FEBS Letters 361 (1995) 265–268

DNA binds neutrophil elastase and mucus proteinase inhibitor and impairs their functional activity

Didier Belorgey, Joseph G. Bieth*

Laboratoire d'Enzymologie, INSERM U392, Université Louis Pasteur de Strasbourg, F-67400 Illkirch, France

Received 30 January 1995; revised version received 11 February 1995

Abstract DNA binds neutrophil elastase and mucus proteinase inhibitor as evidenced by affinity chromatography on elastase–Sepharose, inhibitor–Sepharose and DNA–cellulose. DNA is a potent hyperbolic inhibitor of elastase. The polynucleotide–enzyme complex is partially active on synthetic substrates and on elastin. DNA strongly increases k_{diss} and K_i for the inhibition of elastase by mucus proteinase inhibitor

($E + I \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} EI$, $K_i = k_{diss}/k_{ass}$).

The above effects are all salt-dependent. At physiological ionic strength, DNA is a potent inhibitor of the elastolytic activity of elastase and increases k_{diss} and K_i for the elastase-mucus proteinase inhibitor interaction 160-fold and 100-fold, respectively.

Key words: Elastase; Mucus proteinase inhibitor; Enzyme kinetics; DNA; Cystic fibrosis

1. Introduction

Neutrophil elastase (NE) is a 30-kDa cationic glycoprotein whose crystal structure is known. This serine proteinase cleaves a number of plasma and extracellular matrix proteins including elastin. It is stored in the azurophil granules of neutrophils from which it may be released following excessive phagocytosis or cell death. Its extracellular proteolytic action is normally prevented by protein proteinase inhibitors such as α_1-proteinase inhibitor, α_2-macroglobulin or mucus proteinase inhibitor (MPI) (for a review see [1]). The latter is an 11.7-kDa unglycosylated cationic protein composed of two domains of similar size and architecture ([2] and refs. therein).

MPI is the most abundant NE inhibitor of airways secretions where it occurs in concentrations as high as 5 μM [3]. Despite this high load of inhibitor, bronchial secretions from patients with cystic fibrosis contain significant amounts of active NE [4]. On the other hand, these secretions also contain large quantities of neutrophil-derived DNA [5]. This raises the question as to whether DNA interferes with the inhibition of NE by MPI. The present investigation attempts to answer this question.

*Corresponding author. INSERM U392, Faculté de Pharmacie, 74 route du Rhin, F-67400 Illkirch, France. Fax: (33) 88 67 92 42. E-mail: jgbieth@pharma.u-strasbg.fr

Abbreviations: NE, neutrophil elastase; MPI, mucus proteinase inhibitor = secretory leucoprotease inhibitor (SLPI); Suc-Ala_3-*p*NA, succinyl-Ala_3-*p*-nitroanilide; MeOSuc-Ala_2-Pro-Val-*p*NA, methoxysuccinyl-Ala_2-Pro-Val-*p*-nitroanilide; RBB-elastin, remazol Brilliant-blue elastin.

2. Materials and methods

2.1. Materials

Human NE was purified from purulent sputum as described in [6]. Recombinant MPI was obtained from Synergen, Boulder, CO through the courtesy of Dr. H.P. Schnebli, Ciba-Geigy, Basel, Switzerland. NE and MPI were active site titrated as indicated in [6]. Stock solutions of Suc-Ala_3-*p*NA and MeOSuc-Ala_2-Pro-Val-*p*NA (Bachem, Bubendorf, Switzerland) were made in *N*-methylpyrolidone and dimethylformamide, respectively. The final concentration of organic solvent in the reaction mixtures was 2% (v/v) throughout. Remazol Brilliant-blue elastin (RBB-elastin) was purchased from Elastin Products Company (Owenville, MO). Salmon sperm DNA and DNA–cellulose came from Sigma. NE or MPI were coupled to epoxy-activated Sepharose (Pharmacia) as described by the manufacturer. The three affinity supports (4 ml) were poured into HR 10/10 Pharmacia columns and equilibrated with 50 mM HEPES buffer, pH 7.4.

2.2. Determination of k_{cat} and K_m

The kinetic constants k_{cat} and K_m for the hydrolysis of MeOSuc-Ala_2-Pro-Val-*p*NA by 30 nM free or DNA-bound NE were determined by measuring the enzyme velocities as a function of the substrate concentration and fitting the data to the Michaelis–Menten equation using a nonlinear regression analysis program (ENZFITTER, Biosoft, Cambridge, UK).

