was continued for 3 more hours, after which the medium was removed and the metabolically reduced water-insoluble blue formasan dye was solubilised by acidic isopropanol. Optical densities were determined by an Anthos Labtech 2010 ELISA reader (Wien, Austria) at 550 nm wavelength. All experiments were run in at least 6 parallels and repeated 3 times. Data of Table 1 are the concentration of benzimidazoles (in μM) at which the rate of H_2O_2-induced cell death was inhibited by 50%.

Hydroxyl Radical Scavenging of Benzimidazole Derivates (Antiox): Hydroxyl radical formation was detected using the oxidant-sensitive non-fluorescent probe benzoic acid which is hydroxylated to 2, 3 or 4-hydroxy-benzoic acid[44] Hydroxylation of benzoic acid results in the appearance of intense fluorescence which makes possible the fluorescence spectroscopic monitoring of the hydroxylation reactions (excitation λ=305 nm emission λ =407 nm). The reaction was studied in 2.5 mL reaction volumes containing 20 mM potassium phosphate buffer (pH 6.8), 0.1 mM benzoic acid, 0.1 mM H_2O_2 and 20 μM Fe^{2+}-EDTA. Data of Table I show the concentration of benzimidazoles (in μM) at which the rate of hydroxyl radical induced hydroxylation is inhibited by 50%. The parent ring (4-carboxamido benzimidazole) fluorescence $\lambda_{ex}/\lambda_{em}$ = 267/357 nm (Figure 6 in supporting information) has not influence on determination of hydroxy-benzoic acids. Under Fenton reaction conditions in the presence of 4-carboxamidobenzimidazole (**14**) and further aromatic ring containing compounds **3f, 4f, 4g, 3j, 4j** no species were formed with fluorescence at 407 nm (see Figure 7-12 in Supporting Information).

Acute toxicity studies.

C57BL/6 mice were purchased from Charles River Hungary Breeding LTD. The animals were kept under standardized conditions; tap water and mouse chow was provided *ad libitum* during the whole experimental procedure. Animals were treated in compliance with the approved institutional animal care guidelines. The experiments were conducted by distributing the rodents into groups of eight mice each, that were treated intraperitonially by 100, 200, 300 and 500 mg/kg PARP inhibitors in a single injection. Mice were observed for the presence of respiratory, digestive and neurological alterations. The number of deaths was noted in each 24 h for 14 days.

Statistical Analysis: Data were presented as means: ±S.E.M. For multiple comparison of groups ANOVA was used. Statistical difference between groups was established by paired or unpaired Student's test, with Bonferroni correction. Differences with p-values below 0.05 were considered to be significant.

Modeling PARP inhibitor (3h) and PARP enzyme Interactions: The PARP enzyme catalytic fragment (3pax) was downloaded from protein data bank, and the PDB crystal structure was prepared by removing ligand and water. Structure is visualysed by Accelrys DS Visualizer v2.0.1.7347.

Oxidative metabolism studies with ESR:

Male, 290-350 g Wistar rats (Charles River, Budapest, Hungary) were involved into our study. All animals were housed in wire bottom individual metabolic cages in order to collect urine. Rats were randomly divided into 2 groups. One group were treated with 2mg, ip. HO-3089 (group 1, n=3) once time. Single dose of HO-3088, 2mg, ip were administered to the other group (group 2, n=3). Our agents were completely dissolved in 1,0 ml of 0,9 % NaCl. Urine was collected 3 occasions. First collection was performed before the treatment, the second fraction was collected from right after the injection to 4 hours. The third part of urine was collected from 4 hours to 22 hours after the treatment. The PbO_2 treatment of urine was exploited as follows: to 1 mL urine 50 mg PbO_2 was added and vortexed for 1 min., then the sample was allowed stay for 10 min. and 50 μL from the supernatant was studied by EPR. ESR analysis were performed on Magnettech MS200 spectrometer (X-band) in 50 μL glass micropipettes. Modulation was 1400 mG, sweep 59.5 G, sweep time 60s, gain 500, microwave attention 10dB and urine was measured directly after collection.

Synthesis of compounds 3a-n, 21, 24 (Procedure A):

To a solution of compound **1** or **23** (1.51g, 10.0 mmol) or **20** (1.93g, 10.0 mmol) and aldehyde **2a-n** (10.0 mmol) in toluene (70 mL) *p*-toluenesulfonic acid (100 mg) was added and the mixture was heated to reflux under Dean-Stark apparatus to remove the water formed for a period of 8h. After cooling, the toluene was evaporated off and the residue was dissolved in $CHCl_3$ (50 mL). Activated MnO_2 (4.3 g, 50.0 mmol) was added and the mixture was stirred and refluxed for 2h. After cooling the mixture was filtered through a Celite pad and the filtrate was washed with water (20 mL). The organic phase was separated, dried ($MgSO_4$) and filtered. The residue was purified by flash column chromatography ($CHCl_3/Et_2O$) to afford the title compounds (39-73%) as yellow or white or off-white solids.

2-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-1*H*-benzimidazol-4-carboxylic acid Amide Radical (3a): yellow solid (1.52g, 51%), mp 248-250 ºC. Anal. $C_{16}H_{19}N_4O_2$ C H N. MS m/z(%): 299 (M^+, 19), 269 (37), 223 (51), 41 (100).

