ELSEVIER

International Journal of Pharmaceutics 104 (1994) 151–156

international journal of pharmaceutics

The effect of mesna in reversing, in vitro, the protease-antiprotease imbalance: Its reaction on the MPO-H_2O_2 system and on human leukocyte elastase

Bernard Gressier *, Sophie Lebegue, Claude Brunet, Thierry Dine, Michel Luyckx, Micheline Cazin, Jean Claude Cazin

Faculté des Sciences Pharmaceutiques et Biologiques, Laboratoire de Pharmacologie, Pharmacocinétique et Pharmacie Clinique, Université de Lille II, rue du Professeur Laguesse, B.P. 83, 59006 Lille Cedex, France

(Received 19 July 1993; Accepted 22 September 1993)

Abstract

During inflammatory disorders, in the lung, potentially destructive reactive oxygen species and proteases are produced by activated phagocytic cells. These oxidants and proteases released during inflammation may cause pulmonary tissue injuries either directly or indirectly, through inactivation of α_1-proteinase inhibitor (α_1PI) by a disequilibrium of the protease-antiprotease balance. This inactivation of α_1PI is the result of both oxidation by the myeloperoxidase (MPO)-hydrogen peroxide (H_2O_2) system and a proteolytic process especially by leukocyte elastase. It was demonstrated, in vitro, that mesna, a mucolytic drug, can protect α_1PI against inactivation by stimulated polymorphonuclear neutrophils (PMNs). Mesna, at low concentrations, protected α_1PI against oxidative inactivation by the MPO-H_2O_2 system with a scavenging effect on H_2O_2, whereas a protecting effect against leukocyte elastase was only achieved at high concentrations. This suggests that mesna might reverse the oxidant-antioxidant imbalance by protecting α_1PI from the oxidative inactivation but might not completely reverse the protease-antiprotease imbalance with the usual concentrations, in vivo, via aerosol.

Key words: Mesna; Neutrophil; Leukocyte elastase; Proteolysis; Myeloperoxidase-H_2O_2 system; α_1-Proteinase inhibitor; Antioxidant; Antielastase activity; Lung

1. Introduction

Polymorphonuclear neutrophils (PMNs) play a central role in acute inflammatory processes (Weiss, 1989). Their functions are based partly on their phagocytic capacity and partly on their ability to liberate potent proteinases (elastase, collagenase, etc.) and large amounts of reactive oxygen species as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) via the myeloperoxidase (MPO)-H_2O_2 system. Neutrophil elastase has been shown to digest every major element of the lung's extracellular matrix. Moreover, this protease has been shown to produce emphysema

* Corresponding author. Tel: 20.96.40.40 poste 4364; Fax: 20.96.97.52.

0378-5173/94/$07.00
SSDI 0378-5173(93)E0284-W

in animals; by contrast, installation of collagenases results in minimal structural alterations in the lungs of animals (Gadek and Pacht, 1990).

In the lung, the major inhibitor of leukocyte elastase is α_1-proteinase inhibitor (α_1PI) (Travis and Johson, 1981; Crystal, 1990). However, α_1PI is rapidly oxidized, at the critical methionine residue, by HOCl generated by the MPO-H_2O_2 system (Wallaert et al., 1991), losing its ability to inhibit elastase (Weiss, 1989); thus, the uninhibited action of human leukocyte elastase (HLE) can generate tissue destruction in the lung.

Usually, the lungs exist in an oxygen-rich environment delicately balanced between toxicity of oxidants and protective activities of several intracellular and extracellular antioxidant defense systems. Disequilibrium, either through an increase in oxidant stress or through a compromise of antioxidant resources, can initiate a series of physiopathologic events and may lead eventually to emphysema (Hoidal and Niewoehner, 1983). Moreover, Hubbard et al. (1991) recently demonstrated that nonsmoking α_1PI-deficient individuals with emphysema have more PMNs in bronchoalveolar lavage (BAL) than normal subjects, suggesting that destruction of the alveolar walls occurs with the chronic presence of neutrophils within the lungs.

Over the last few years, several experimental studies have demonstrated some antioxidant properties of several drugs such as *N*-acetylcysteine (NAC) (Bernard, 1991), enprofylline (Kaneko et al., 1990) and fenspiride (Carre et al., 1991). In particular, for NAC, antioxidant properties were conferred by a thiol group (Bernard, 1991). However, during inflammatory disorders, especially in the lung, generation of tissue injuries may be a result of both oxidation and proteolytic processes with the presence of neutrophils (Gadek and Pacht, 1990).