2.3. Elastolytic activity

The effect of DNA on the elastolytic activity of NE was assessed as follows. Constants concentrations of NE were reacted with increasing concentrations of DNA in a total volume of 600 μl. After 15 min at 25°C, 500 μl aliquots were withdrawn from these mixtures and added to 1,500 μl of continuously stirred RBB–elastin suspensions. From time to time a 450 μl sample was withdrawn from these suspensions, diluted with 550 μl of 0.75 M acetate buffer pH 4.5, centrifuged at 10,000 × *g* and read at 595 nm to assay the concentration of soluble elastin peptides. A total of four aliquots were removed over a period of ca. 1 h. The rate of elastolysis was calculated from the slope of the linear relationship between A_{595} and time. The final concentration of NE and RBB–elastin were 2.5 μM and 3 mg/ml, respectively.

2.4. Kinetics of inhibition of NE

The rate of inhibition of NE by MPI was measured by reacting free or DNA-bound NE with a mixture of MPI and MeO-Suc-Ala_2-Pro-Val-*p*NA and recording the release of *p*-nitroaniline at 410 nm. A SFA-12 rapid mixing accessory (Hi-Tech Scientific, Salisbury, UK) with a dead time of ca. 1 s was used to mix the reagents. Nonlinear regression analysis of the progress curves was done using the ENZFITTER software. The final concentrations of NE, MPI and substrate were 30 nM, 300 nM and 3 mM, respectively.

All other technical details are given in the legends to the figures.

3. Results

3.1. DNA binds NE and MPI

Affinity chromatography was used to assess the interaction of DNA with NE and MPI. At low ionic strength (50 mM HEPES, pH 7.4) DNA binds to NE-Sepharose (Fig. 1A) and to MPI-Sepharose (Fig. 1B). A linear NaCl gradient desorbs the polynucleotide from each column. On the other hand, NE

SSDI 0014-5793(95)00173-5

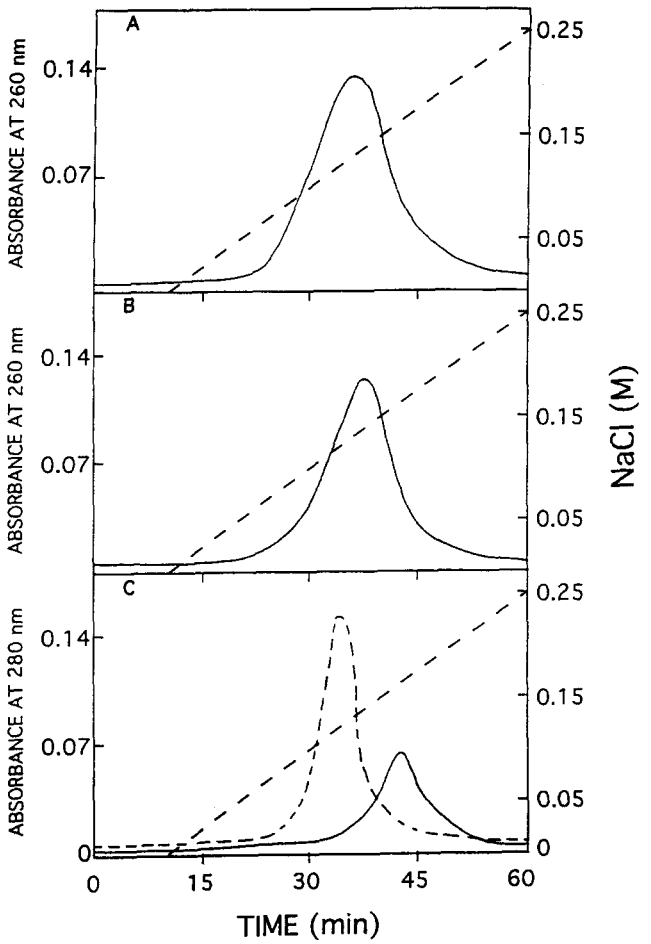


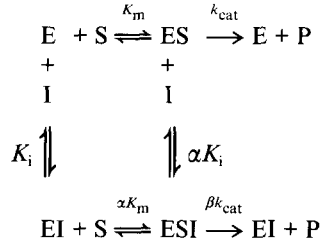
Fig. 1. Affinity chromatography of 50 μg of DNA on NE-Sepharose (panel A) and MPI-Sepharose (panel B) and of 300 μg of NE (——) and 210 μg of MPI (----) on DNA–cellulose (panel C). The columns were equilibrated with 50 mM HEPES, pH 7.4. DNA, NE, and MPI were dissolved in the same buffer and eluted from the columns with a linear NaCl gradient at a flow-rate of 0.5 ml·min^{-1}.