Synthesis of compounds 4a-p by reduction of nitroxide with Fe in AcOH (4a-k, 13a, 13b, 22, 25) (Procedure B): To a solution of nitroxide **3a-k** or **12a** or **12b** or **21** or **24** (5.0 mmol) in AcOH (15 mL) iron powder (1.4g, 25.0 mmol) was added and the mixture was stirred at 60 ºC for 30 min. After cooling, the reaction mixture was diluted with water (40 mL) and filtered. The filtrate was basified with solid K_2CO_3 to pH=8 (intensive foaming). The aq. phase was extracted with $CHCl_3$ containing 10% MeOH (2 x 30 mL) and the combined organic phase was dried ($MgSO_4$), filtered and evaporated. The residue was purified by flash column chromatography ($CHCl_3$ / MeOH) to give the title amines as white solids (48-65%).

2-(2,2,5,5-Tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-1*H*-benzimidazol-4-carboxylic acid Amide (4a): white solid (923 mg, 65%), mp 239-241 ºC. Anal. $C_{16}H_{20}N_4O$ C H N. MS m/z(%): 284 (M^+, 1), 269 (100), 252 (91), 224 (14). ^{1}H NMR (399.9 MHz, DMSO-d_6): δ 1.27 (s, 6 H), 1.52 (s, 6 H), 6.65 (s, 1 H), 7.29 (t, J = 7.7 Hz, 1 H), 7.62 (d, J = 7.9 Hz, 1 H), 7.67 (s, 1 H), 7.80 (d, J = 7.4 Hz, 1 H), 9.18 (s, 1 H), 12.91 (s, 1 H). ^{13}C NMR (100.5 MHz, DMSO-d_6): δ 30.4, 30.5, 63.7, 66.4, 114.4, 122.3, 122.4, 122.5, 134.3, 136.5, 141.0, 141.1, 148.1, 166.1.

One-pot procedure for synthesis of amines 4h, 4o, 4p, 17 (Procedure C): A solution of compound **1** (750 mg, 5.0 mmol) or *o*-phenylenediamine (**16**)(540 mg, 5.0 mmol) and paramagnetic aldehyde **2h** or **2o** or **2p** (5.0 mmol) in DMF (10 mL) in tube was deoxygenated with N_2 for 10 min. Afterwards $Na_2S_2O_5$ (1.14g, 6.0 mmol) was added, the tube was sealed, and the solution heated for 4h by immersing the tube into an oil bath heated to 120 ºC. After cooling, the mixture was poured onto ice-water (200 mL) and the precipitated solid was filtered, air dried and purified further by flash column chromatography for analysis. The crude product was re-crystallysed from methanol/ether or methanol/acetone and used for HCl salt formation, yields ranged from 45-79%.

2-(2,2,6,6-Tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)-1*H*-benzimidazol-4-carboxylic Acid Amide (4h): white solid (1.17g, 79%), mp 292-295 ºC. Anal. $C_{17}H_{22}N_4O$ C H N. MS m/z(%): 298 (M^+, 23), 283 (81), 266 (29), 42 (100). ^{1}H NMR (399.9 MHz, DMSO-d_6): δ 1.13 (s, 6 H), 1.22 (s, 6 H), 2.39 (s, 2 H), 6.81 (s, 1 H), 7.26 (t, J = 7.7 Hz, 1 H), 7.50 – 7.70 (m, 2 H), 7.79 (d, J = 7.3 Hz, 1 H), 9.29 (s, 1 H), 12.82 (s, 1 H). ^{13}C NMR (100.5 MHz, DMSO-d_6): δ 29.8, 31.0, 36.8, 48.7, 51.1, 114.4, 121.9, 122.1, 122.3, 123.2, 134.8, 138.3, 141.1, 153.3, 166.3.

2-Mercapto-4-carboxamidobenzimidazole (**10**):

To a solution of compound **1** (1.51g 10.0 mmol) and CS_2 (760 mg, 10.0 mmol) in THF (20 mL) NaOMe solution (0.5 mL, 1.0 M stock solution in MeOH) was added. After refluxing for 1h, the reaction mixture was allowed to stay at rt. overnigt. The precipitated crystals were filtered, washed with Et_2O (5 mL), and dried. Yellow solid: 900 mg (46%), mp: 354-356 ºC. Anal. $C_8H_7N_3OS$ C H N. MS m/z (%): 193 (M^+, 90), 176 (100), 148 (33), 105 (20), 90 (33). ^{1}H NMR (399.9 MHz, DMSO-d_6): δ 7.13 (t, J = 7.8 Hz, 1 H), 7.25 (d, J = 7.8 Hz, 1 H), 7.55 (s, 1 H), 7.58 (d, J = 7.8 Hz, 1 H), 8.15-8.35 (br s, 1 H), 10.80-12.30 (br s, 1H). ^{13}C NMR (100.5 MHz, DMSO-d_6): δ 111.9, 115.9, 120.6, 121.4, 132.6 (br), 133.5, 167.4, 169.3.

Alkylation of 2-Mercapto-4 carboxamidobenzimidazole, General procedure for 12a and 12b: To a solution of compound **10 (**1.93 g, 10.0 mmol) and powdered KOH (560 mg, 10 mmol) in methanol (20 mL) compound **9** (2.33 g, 10.0 mmol) or compound **11** (2.47 g, 10.0 mmol) was added and the solution was refluxed for 2h. After cooling the inorganic salts were filtered off, the filtrate was evaporated, and the residue was dissolved in $CHCl_3$ (30 mL). The organic phase was washed with water (10 mL), the organic phase was separated, dried ($MgSO_4$), filtered and evaporated. The crude product was purified by flash column chromatography ($CHCl_3$/Et_2O) to afford compound **12a** or **12b**.

2-(1-Oxyl-2,2,5,5-Tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethylsulfanyl)-1*H*-benzimidazole-4-carboxylic Acid Amide (12a): yellow solid, 1.55 g (45%), mp 249-251 ºC. Anal. $C_{17}H_{21}N_4O_2S$ C H N. MS m/z (%): 345 (M^+, 20), 315 (18), 300 (13), 193 (100).