Thus, a drug which can decrease elastase production by PMNs and reduce the production of reactive oxygen metabolites (MPO-H_2O_2 system) by PMNs may reverse the protease-antiprotease imbalance in the lung either directly or indirectly via the oxidant-antioxidant balance by protecting α_1PI against oxidative inactivation.

In this paper, the capacity of a mucolytic agent, mesna (sodium 2-mercaptoethanesulfonate), to reverse, in vitro, the protease-antiprotease imbalance, by inhibiting elastase and the MPO-H_2O_2 system, has been investigated.

2. Materials and methods

2.1. Purification of α_1PI

Human α_1PI was purified from pooled plasma of healthy donors, using the immunoaffinity chromatography procedure of Sugiura et al. (1981).

2.2. Isolation of human polymorphonuclear neutrophils (PMNs)

PMNs were purified from the fresh heparinized venous blood of healthy human subjects by standart techniques of centrifugation through Ficoll (Histopaque, Sigma Chemical), dextran sedimentation and sodium chloride haemolysis (Boyum, 1968).

PMNs isolated through this technique were always $> 95\%$ viable as determined by trypan blue exclusion, and $> 95\%$ of cells were PMNs. Cells were suspended in Hanks' Hepes pH 7.4 buffer.

2.3. Evaluation of the ability of mesna to protect α_1PI against inactivation by stimulated PMNs

α_1PI (0.37 μM) was incubated for 45 min at 37°C with PMNs (2×10^6), PMA (160 nm) (Wallaert et al., 1991), and various concentrations of mesna (10 μM–300 mM) (Sigma Chemical).

Supernatants were overloaded with bovine serum albumin (BSA) for a final concentration of 200 mg/l in order to prevent inactivation of α_1PI by freezing (Wallaert et al., 1991). Samples were kept frozen (−20°C) until determination of elastase inhibitory capacity (EIC) as described below.

The EIC of α_1PI was determined using an ABA 100 analyser (Abbot Bichromatic Analyser) (El Yamani et al., 1986). First, 25 μl of sample and 250 μl of porcine pancreatic elastase (PPE) (3.35×10^{-8} M in 0.2 M Tris-HCl pH 8 buffer) (Biosys SA) were mixed. After 20 min incubation

at 30°C, 250 μl of specific substrate Suc-$(Ala)_3$-pNA (2 mM in 0.2 M Tris-HCl pH 8 buffer) (Biosys SA) were added to the mixture. The EIC of each sample was estimated by subtraction of its measured activity from an elastase reference value obtained with a 200 mg/l BSA solution. The effects of mesna were expressed as the ability to protect α_1PI against inactivation by PMNs.

Results were expressed as the percentage of the functional α_1PI as follows:

$$\%\ \text{functional}\ \alpha_1\text{PI} = \frac{\text{EIC}_{\text{studies}\ \alpha_1\text{PI}}}{\text{EIC}_{\text{control}\ \alpha_1\text{PI}}} \times 100$$

where $\text{EIC}_{\text{control}\ \alpha_1\text{PI}}$ denotes the EIC of α_1PI incubated without PMNs and $\text{EIC}_{\text{studies}\ \alpha_1\text{PI}}$ is the EIC of α_1PI incubated in the presence of PMNs.

2.4. Evaluation of the ability of mesna to protect α_1PI against oxidation by the MPO-H_2O_2 system

α_1PI (0.37 μM) was incubated for 45 min at 37°C with H_2O_2 (12.5 μM), MPO (25 mU) (Calbiochem), NaCl (25 μM) and various concentrations of mesna (10–1000 μM) (Sigma Chemical). Tubes were overloaded with BSA for a final concentration of 200 mg/l in order to prevent inactivation of α_1PI by freezing (Wallaert et al., 1991). Samples were kept frozen (−20°C) until EIC determination as described previously.