and MPI bind to DNA–cellulose at low ionic strength and are eluted from the affinity column by a linear salt gradient (Fig. 1C). NE has apparently a better affinity for DNA than MPI since its retention time on the DNA–cellulose column (42 s) is significantly longer than that of MPI (33 s). The data of Fig. 1C also suggest that at a physiological ionic strength (0.15 M NaCl) the affinity of DNA for MPI is relatively weak whereas it is fairly strong for NE.

3.2. Hyperbolic inhibition of NE by DNA

Fig. 2 shows the effect of DNA on the rate of the NE-catalyzed hydrolysis of Suc-Ala$_3$-*p*NA, a synthetic elastase substrate [7]. The enzyme velocity decreases sharply and then levels off, indicating that the NE–DNA complex is partially active, i.e. that the inhibition is hyperbolic. The enzyme–polynucleotide complex retains 20% and 70% activity of the free enzyme in the presence of 50 mM and 150 mM NaCl, respectively (see Fig. 2) and 30% activity in the presence of 100 mM NaCl (data not shown).

Hyperbolic inhibition [8] may be analyzed using Scheme 1



Scheme 1.

where E, S and I stand for enzyme, substrate and inhibitor, respectively and α and β are dimensionless numbers. The conditions under which hyperbolic inhibition takes place may be summarized by $0 < \beta \leq 1$; $\infty > \alpha \geq 1$. We have determined α and β for the effect of DNA on the NE-catalyzed hydrolysis of MeO-Suc-Ala$_2$-Pro-Val-*p*NA, a more specific NE substrate [9]. The results are reported in Table 1. It can be seen that DNA has two effects: (i) it increases K_m ($\alpha > 1$), i.e. it apparently acts as a 'competitive inhibitor' of NE, (ii) it forms an ESI complex that decomposes at a significant rate ($0 < \beta < 1$), i.e. it is a hyperbolic inhibitor of NE. Ionic strength strongly decreases α but does not significantly change β. As a consequence, the lower the ionic strength, the better the inhibition, a feature that also characterises the NE-catalyzed cleavage of Suc-Ala$_3$-*p*NA (see Fig. 2).

Fig. 3 shows the effect of DNA on the activity of NE on fibrous elastin, its natural substrate. The inhibition again appears to be hyperbolic although the residual activities do not reach a true plateau. Higher DNA concentrations could not be used because the reaction media became cloudy and viscous. The inhibition curve obtained in the presence of 100 mM NaCl was found to lie between those obtained at 50 mM and 150 mM NaCl (data not shown). The most striking feature observed with elastin is that at a physiological ionic strength, DNA is a potent inhibitor of the elastolytic activity of NE (Fig. 3) whereas it has a minor effect on the NE-catalyzed hydrolysis of the synthetic substrates (Fig. 2 and Table 1).

3.3. DNA impairs the functional activity of MPI

The kinetics of inhibition of NE by MPI was studied using

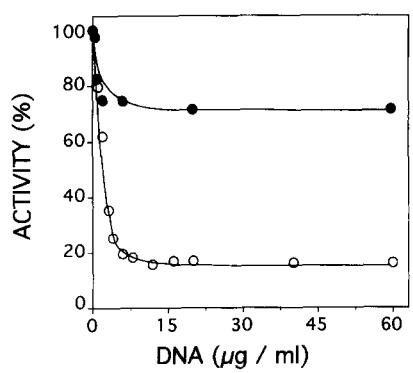


Fig. 2. Effect of DNA on the activity of NE on Suc-Ala$_3$-*p*NA. Constant concentrations of NE were reacted with increasing concentrations of DNA in a total volume of 980 μl of 50 mM HEPES, pH 7.4 containing 50 mM NaCl (○) or 150 mM NaCl (●). After 15 min at 25°C, 20 μl of 50 mM Suc-Ala$_3$-*p*NA were added and the absorbance was recorded at 25°C. The final concentration of NE was 0.5 μM throughout.