2-(1-Oxyl-2,2,6,6-Tetramethyl-1,2,3,6-tetrahydro-pyridin-4-ylmethylsulfanyl)-1*H*-benzimidazole-4-carboxylic Acid Amide (12b): pink solid, 1.36 g (38%), mp 102-104 ºC. Anal. $C_{18}H_{23}N_4O_2S$ C H N. MS m/z (%): 359 (M^+, 2), 329 (18), 196 (42), 41 (100).

1-(1-Oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-1*H*-benzimidazole-4-carboxamide (15): To a solution of compound **14** (805 mg, 5.0 mmol) and powdered K_2CO_3 (690 mg, 5.0 mmol) in acetonitrile (20 mL) compound **9** (1.16 g, 5.0 mmol) was added and the solution was refluxed for 3h. After cooling the inorganic salts were filtered off, the filtrate was evaporated, and the residue was dissolved in $CHCl_3$ (30 mL). The organic phase was washed with water (10 mL), the organic phase was separated, dried ($MgSO_4$), filtered and evaporated. The crude product was purified by flash column chromatography ($CHCl_3/Et_2O$) to afford compound **15** yellow solid, 970 mg (62%), mp 247-249 ºC. Anal. $C_{16}H_{20}N_3O$ C H N. MS m/z(%): 313 (M^+, 48), 299 (15), 283 (24), 41 (100).

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Supporting Information Available: Table of micronalytical data of compounds **2-25** and physicochemical and spectral data of compounds **2e, 2f, 2j, 2n, 2m, 2p, 3b-3n, 4b-4k, 4o, 4p, 13a, 13b, 17, 19, 20, 21, 22, 24, 25** and blank fluorescence measurement under Fenton conditions**.** This material is available free of charge via the Internet at http://pubs.acs.org.

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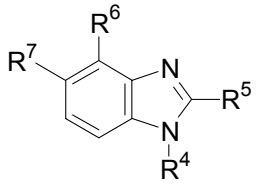
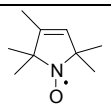
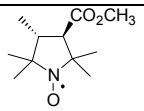
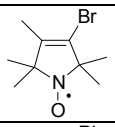
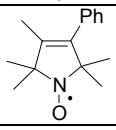
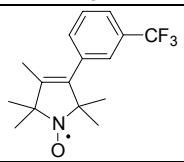
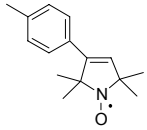
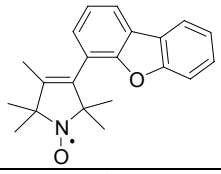
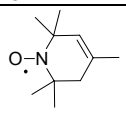
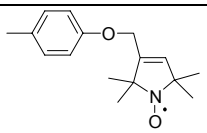
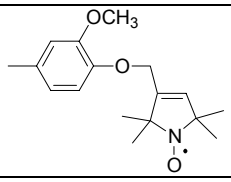
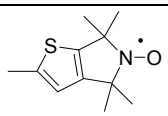
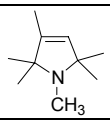
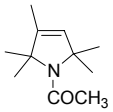
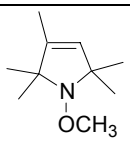
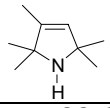
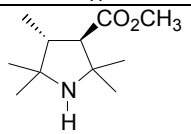
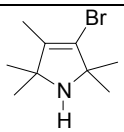
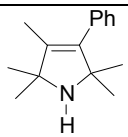
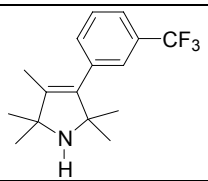
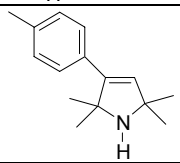
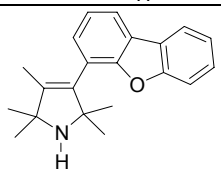
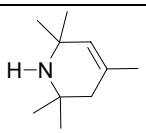
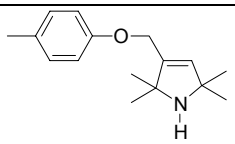
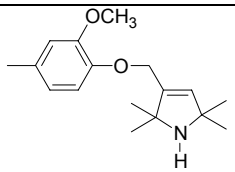
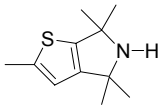
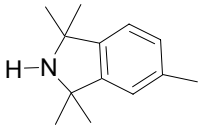
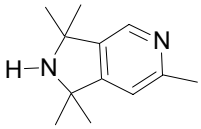
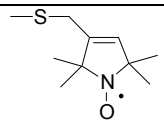
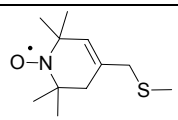
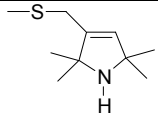
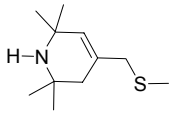
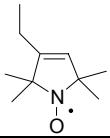
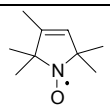
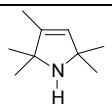
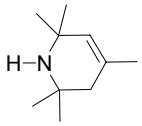
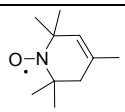
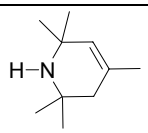