2.5. Scavenging of human leukocyte elastase (HLE)

Initially, in order to calculate the HLE concentration released by 2×10^6 PMNs, cells were incubated for 30 min at 37°C with PMA (160 nm). Then, 400 μl of supernatant and 400 μl of specific substrate methoxy-Suc-$(Ala)_2$-Pro-Val-pNA (0.5 mM in 0.1 M Hepes-0.5 M NaCl pH 7.5 buffer) (Sigma Chemical) were mixed in a final volume of 1 ml. After 5 min incubation at 37°C, the reaction was stopped with phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical) and absorbance was measured at 405 nm in a spectrophotometer (Kontron Uvikon 860). HLE concentration was based on a standard curve using various concentrations of reagent grade HLE (Biosys SA).

Subsequently, since PMA stimulated PMNs release 20 nM ELH, 400 μl of HLE (20 nm in 0.1 M Hepes-0.5 M NaCl pH 7.5 buffer containing 0.05% Brij) (Biosys SA) and 50 μl of increasing concentrations of mesna (1–300 mM) in 0.1 M Hepes-0.5 M NaCl pH 7.5 buffer were incubated for 15 min at 37°C in a final volume of 500 μl.

Table 1
Protection of α_1PI against inactivation by stimulated PMNs

Supplements	EIC studies (%)	Inhibited elastase activity (%)
Buffer alone	0	0
Mesna (M) 1000 μM	1.9 ± 0.5	1.1 ± 0.3
α_1PI + M	167 ± 0.5	99.4 ± 0.3
α_1PI + PMNs + PMA	1.8 ± 0.9	1.1 ± 0.5
α_1PI + PMNs + PMA + catalase	116 ± 12.7	69.3 ± 7.6
α_1PI + PMNs + PMA + M (300 mM)	161 ± 15.8	95.8 ± 9.2
α_1PI + PMNs + PMA + M (200 mM)	149 ± 14.1	88.7 ± 8.1
α_1PI + PMNs + PMA + M (100 mM)	135 ± 13.2	80.4 ± 7.9
α_1PI + PMNs + PMA + M (1 mM)	117 ± 12.9	69.6 ± 7.2
α_1PI + PMNs + PMA + M (500 μM)	111 ± 10.4	66.1 ± 6.1
α_1PI + PMNs + PMA + M (250 μM)	54.5 ± 8.2	32.4 ± 4.3
α_1PI + PMNs + PMA + M (100 μM)	4.2 ± 1.1	2.5 ± 0.6
α_1PI + PMNs + PMA + M (50 μM)	1.8 ± 0.9	0.01 ± 0.005
α_1PI + PMNs + PMA + M (25 μM)	1.3 ± 0.5	0.007 ± 0.003
α_1PI + PMNs + PMA + M (10 μM)	0.8 ± 0.5	0.004 ± 0.002

$\text{EIC}_{\text{control}\ \alpha_1\text{PI}} = 168$. 100% functional α_1PI indicates that 100% elastase was inhibited by α_1PI. Supplements were incubated in a total volume of 1 ml for 45 min at 37°C. Then, 25 μl was taken for assay of elastase inhibitory capacity as described in section 2. Results were expressed as % of the inhibited elastase activity in the same experiment. Number of experiments was $n = 4$.

Then, as described above, 400 μl of the same specific substrate were added to the mixture and after 5 min incubation at 37°C, the reaction was stopped. The absorbance of each sample was measured at 405 nm against a reference cuvette containing substrate, increasing amounts of mesna and buffer. The concentration of HLE was based on a standard curve using various concentrations of reagent grade HLE (Biosys SA). Results were expressed as the percentage of HLE inhibited by mesna.

3. Results and discussion

3.1. Cellular viability after mesna reaction

In order to control cellular viability after mesna reaction, the viability test by trypan blue exclusion was carried out at the end of every cellular experiment. Mesna (10 μM–300 mM) was found to have no effect on cellular viability.

3.2. Protection of α_1PI by mesna (10 μM–300 mM) against inactivation by stimulated PMNs

When α_1PI was incubated with PMA stimulated PMNs, complete inactivation of α_1PI was observed (Table 1). This inactivation is the result of oxidation (MPO-H_2O_2 system) and proteolysis (HLE) by PMNs as previously reported (Wallaert et al., 1991).