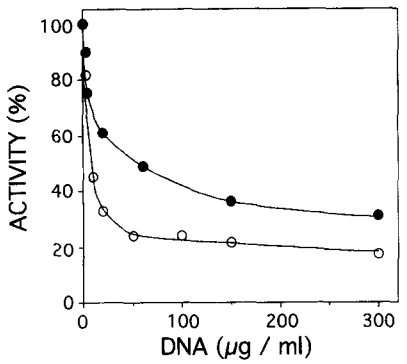


Fig. 3. Effect of DNA on the elastolytic activity of NE. Constant concentrations of NE were reacted with increasing concentrations of DNA. The residual enzymatic activities were then tested with RBB–elastin as described in the experimental section. The buffer was 50 mM HEPES, pH 7.4, containing 50 mM NaCl (○) or 150 mM NaCl (●). The final concentration of NE was 2.5 μM throughout.

the progress curve method which consists of adding enzyme to a mixture of inhibitor and substrate and recording the release of product as a function of time [10]. Fig. 4 shows typical progress curves run in the absence and presence of 200 μg/ml DNA and at a physiological ionic strength. Both curves are characterised by a pre-steady-state followed by a steady-state (linear) release of product. DNA significantly shortens the duration of the pre-steady-state phase and dramatically increases the steady-state velocity so that after a certain time, there is more product released in the presence of DNA than in its absence. With lower DNA concentrations, milder effects were observed (data not shown).

The progress curves, analyzed as described previously [11], yielded k, the pseudo-first-order rate constant describing the pre-steady-state, v_z and v_s, the initial and steady-state velocities, respectively. These three parameters were used to calculate the kinetic constants corresponding to the enzyme-inhibitor equilibrium [12]: $E + I \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} EI$ where E and I stand for free or DNA-bound NE and MPI, respectively. For each DNA concentration we calculated k_{ass}, k_{diss} and K_i using the following relationships [10]:

$$k_{ass} = \frac{k}{[I]_0}\left(1 - \frac{v_s}{v_z}\right)\left(1 + \frac{[S]_0}{K_m}\right) \quad (1)$$

$$k_{diss} = k \cdot \frac{v_s}{v_z} \quad (2)$$

$$K_i = k_{diss}/k_{ass} \quad (3)$$

Fig. 5 shows the influence of the concentration of DNA on k_{ass}, k_{diss} and K_i at physiological ionic strength. There is a moderate increase in k_{ass} but a very strong increase in k_{diss} which varies from 1.1×10^{-4} s^{-1} in the absence of DNA to 1.8×10^{-2} s^{-1} in the presence of 200 μg/ml DNA. This 164-fold increase is responsible in part for the ca. 100-fold increase in K_i. At lower ionic strength the effect of DNA on the kinetic parameters was less pronounced (data not shown). For example, the largest increase in k_{diss} was 43-fold and 67-fold in the presence of 50 mM NaCl and 100 mM NaCl, respectively.

Table 1
Effect of DNA on the kinetic parameters (see Scheme 1) describing the NE-catalysed hydrolysis of MeOSuc-Ala_2-Pro-Val-*p*NA at pH 7.4 and 25°C

NaCl (mM)	αK_m (μM)	βk_{cat} (s^{-1})	α	β
50	330 ± 44	5.7 ± 0.3	3.2 ± 0.7	0.62 ± 0.06
100	156 ± 25	6.2 ± 0.3	1.8 ± 0.4	0.66 ± 0.05
150	176 ± 21	6.8 ± 0.3	1.3 ± 0.3	0.72 ± 0.05

4. Discussion

The binding of NE and MPI to DNA strongly decreases with the ionic strength. Therefore, it probably involves ionic interactions between some basic amino acid residues of the cationic proteins and some phospho-deoxyribosyl moieties of the anionic polymer. This binding impairs the functional activity of both NE and MPI: (i) DNA is a hyperbolic inhibitor of NE, i.e. the DNA–NE complex has a low but significant enzymatic activity, (ii) DNA strongly decreases the stability of the NE–MPI complex by increasing k_{diss} and K_i, i.e. the enzymatic activity of the DNA–NE complex is much more resistant to inhibition by MPI than free NE. The effect of DNA on the NE/MPI system is reminiscent of that observed with heparin, another anionic polymer. Heparin is also a hyperbolic inhibitor of NE [13] which forms a tight complex with MPI [11]. However, unlike DNA, heparin strongly potentiates the inhibition of NE by MPI [11].