Cpd	R^4	R^5	R^6	R^7	PARP Inh. IC_{50} μM	Inh. Cell death IC_{50} μM	Antiox IC_{50} μM
3a	H		$CONH_2$	H	0.721	0.482±0.023	0.00048
3b	H		$CONH_2$	H	ND	11.141±1.120	0.0009
3c	H		$CONH_2$	H	0.201	8.21±0.51	0.0028
3d	H		$CONH_2$	H	1.5	0.240±0.0120	0.00125
3e	H		$CONH_2$	H	0.149	60.92±7.180	0.0112
3f	H		$CONH_2$	H	0.078	0.033±0.015	0.0072
3g	H		$CONH_2$	H	1.8	26.76±1.130	0.0053
3h	H		$CONH_2$	H	0.026	15.57±0.220	0.0096
3i	H		$CONH_2$	H	0.564	1.614±0.055	0.0132
3j	H		$CONH_2$	H	0.472	10.12±0.430	0.0141

Cpd	R^4	R^5	R^6	R^7	PARP Inh. IC_{50} μM	Inh. Cell death IC_{50} μM	Antiox IC_{50} μM
3k	H		$CONH_2$	H	3.4	40±1.680	0.027
3l	H		$CONH_2$	H	0.042	0.095±0.007	0.073
3m	H		$CONH_2$	H	0,049	3.15±0.081	0.081
3n	H		$CONH_2$	H	0.061	0.0094±0.002	0.113
4a	H		$CONH_2$	H	0.345	0.62±0.030	0.0021
4b	H		$CONH_2$	H	0.216	0.33±0.022	0.0031
4c	H		$CONH_2$	H	0.137	3.51±0.240	0.0034
4d	H		$CONH_2$	H	0.310	3.98±0.190	0.00038
4e	H		$CONH_2$	H	0.133	32.01±0.680	0.0134
4f	H		$CONH_2$	H	0.098	0.341±0.011	0.0092
4g	H		$CONH_2$	H	1.8	0.324±0.025	0.0053

Cpd	R^4	R^5	R^6	R^7	PARP Inh. IC_{50} μM	Inh. Cell death IC_{50} μM	Antiox IC_{50} μM
4h	H		$CONH_2$	H	0.014	0.098±0.009	0.0016
4i	H		$CONH_2$	H	0.572	1.614±0.074	0.0043
4j	H		$CONH_2$	H	0.432	0.101±0.019	0.0038
4k	H		$CONH_2$	H	0.354	6.92±0.530	0.0105
4o	H		$CONH_2$	H	0.450	0.19±0.0120	0.14
4p	H		$CONH_2$	H	0.47	0.08±0.020	0.15
13a	H		$CONH_2$	H	6	20.95±2.512	0.2
13b	H		$CONH_2$	H	5	12.67±1.457	0.18
14a	H		$CONH_2$	H	3	3.14±0.860	0.2
14b	H		$CONH_2$	H	4	5.57±0.765	0.35
15		H	$CONH_2$	H	10	1.985±0.071	0.0018

Cpd	R^4	R^5	R^6	R^7	PARP Inh. IC_{50} μM	Inh. Cell death IC_{50} μM	Antiox IC_{50} μM
21	H		CONH-*i*-Pr	H	10	3.347±0.584	0.0021
22	H		CONH-*i*-Pr	H	10	56.66±2.573	0.0023
17	H		H	H	No inh	No effect	0.0055
24	H		H	$CONH_2$	No inh.	28.31±1.64	0.17
25	H		H	$CONH_2$	No inh	No effect	0.045

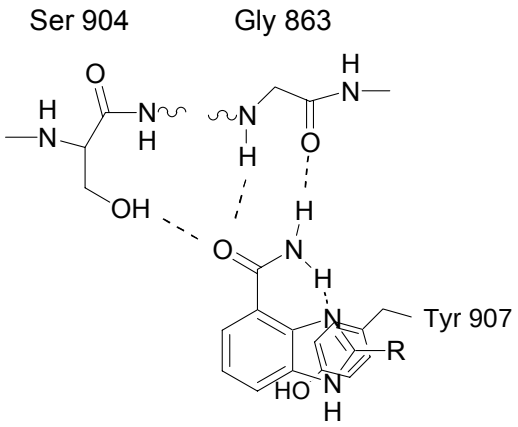


Figure 1: Interactions of 4-carboxamidobenzimidazoles with PARP-1 enzyme active site.