Table 2
Inactivation of α_1PI by the MPO-H_2O_2 system

Supplements	EIC studies (%)	Inhibited elastase activity (%)
Buffer alone	0	0
MPO	2.9 ± 0.4	1.5 ± 0.2
H_2O_2	0.9 ± 0.2	0.5 ± 0.1
MPO + H_2O_2	1.9 ± 0.2	1 ± 0.1
Mesna (1000 μM)	2.8 ± 0.8	5 ± 0.4
α_1PI + mesna (1000 μM)	196 ± 2.3	99.5 ± 1.2
α_1PI + MPO	194 ± 3.5	98.5 ± 1.8
α_1PI + H_2O_2	196 ± 3.5	99.8 ± 1.8
α_1PI + MPO + H_2O_2	6.1 ± 0.5	3.1 ± 0.3

$EIC_{control\ \alpha_1PI} = 197$. 100% functional α_1PI indicates that 100% elastase was inhibited by α_1PI. Supplements were incubated in a total volume of 1 ml for 45 min at 37°C. Then, 25 μl was taken for assay of elastase inhibitory capacity as described in section 2. Results were expressed as % of the inhibited elastase activity in the same experiment. Number of experiments was $n = 4$.

Addition of catalase (antioxidant) was associated with a significant but incomplete protection of α_1PI, which corresponds with a protective effect against oxidation by the MPO-H_2O_2 system. Addition of low concentrations of mesna (10–1000 μM) was associated with a similar protective effect to that of catalase and the 50% inhibitory concentration (IC_{50}), calculated with a computer program (Boniface et al., 1972), was 340 μM; in contrast, addition of high concentrations of mesna (1–300 mM) was associated with complete protection of α_1PI (oxidation and proteolysis).

3.3. Protection of α_1PI by mesna (10–1000 μM) against oxidation by the MPO-H_2O_2 system

Initially, the assay was set up for measuring inactivation of α_1PI by the MPO-H_2O_2 system. α_1PI was incubated with MPO and supplements for inactivation to take place and activity of α_1PI was measured as the ability to inhibit elastase activity. As seen in Table 2, inactivation of α_1PI was dependent on the complete system containing MPO and H_2O_2. Indeed, the absence of one of these compounds resulted in failure to inactivate α_1PI. Mesna (10–1000 μM) was found to have a direct effect on neither the activity of elastase nor the ability of α_1PI to inhibit elastase activity.

As seen in Fig. 1, when α_1PI was incubated with H_2O_2, MPO and mesna in various concentrations (10–1000 μM) at 37°C for 45 min, this agent was able to protect α_1PI from inactivation by the MPO-H_2O_2 system. This protection was nearly complete for concentrations > 250 μM of mesna and the concentration at which 50% protection occurred was 55 μM.

The mechanism which governs mesna to protect α_1PI against the MPO-H_2O_2 system may be a result of either MPO inhibition or reduced production of hypochlorous acid (HOCl). The latter possibility seems to be more probable, since mesna reacts by scavenging H_2O_2 directly as previously reported (Gressier et al., 1993). This ac-

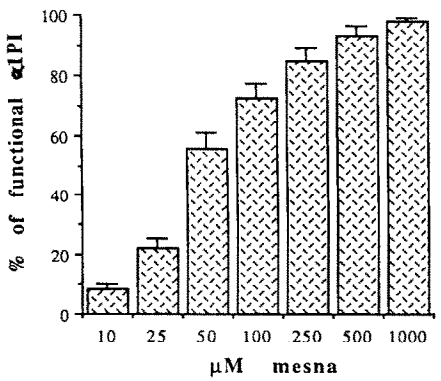

Fig. 1. Protection against MPO mediated inactivation of α_1PI by mesna. α_1PI incubated with the complete MPO-H_2O_2 system and increasing concentrations of mesna was protected in a dose-dependent manner. $IC_{50} = 55$ μM (* mean ± SD with 4 separate experiments).

tion may be complemented by the direct scavenging of HOCl, as in the case of ergothioneine (Akanmu et al., 1991).

3.4. Scavenging of human leukocyte elastase (HLE) by mesna

When purified HLE was incubated with mesna, inhibition of its amount was observed to occur in a dose-dependent manner, as shown in Fig. 2, with an IC_{50} of 200 mM. This significant decrease in elastase activity is consistent with results previously reported on elastase from crude PMN extracts incubated with mesna (Stolk et al., 1986). This scavenging effect of HLE by mesna may explain the protection of α_1PI against proteolysis by HLE released from stimulated human PMNs.