Our findings may have important pathophysiological bearing. Cystic fibrosis is a disease whose major clinical manifestations are in the lung with accumulation of purulent mucus, bacterial infections, airway inflammation and obstruction and ultimately lung tissue destruction due to NE and other neutrophil proteinases ([4] and refs. therein). Cystic fibrosis sputum contains high amounts of DNA (4 mg/ml on the average) [14]. This DNA originates from neutrophils, forms a complex with mucus glycoproteins, is entrapped in the water-insoluble fraction of sputum and is responsible in part for the high viscosity of airways secretions which leads to airways obstruction [5,15].

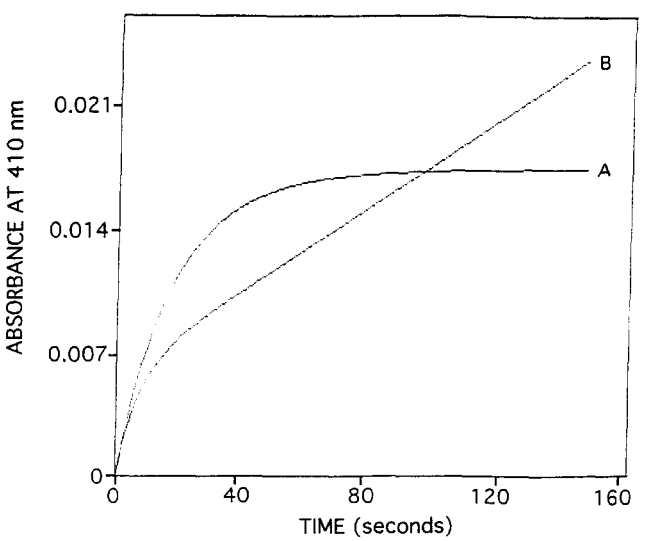


Fig. 4. Progress curves for the inhibition of NE by MPI in the absence (A) or presence (B) of 200 μg/ml DNA. The concentrations of NE, MPI and substrate were as indicated in the experimental section. The buffer was: 50 mM HEPES, 150 mM NaCl, pH 7.4, 25°C.

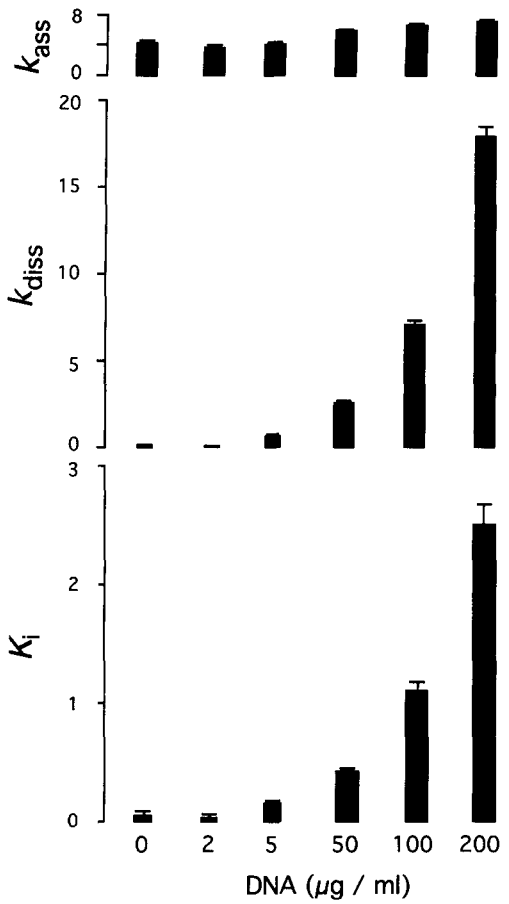


Fig. 5. Kinetic constants describing the inhibition of NE by MPI in absence or presence of variable concentrations of DNA at 25°C and pH 7.4 (50 mM HEPES, 150 mM NaCl). The constants k_{ass}, k_{diss} and K_i are given in ($M^{-1} \cdot s^{-1} \times 10^{-6}$), ($s^{-1} \times 10^{-3}$) and ($M \times 10^{9}$), respectively. The bars represent the standard errors calculated from repeated determinations.