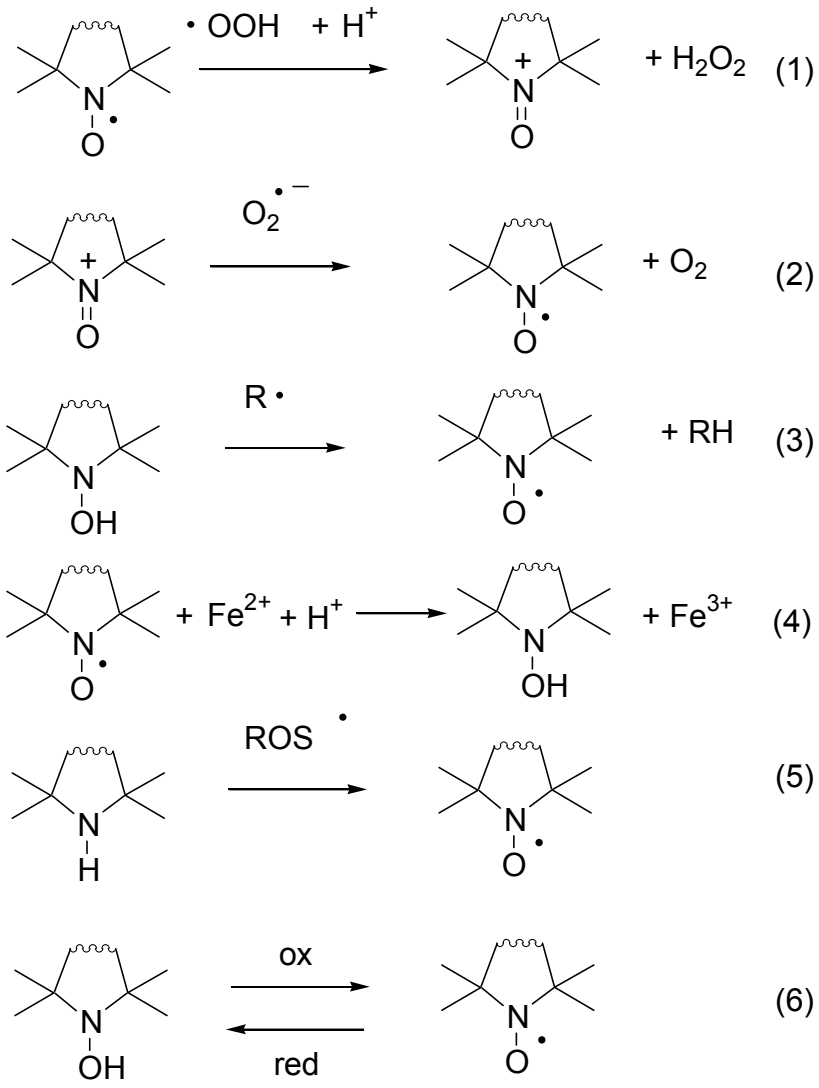


Figure 2. Possible radical scavenging mechanisms and transformations of nitroxides and pre-nitroxides.

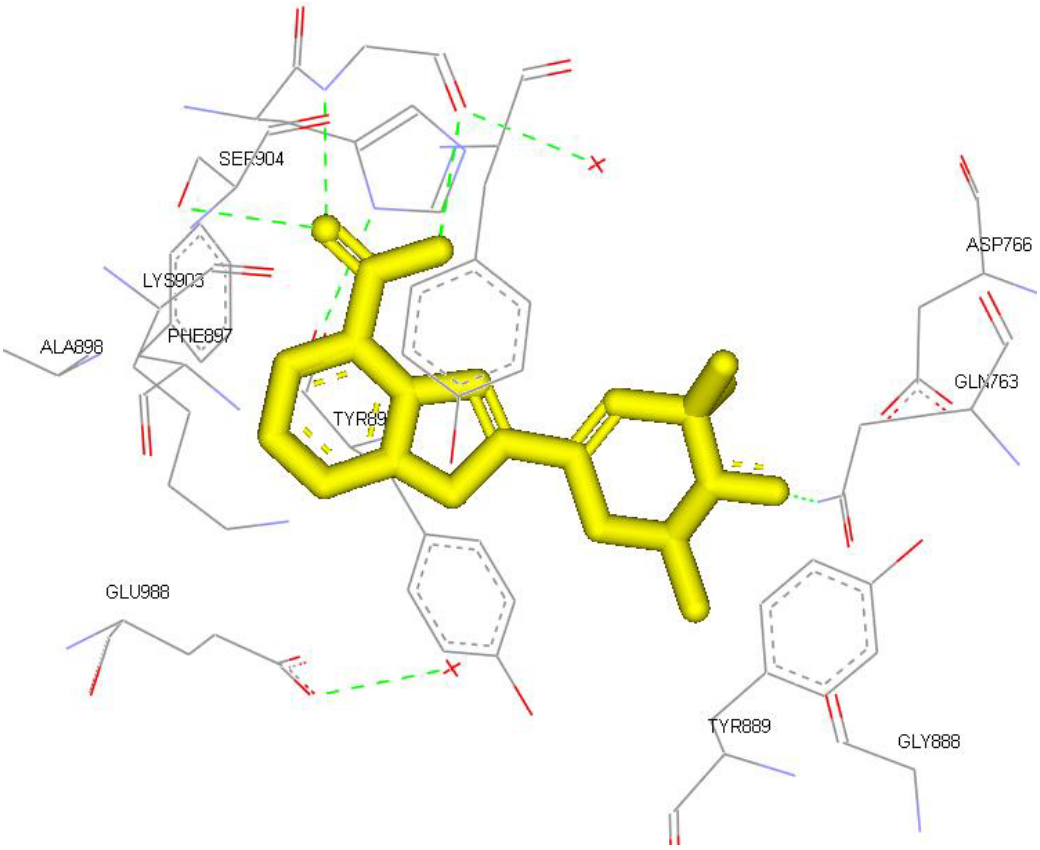


Figure 3: Compound **3h** (yellow) docked in the active site of PARP1 enzyme.