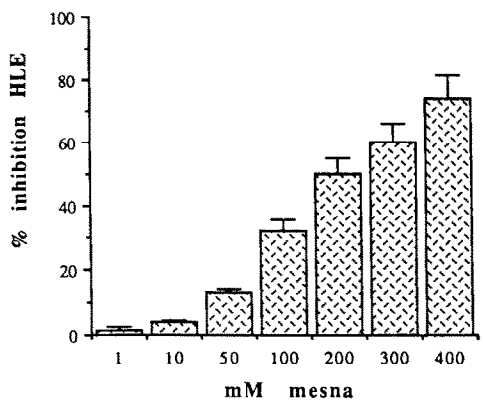

Fig. 2. Scavenging of human leukocyte elastase (HLE). HLE incubated with increasing concentrations of mesna was inhibited in a dose-dependent manner. $IC_{50} = 200$ mM (* mean ± SD with 4 separate experiments).

Therefore, complete protection of α_1PI by mesna against inactivation by PMNs may be a result of both a protective effect against the MPO-H_2O_2 system by scavenging H_2O_2, this effect being associated with low concentrations of mesna, and a protective effect against the proteolysis by HLE, the latter effect being associated with high concentrations of mesna.

4. Conclusion

α_1PI, an agent of major importance in the defense against proteolytic tissue injury (Carell, 1986), is inactivated by secretory products of human phagocytes (Henson and Johson, 1987); oxidation by the MPO-H_2O_2 system and proteolysis by leukocyte elastase are two major effects of this inactivation.

Over the last few years, several experimental studies have demonstrated antioxidant properties of thiol-containing drugs, indicating they may exert a beneficial effect in subjects with human lung diseases (Moldeus et al., 1986; Bernard, 1991; Crystal, 1991). Thus, these thiol-containing drugs might reverse the oxidant-antioxidant imbalance. This is in agreement with the results obtained for mesna, which protects α_1PI in a dose-dependent manner against oxidation by the MPO-H_2O_2 system with low concentrations of this drug. However, these concentrations do not completely reverse the protease-antiprotease imbalance when α_1PI is incubated with stimulated PMNs. A complete reversal of this imbalance was observed when α_1PI and stimulated PMNs were incubated with high concentrations of mesna, which reacts by scavenging HLE and protects α_1PI against proteolysis by stimulated PMNs.

In the case of increased formation of reactive oxygen species in some human lung diseases such as adult respiratory distress syndrome (Repine, 1992) and asthma (Doelman and Bast, 1990), mesna, via aerosol administration, might reverse

the oxidant-antioxidant imbalance in the lower respiratory tract as has been suggested for glutathione by Borok et al. (1991) in patients with idiopathic fibrosis, whereas complete reversal of the protease-antiprotease imbalance with mesna is not possible, in vivo, since the concentrations required to achieve this effect, in vitro, are too high for therapy. Only a reduction of the protease-antiprotease imbalance can be expected in some lung diseases by protecting α_1PI against the MPO-H_2O_2 system.

5. Acknowledgement

The authors are grateful to Annie Legars for technical assistance.

6. References

Akanmu, D., Cecchini, R., Aruoma, O. and Halliwell, B., The antioxidant action of ergothioneine. *Arch. Biochem. Biophys.*, 288 (1991) 10–16.

Bernard, G.R., *N*-Acetylcysteine in experimental and clinical acute lung injury. *Am. J. Med.*, 91 (Suppl. 3C) (1991) 54–59.

Boniface, M., Cazin, J.C., Cazin, M. and Luyckx, M., Calcul sur ordinateur de la dose efficace par la méthode des probits. Application au calcul de la dose léthale. *Bull. Soc. Pharm. Lille*, 4 (1972) 187.

Borok, Z., Buhl, R., Grimes, G.J., Bokser, A.D., Hubbard, R.C., Holroyd, K.J., Roum, J.H., Czerski, D.B., Cantin, A.M. and Crystal, R.G., Effect of glutathione aerosol on oxidant-antioxidant imbalance in idiopathic pulmonary fibrosis. *Lancet*, 338 (1991) 215–216.

Boyum, A., Isolation of mononuclear cells and granulocytes from human blood. *Scan. J. Clin. Lab. Invest.* 97 (Suppl. 21) (1968) 77–89.

