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Original article

An elastase activity reporter for Electronic Paramagnetic Resonance (EPR) and Overhauser-enhanced Magnetic Resonance Imaging (OMRI) as a line-shifting nitroxide

Natacha Jugniot[a,1], Indranil Duttagupta[b,1], Angélique Rivot[a], Philippe Massot[a], Colleen Cardiet[a], Anne Pizzoccaro[c], Marion Jean[d], Nicolas Vanthuyne[d], Jean-Michel Franconi[a], Pierre Voisin[a], Gilles Devouassoux[c], Elodie Parzy[a], Eric Thiaudiere[a], Sylvain R.A. Marque[b,e], Abderrazzak Bentaher[c], Gérard Audran[b], Philippe Mellet[a,f,*]

[a] Centre de Résonance Magnétique des Systèmes Biologiques, UMR5536, CNRS, Université de Bordeaux, F-33076 Bordeaux, France
[b] Aix Marseille Univ., CNRS, ICR, UMR 7273, case 551, Avenue Escadrille Normandie-Niemen, 13397 Marseille Cedex 20, France
[c] Equipe "Inflammation et Immunité de l'Epithélium Respiratoire" - EA7426 Faculté de Médecine Lyon Sud, 165, Chemin du Grand Revoyet, 69495 Pierre Bénite, France
[d] Aix Marseille Univ., CNRS, Centrale Marseille, iSm2, Marseille, France
[e] Vorozhtsov Novosibirsk Institute of Organic Chemistry SB RAS, Pr. Lavrentjeva 9, 630090 Novosibirsk, Russia
[f] INSERM, 33076 Bordeaux Cedex, France

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ABSTRACT

Pulmonary inflammatory diseases are a major burden worldwide. They have in common an influx of neutrophils. Neutrophils secrete unchecked proteases at inflammation sites consequently leading to a protease/inhibitor imbalance. Among these proteases, neutrophil elastase is responsible for the degradation of the lung structure *via* elastin fragmentation. Therefore, monitoring the protease/inhibitor status in lungs non-invasively would be an important diagnostic tool.

Herein we present the synthesis of a MeO-Suc-$(Ala)_2$-Pro-Val-nitroxide, a line-shifting elastase activity probe suitable for Electron Paramagnetic Resonance spectroscopy (EPR) and Overhauser-enhanced Magnetic Resonance Imaging (OMRI). It is a fast and sensitive neutrophil elastase substrate with $K_m = 15 \pm 2.9\,\mu M$, $k_{cat}/K_m = 930{,}000\,s^{-1}\,M^{-1}$ and $K_m = 25 \pm 5.4\,\mu M$, $k_{cat}/K_m = 640{,}000\,s^{-1}\,M^{-1}$ for the *R* and *S* isomers, respectively. These properties are suitable to detect accurately concentrations of neutrophil elastase as low as 1 nM. The substrate was assessed with broncho-alveolar lavages samples derived from a mouse model of *Pseudomonas* pneumonia. Using EPR spectroscopy we observed a clear-cut difference between wild type animals and animals deficient in neutrophil elastase or deprived of neutrophil Elastase, Cathepsin G and Proteinase 3 or non-infected animals.

These results provide new preclinical *ex vivo* and *in vivo* diagnostic methods. They can lead to clinical methods to promote in time lung protection.

1. Introduction

Pulmonary inflammatory diseases represent a major health concern worldwide as well as an economic burden. They include asthma, cystic fibrosis (CF), chronic obstructive pulmonary disorder (COPD) (e.g., emphysema), acute respiratory distress syndrome and alpha-1-anti-trypsin deficiency. For instance, COPD alone concerns a population estimated to 175 million people and accounts for 3.2 million deaths ranking it to the fifth cause of mortality worldwide [1]. Among the precipitating factors for disease development are tobacco smoking, urban air pollution and wood fire smoke. A common denominator of pulmonary inflammatory diseases is the high neutrophil influx as seen in CF or during the exacerbation phase of COPD. At inflammation sites, neutrophils discharge four serine proteases into the extracellular environment whose concentrations surpass that of their corresponding physiologic inhibitors resulting in protease/anti-protease imbalance

* Corresponding author.
E-mail addresses: eric.thiaudiere@rmsb.u-bordeaux.fr (E. Thiaudiere), sylvain.marque@univ-amu.fr (S.R.A. Marque), azzak.bentaher@inserm.fr (A. Bentaher), g.audran@univ-amu.fr (G. Audran), philippe.mellet@rmsb.u-bordeaux.fr (P. Mellet).
[1] These authors contributed equally to this work.