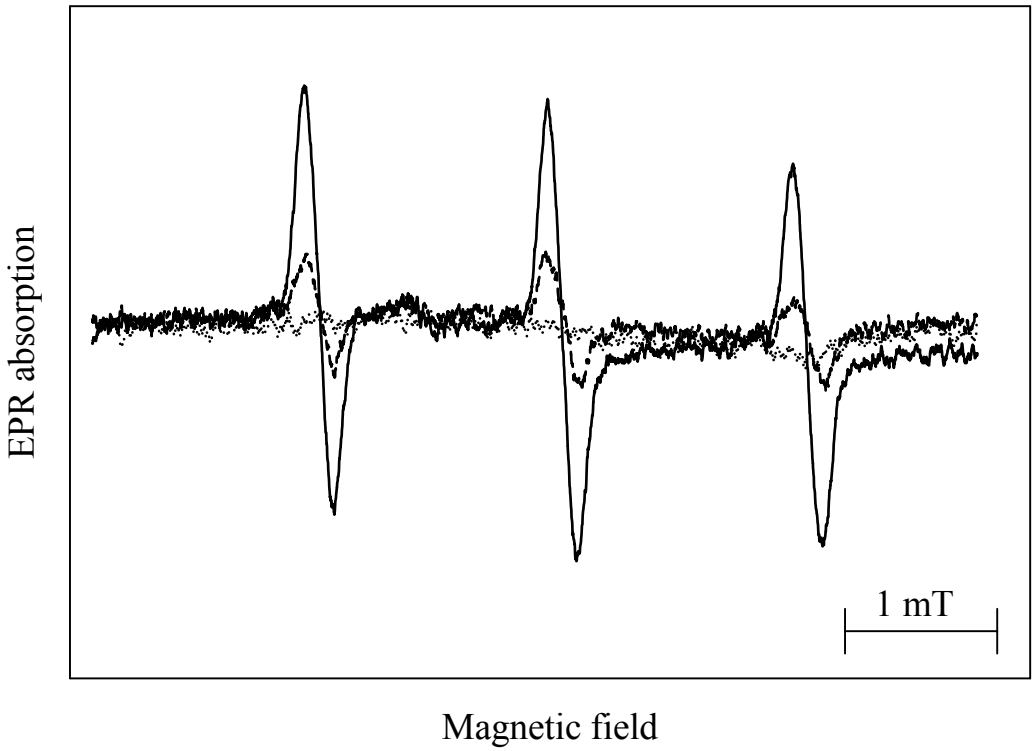


Figure 4: EPR spectra of collected urine before administration of **4h** (⋯⋯), EPR spectra of collected urine (between 0-4 hour) after administration of **4h** (----), EPR spectra of collected urine (between 0-4 hour) after administration of **4h** (——) and oxidation further with PbO2.

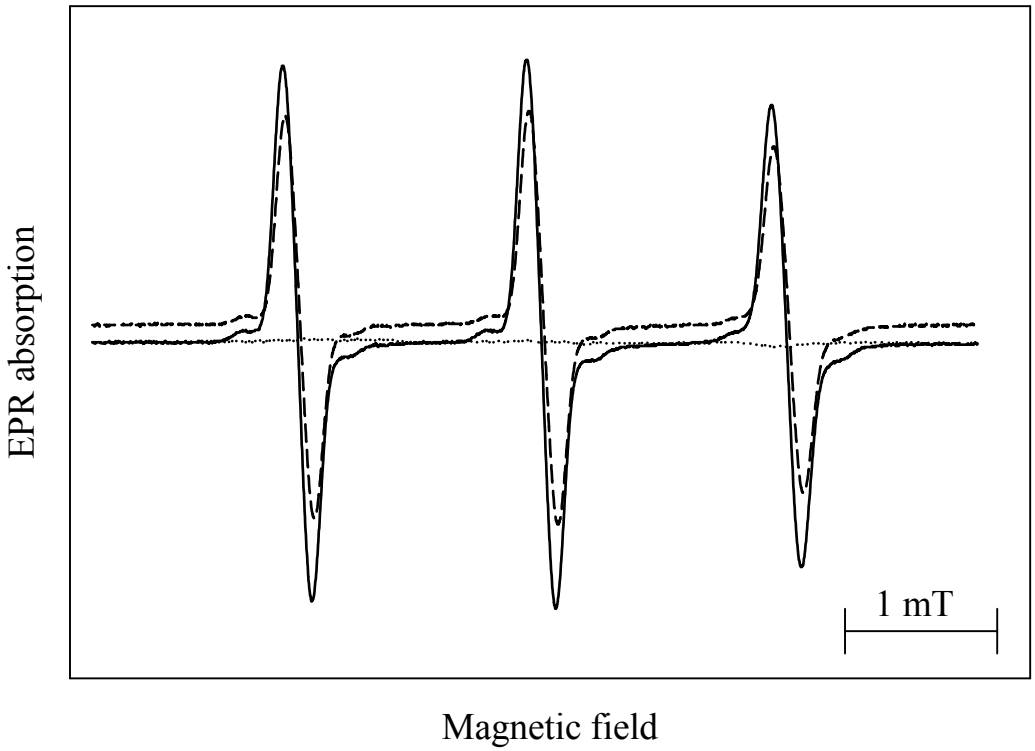
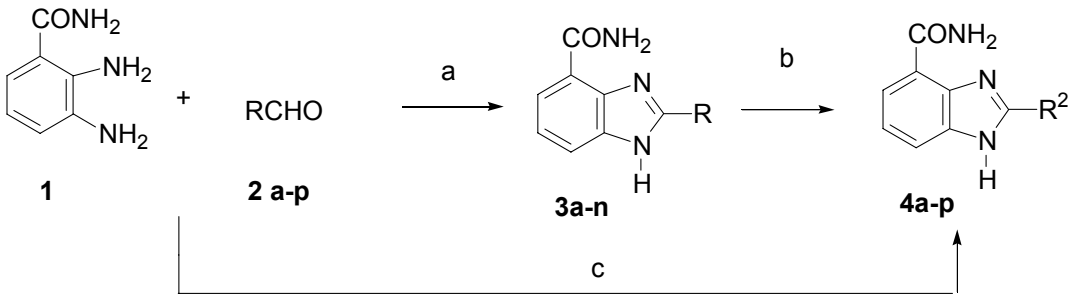