Carell, R.W., α1 antitrypsin: molecular pathology, leukocytes and tissue damage. *J. Clin. Invest.*, 78 (1986) 1427–1431.

Carre, P., Pipy, B., Pinelli, E., Forgue, M.F., Beraud, M., Didier, A. and Leophonte, P., In vitro effects of fenspiride on the production of free oxygen radicals, prostaglandins and leukotrienes by guinea-pig alveolar macrophages. *Eur. Respir. Rev.*, 1 (1991) 79–85.

Crystal, R.G., α_1-Antitrypsin deficiency, emphysema and liver disease. Genetic basis and strategies for therapy. *J. Clin. Invest.*, 85 (1990) 1343–1352.

Crystal, R.G., Oxidants and respiratory tract epithelial injury: pathogenesis and strategies for therapeutic intervention. *Am. J. Med.*, 91 (Suppl. 3C) (1991) 39–44.

Doelman, C.J.A. and Bast, A., Oxygen radicals in lung pathology. *Free Radicals Biol. Med.*, 9 (1990) 381–400.

El Yamani, Y., Hayem, A., Laffite, J.J., Gressier, B. and Mizon, J., Functional and immunoreactive α_1-proteinase inhibitor in bronchoalveolar lavages: methodological studies. *Bull. Eur. Physiopathol. Respir.*, 22 (1986) 359–363.

Gadek, J.E. and Pacht, E.R., The protease-antiprotease balance within the human lung: implications for the pathogenesis of emphysema. *Lung*, (Suppl.) (1990) 552–564.

Gressier, B., Cabanis, A., Lebegue, S., Brunet, C., Dine, T., Luyckx, M., Cazin, M. and Cazin, J.C., Comparison of effect in vitro of two thiol containing drugs on human neutrophils hydrogen peroxide production. *Methods Find. Exp. Clin. Pharmacol.*, 15 (1993) 101–105.

Henson, P.M. and Johson, R.B., Tissue injury in inflammation. Oxidants, proteinases and cationic proteins. *J. Clin. Invest.*, 79 (1987) 669–674.

Hoidal, J.R. and Niewoehner, D.E., Pathogenesis of emphysema. *Chest*, 83(4) (1983) 679–685.

Hubbard, R.C., Fells, G., Gadek, J., Pacholok, S., Humes, J. and Crystal, R.G., Neutrophils accumulation in the lung in α_1-antitrypsin deficiency. Spontaneous release of leukotriene B_4 by alveolar macrophages. *J. Clin. Invest.*, 88 (1991) 891–897.

Kaneko, M., Suzuki, K., Furui, H., Takagi, K. and Satake, T., Comparaison of theophylline and enprofylline effects on human neutrophil superoxide production. *Clin. Exp. Pharmacol. Physiol.*, 17 (1990) 849–859.

Moldeus, P., Cotgreave, I.A. and Berggreir, M., Lung protection by a thiol-containing antioxidant: *N*-acetylcysteine. *Respiration*, 50 (Suppl. 1) (1986) 31–42.

Repine, J.E., Scientific perspectives on adult respiratory distress syndrome. *Lancet*, 339 (1992) 466–472.

Stolk, J., Kramps, J.A. and Dijkman, J.H., In vitro effect of a mucolytic thiol agent on the activity of polymorphonuclear leukocyte elastase and antileucoprotease. *Thorax*, 41 (1986) 840–845.

Sugiura, M., Hayakawa, S., Adachi, T., Ito, Y., Hirano, K. and Sawaki, S., A simple one-step purification of human α1 proteinase inhibitor by immunoadsorbent column chromatography. *J. Biochem. Biophys. Methods*, 5 (1981) 243–249.

Travis, J. and Johson, D., Human α_1-proteinase inhibitor. *Methods Enzymol.*, 80 (1981) 754–765.

Wallaert, B., Gressier, B., Aerts, C., Mizon, C., Voisin, C. and Mizon, J., Oxidative inactivation of α_1-proteinase inhibitor by alveolar macrophages from healthy smokers requires the presence of myeloperoxidase. *Am. J. Respir. Cell. Mol. Biol.*, 5 (1991) 437–444.

Weiss, S.J., Tissue destruction by neutrophils. *N. Engl. J. Med.*, 320 (1989) 365–376.