Recombinant DNase I has been shown in vitro to reduce the viscoelasticity of cystic fibrosis sputum [16]. In addition, administration of aerosolized enzyme improves the lung function of cystic fibrosis patients [17]. Our in vitro data strongly suggest that most of the NE released from neutrophils in cystic fibrosis airways secretions is entrapped in insoluble DNA and therefore has little, if any, proteolytic activity. We therefore believe that long-term administration of DNase may have deleterious effects on lung tissue proteins because it will release free and active NE from its complex with DNA. Since the concentration of MPI is much lower than that of NE in cystic fibrosis secretions [3,18], NE may attack lung tissue proteins in an unimpaired way.

At physiological ionic strength the binding of MPI to DNA is relatively weak. In cystic fibrosis airways secretions this poor affinity may, however, be largely compensated for by the enormous DNA concentration present in these fluids. Hence, it is likely that a large proportion of the MPI administered by aerosol to inhibit NE in lung secretions of cystic fibrosis patients [4] will be entrapped in insoluble DNA and will, therefore, not be available for NE inhibition. Also, the fraction of administered MPI which reacts with DNA-bound NE will form an unstable inhibitory complex as we have shown in vitro. The effects discussed here may explain why considerably high amounts of recombinant MPI are required to observe significant NE inhibition in lung secretion of cystic fibrosis patients [4].

Acknowledgements: We thank Synergen (Boulder, CO, USA) for the gift of recombinant MPI.

References

[1] Bode, W., Meyer Jr., E. and Powers, J.C. (1989) Biochemistry 28, 1951–1963.
[2] Grütter, M.G., Fendrich, G., Huber, R. and Bode, W. (1988) EMBO J. 7, 345–351.
[3] Tournier, J.M., Jacquot, J., Sadoul, P. and Bieth, J.G. (1983) Anal. Biochem. 131, 345–350.
[4] McElvaney, N.G., Nakamura, H., Birrer, P., Hebert, C.A., Wong, W.L., Alphonso, M., Baker, J.B., Catalana, M.A. and Crystal, R.G. (1992) J. Clin. Invest. 90, 1296–1301.
[5] Potter, J.L., Spector, S., Matthews, L.W. and Lemm, J. (1969) Am. Rev. Respir. Dis. 99, 909–916.
[6] Boudier, C. and Bieth, J.G. (1994) Biochem. J. 303, 61–68.
[7] Bieth, J.G., Spiess, B. and Wermuth, C.G. (1974) Biochem. Med. 11, 350–357.
[8] Baici, A., Salgam, P., Fehr, K. and Böni, A. (1981) Biochem. Pharmacol. 30, 703–708.
[9] Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmerman, M. (1979) J. Biol. Chem. 254, 4027–4032.
[10] Morrison, J.F. and Walsh, C.T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–301.
[11] Faller, B., Mely, Y., Gérard, D. and Bieth, J.G. (1992) Biochemistry 31, 8285–8290.
[12] Boudier, C. and Bieth, J.G. (1989) Biochim. Biophys. Acta 995, 36–41.
[13] Redini, F., Tixier, J.-M., Petitou, M., Choay, J., Robert, L. and Hornebeck, W. (1988) Biochem. J. 252, 515–519.
[14] Matthews, L.W., Spector, S., Lemm, J. and Potter, J.L. (1963) Am. Rev. Respir. Dis. 88, 199–204.
[15] Lethem, M.I., James, S.L., Mariott, C. and Burke, J.F. (1990) Eur. Resp. J. 3, 19–23.
[16] Shak, S., Capon, D.J., Hellmiss, R., Marsters, S.A. and Baker, C.L. (1990) Proc. Natl. Acad. Sci. USA 87, 9188–9192.
[17] Ranasinha, C., Assoufi, B., Shak, S., Christiansen, D., Fuchs, H., Empey, D., Geddes, D. and Hodson, M. (1993) Lancet 342, 199–202.
[18] McElvaney, N.G., Doujaiji, B., Moan, M.J., Burnham, M.R., Wu, M.C. and Crystal, R.G. (1993) Am. Rev. Respir. Dis. 148, 1056–1060.