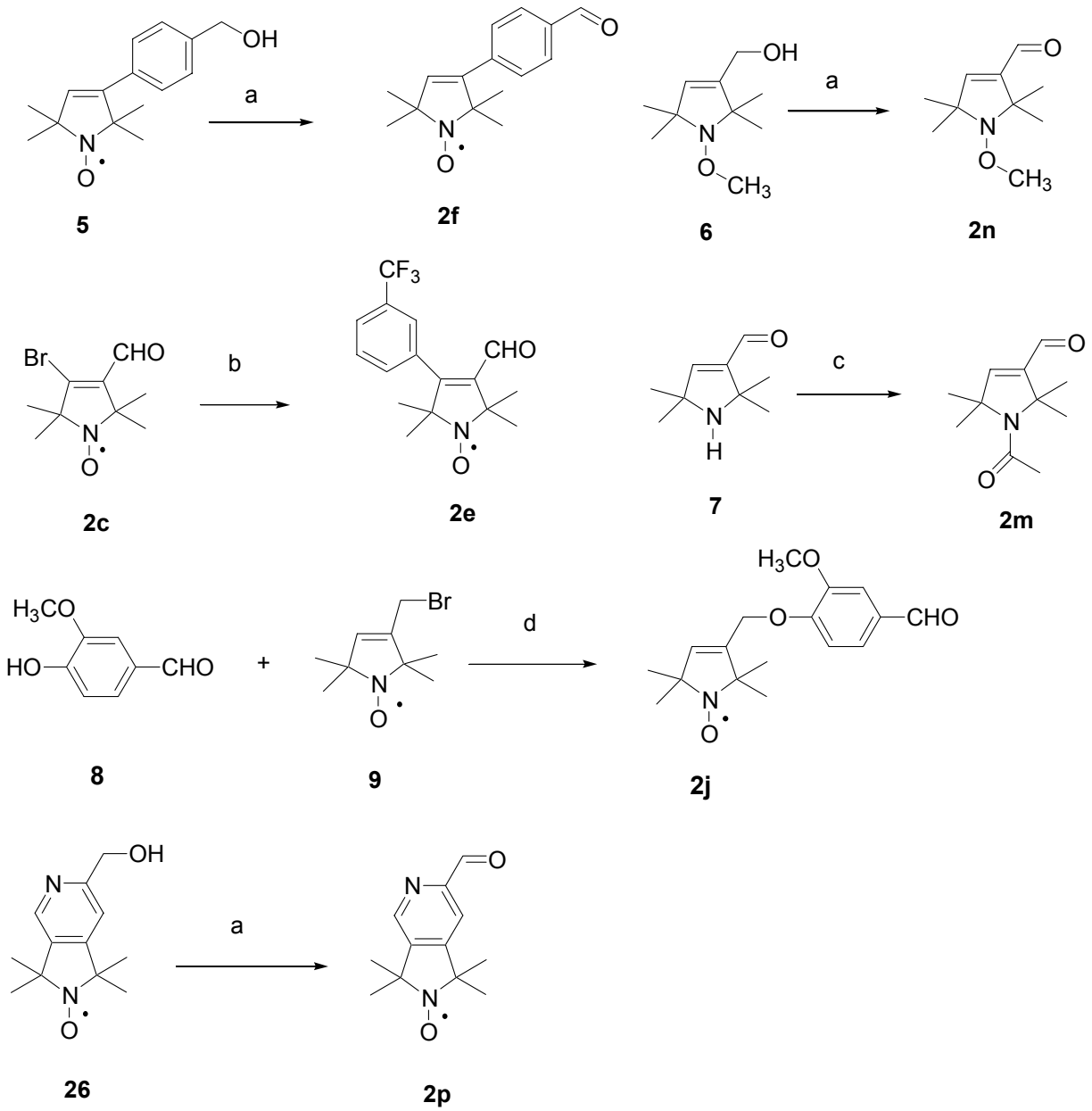
Figure 5: EPR spectra of collected urine before administration of **3h** (⋯⋯), EPR spectra of collected urine (between 0-4 hour) after administration of **4h** (----), EPR spectra of collected urine (between 0-4 hour) after administration of **4h** and (——) and oxidation further with PbO2.

Scheme 1. Synthesis of 2-substituted-4-carboxamidobenzimidazoles.[a]



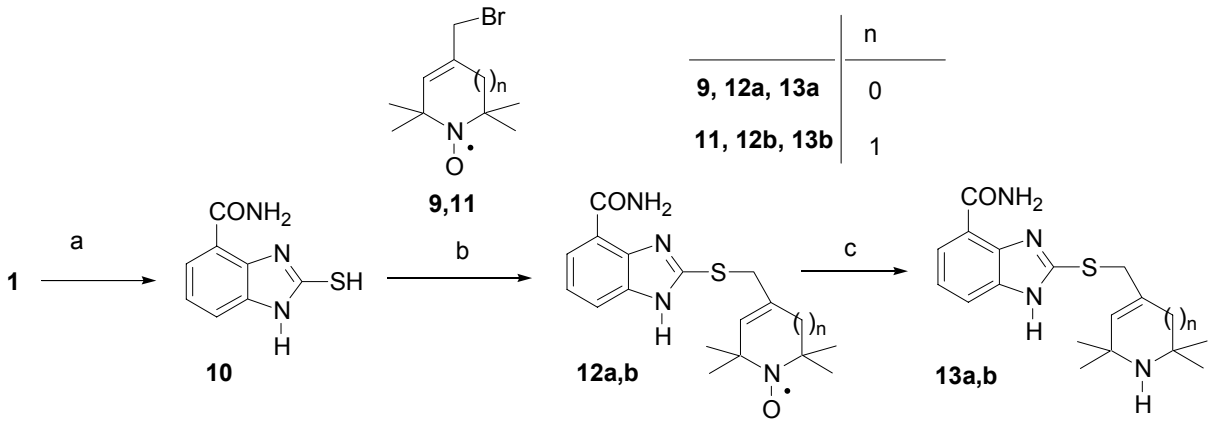
[a] Reagents and conditions: (a) (i) TosOH (cat.), benzene, reflux, 8h; (ii) activated MnO_2, $CHCl_3$, reflux, 2h; (b) Fe, AcOH, heated to reflux, 30 min.,then K_2CO_3 to pH=8; (c) $Na_2S_2O_5$, under N_2, sealed tube, DMF, 120 °C, 4h.