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causing tissue damage, hence progressive loss of lung functions.

Neutrophil elastase (NE), a potent protease, was shown to be the main tissue-destructive actor because of its large repertoire of substrate including structural proteins particularly elastin [2,3], a major lung structural protein, although the four neutrophil proteases can act synergistically [4].

Therapeutically, lungs protection needs an adapted treatment with protease inhibitors. However, preclinical research to setup such protocols is impaired by the absence of a reliable imaging method to localize deleterious enzyme activities in order to assess the actual protease/antiprotease balance status before and after treatment. Ultimately, an elastase activity imaging method valid for humans would detect lung inflammation long before any irreversible tissue damage could occur. Thus a treatment with inhibitors [5] or a change in habits could be proposed "in time" to save the lungs.

Molecular imaging of the proteolytic activity is most easily done using internally quenched fluorescent substrates. These substrates have good enzymatic constants because they can encompass both the P and P′ regions in the Schechter and Berger nomenclature [6]. There are however several drawbacks of this method: substrate fluorescence quenching is not complete thus causing long waiting times to eliminate nonspecific "blinding" light, light tissue penetration is limited and prevents imaging of deeply seated tissues or skull and three-dimensional images are obtained by reconstruction.

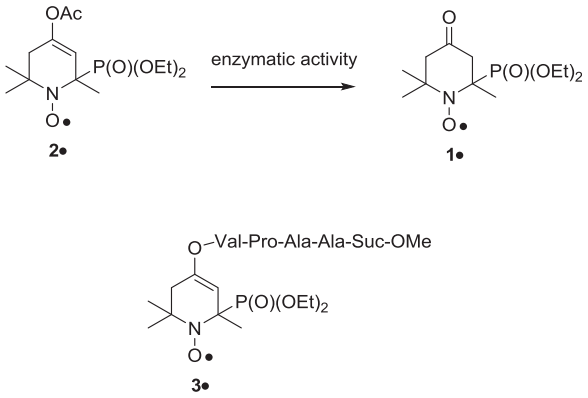
Magnetic Resonance Imaging (MRI) methods have a superior true 3D space encoding and use wavelengths that only weakly interfere with tissues. Electronic Paramagnetic Resonance (EPR) is a sensitive free radical detection method suitable in visible light-opaque media. Stable free radicals such as nitroxides or trityls can be detected or imaged in vitro and in vivo. Since unpaired electrons are particularly sensitive to the electronic environment some have been designed to display resonance line broadening or shifting to detect various parameters. Hence free radicals have been used for oximetry [7,8], redox status imaging [9,10], pH measurement [11–14], water content measurement [15] or to report on enzymatic activity [16–18]. EPR imaging (EPRI) can be used with these free radicals. Unfortunately, due to the very fast relaxation of free electrons EPRI still remains slow and insufficiently resolved. Magnetic Resonance Imaging (MRI) is the method of choice to deliver exquisite anatomical details but its low sensitivity so far prevented molecular imaging such as enzyme activity imaging. Interestingly, a line shifting substrate can also be monitored by Overhauser-enhanced Magnetic Resonance Imaging (OMRI). OMRI is an emerging imaging method designed to enhance NMR sensitivity. It is a double resonance experiment transferring a part of the higher spin polarization of an unpaired electron to the environing water protons (through the electron-proton Overhauser effect) which enhances the MRI signal that appears brighter [19].

It has been shown recently that OMRI at 0.2 T was able to reveal brain tumors in mice models of glioma through intravenous injection of a nonspecific nitroxide with high contrast on three-dimensional images [20]. Moreover, a nonspecific prototype of the line-shifting nitroxide later described in this study was able to reveal stomach and intestinal enzymatic activity [18] (see reaction in Scheme 1). Both studies showed that high contrast and high resolution images are possible in mice with short recording times.

In this paper the synthesis of MeO-Suc-$(Ala)_2$-Pro-Val-nitroxide (molecule 3• in Scheme 1), the first of a new family of dedicated protease substrates based on line-shifting nitroxides is reported. Its catalytic properties with neutrophil elastase and various enzymes were studied using EPR spectroscopy.

The substrate was probed in broncho-alveolar lavages from a mouse model of *Pseudomonas aeruginosa* lung infection with wild type and several mice knocked-out for neutrophil serine proteases.

It is also shown that this substrate is suitable for Overhauser-enhanced Magnetic Resonance Imaging.



Scheme 1. Enzymatic activity on enol acetate 2• releasing 1•. Elastase targeting substrate 3•.

2. Material and methods

2.1. Organic synthesis

2.1.1. General remarks

1H nuclear magnetic resonance (NMR) spectra were recorded using an internal deuterium lock at ambient temperatures on the following instruments: Bruker AC 400 (400 MHz) and Bruker AC 300 (300 MHz). Data are presented as follows: chemical shift (in ppm), integration, multiplicity (*s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet, *br* = broad, *dd* = doublet of doublets), coupling constant (*J* in Hz) and integration. ^{31}P NMR spectra were recorded on a Bruker AC 300 (122 MHz) and on a Bruker AC 400 (162 MHz) spectrometers with complete proton decoupling. Chemical shifts (δ) were reported in ppm using TMS as internal reference for ^{1}H and ^{13}C NMR spectra, and 85% H3PO4 for ^{31}P NMR spectra. High-resolution mass spectra (HRMS) were performed on a SYNAPT G2 HDMS (Waters) spectrometer equipped with atmospheric pressure ionization source (API) pneumatically assisted. Samples were ionized by positive electrospray mode as follows: electrospray tension (ISV): 2800 V; opening tension (OR): 20 V; nebulization gas pressure (nitrogen): 800 L/h. Low resolution mass spectra were recorded on ion trap AB SCIEX 3200 QTRAP equipped with an electrospray source. The parent ion (M^+, $[M+H]^+$, $[M+Na]^+$ or $[M+NH_4]^+$) is quoted. Analytical thin layer chromatographies (TLC) were carried out on Merck Kieselgel 60 F254 plates. Flash column chromatographies were carried out on Merck Kieselgel 60 (230–400 mesh). Solvent system: gradients of DCM/MeOH; EtOAc/EtOH. All experiments were performed under anhydrous conditions and an inert atmosphere of argon and, except where stated, using dried apparatus and employing standard techniques for handling air-sensitive materials. For EPR measurements, samples with 0.5 mM concentration of nitroxide were prepared in non-degassed solvents. Experiments were performed indifferently on Elexsys, EMX or ER 100D Bruker machines (a difference smaller than 0.1 G was noticed). EPR spectra were recorded with a gain of 2 105 (72 dB for Elexsys), a modulation amplitude of 1.0 G, a sweep width of 150 G, a sweep time of 21 s, and a power of 20 mW.

2.1.2. (9H-fluoren-9-yl)methyl-(S)-2-(((S)-1-(benzyloxy)-3-methyl-1-oxobutan-2-yl)carbamoyl) pyrrolidine-1-carboxylate (2)

DIPEA (2 mL, 11.5 mmol) was added dropwise to a stirred suspension of L-Val-OBn·HCl (2.8 g, 11.5 mmol) in dichloromethane (30 mL) at room temperature under an atmosphere of nitrogen. On dissolution, the solution was cooled to 0 °C and Fmoc-L-Pro (4.26 g, 12.6 mmol) and 1-hydroxybenzotriazole (1.86 g, 13.8 mmol) were added successively, each in one portion. The suspension was stirred at 0 °C for a further 15 min, and then DCC (2.85 g, 13.8 mmol) was added in one portion. The mixture was allowed to warm to room temperature over the course