Scheme 2. Synthesis of new paramagnetic aldehydes.[a]



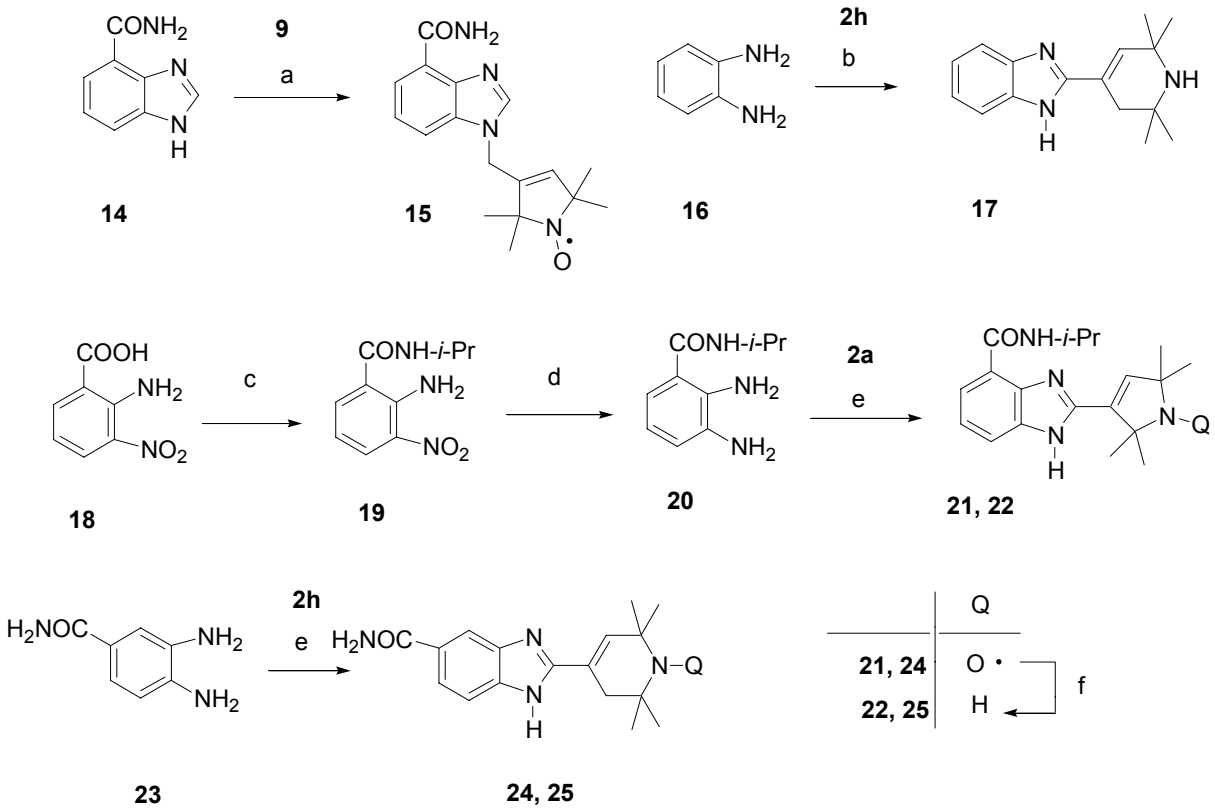
[a] Reagents and conditions: (a) activated MnO_2, $CHCl_3$, reflux, 2h; (b) 3-$(CF_3)C_6H_4B(OH)_2$, $Ba(OH)_2 \cdot 8\ H_2O$, $PdCl_2(PPh_3)_2$, dioxan/water 4:1, reflux, 5h; (c) AcCl, Et_3N, CH_2Cl_2, 0 °C to rt.; (d) acetone, K_2CO_3, reflux, 2h.

Scheme 3. Synthesis of 2-*S*-alkyl-4-carboxamidobenzimidazoles.[a]



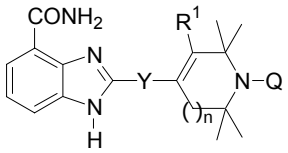
[a] Reagents and conditions: (a) THF, CS_2, NaOMe, reflux 1h then 12h rt; (b) MeOH, KOH, reflux, 2 h (c) Fe, AcOH, heated to reflux, 30 min., then K_2CO_3 to pH=8.

Scheme 4. Synthesis of isomers of 2-substituted-4-carboxamidobenzimidazoles.[a]



[a] Reagents and conditions: (a) **9**, K_2CO_3, acetonitrile, reflux, 3h (b) **2h**, $Na_2S_2O_5$, under N_2, sealed tube, DMF, 120 °C, 4h; (c) CDI, THF, reflux 15 min. then *i*-Pr-NH_2 (d) Pd/C, HCO_2NH_4, MeOH, under N_2, 40 °C, 2h; (e) (i) TosOH (cat.), benzene, reflux, 8h; (ii) activated MnO_2, $CHCl_3$, reflux, 2h; (f) Fe, AcOH, heated to reflux, 30 min., then K_2CO_3 to pH=8.

Table of Contents Graphic



n: 0, 1 R^1: H, Ph, Br

Y: