CHEMISTRY
A EUROPEAN JOURNAL

COMMUNICATION

DOI: 10.1002/chem.201301289

A Detective Story in Drug Discovery: Elucidation of a Screening Artifact Reveals Polymeric Carboxylic Acids as Potent Inhibitors of RNA Polymerase

Weixing Zhu, Matthias Groh, Jörg Haupenthal, and Rolf W. Hartmann*[a]

Scientific progress is hampered by publications with erroneous results.[1] An improvement of this nuisance will depend on an enhanced alertness of researchers and reviewers of experimental artifacts. In the field of drug discovery high-throughput screening traditionally performed by the pharmaceutical industry, has found its way into academic research.[2] The hit compounds are reported to the scientific community as good starting points for drug discovery. However, in many cases these compounds act by unspecific inhibition modes, like aggregation,[3] or interfere with the bioassay,[4] meaning that they represent false starts.[5,6] During our quest for novel bacterial RNA polymerase (RNAP) inhibitors for the treatment of infectious diseases, we recently performed a fragment screening using a small commercial compound library and thereby identified compound **1** as being fairly active. Literature search revealed that three structurally related compounds (**2**–**4**) have already been described as RNAP inhibitors.[7,8] Resynthesis of compound **1** resulted in an inactive compound. In the subsequent elucidation of this phenomenon we discovered that a polymeric side product was responsible for RNAP inhibition. During this process it was disclosed that the negatively charged macromolecule interacts with the positively charged protein surface.

Due to the increasing appearance of resistant microbes against clinically used drugs, novel antibiotics are urgently needed.[9] Since the therapeutic success of rifamycins, bacterial RNA polymerase is regarded as a promising target for broad-spectrum antibacterial therapy. However, their efficiency has been reduced by an upcoming rifampicin resistance.[10–12] Biological screening using low-molecular-weight fragments has recently become a promising alternative to high-throughput screening using drug-like molecules since the hit rate is much higher. By subsequent fragment growing or fragment linking, the initial hits can be optimized to lead compounds, which ideally fit into the target binding site.[13,14]

In a recent project we determined the in vitro inhibitory activity of 500 commercially available fragments (molecular weight, MW < 300) towards *E. coli* RNAP. During that process compound **1** was identified, showing 85% *E. coli* RNAP inhibition at a concentration of 200 μM. Its purity was 91%, determined by HPLC-UV. Surprisingly, compound **1** synthesized in our laboratory (>96% HPLC-UV) exhibited no RNAP inhibition. Remarkably, the closely related compounds **2**–**4** have already been reported as inhibitors of bacterial RNAP (Figure 1).[7,8] Synthesis of these compounds and subsequent testing in our laboratory revealed no activity (Table 1).

As we had observed that the color of compounds **1**–**4** turned to red under "bench conditions" (exposed to air at room temperature for several hours), we considered the possibility that they might be unstable and their inhibitory activities are due to the formation of decomposition products. To speed up the degradation process, compound **1** was

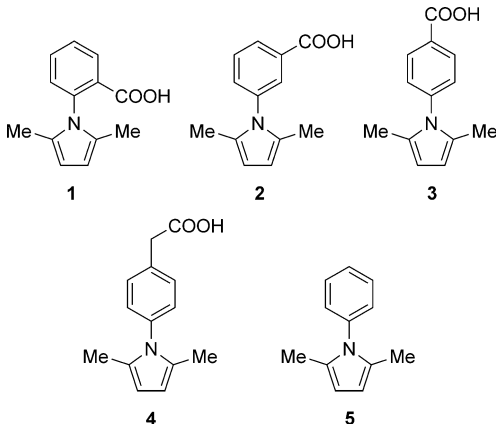


Figure 1. Compounds **1**–**5**.

Table 1. *E. coli* RNAP inhibition of compounds **1**–**5** and **P1**–**P5**.

Compound	Inhibition at 200 μM	Compound	IC_{50} [μg mL^{-1}]
1	n.i.[a]	**P1**	0.6
2	n.i.[a]	**P2**	1.0
3	n.i.[a]	**P3**	0.8
4	n.i.[b]	**P4**	2.5
5	n.i.[c]	**P5**	n.i.[d]

[a] 200 μM = 43.0 μg mL^{-1}. [b] 200 μM = 45.8 μg mL^{-1}. [c] 200 μM = 34.2 μg mL^{-1}. [d] 20.0 μg mL^{-1}; n.i. = no inhibition (inhibition < 15%).

[a] W. Zhu,[+] Dr. M. Groh,[+] Dr. J. Haupenthal, Prof. Dr. R. W. Hartmann
Helmholtz Institute for Pharmaceutical Research Saarland and
Pharmaceutical and Medicinal Chemistry, Saarland University
Campus C2.3, 66123 Saarbrücken (Germany)
Fax: (+49) 681-302-70308
E-mail: rolf.hartmann@helmholtz-hzi.de

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201301289.

heated and analyzed by HPLC-UV. The results revealed that it was decomposed (Figure 2a). The effect of decomposition on RNAP inhibition was determined and an increase of activity was observed with enhanced degradation (Figure S1 in Supporting Information). From the ^{1}H NMR spectra it becomes apparent that the signal of the β-C–H on the pyrrole ring ($\delta = 5.7$ ppm) of compound **1** disappears whereas in the aromatic range ($\delta = 8.5$ to 7.0 ppm) a broad new signal can be observed (Figure 2b). The appearance of a broad peak in the chromatogram and broad signals in the NMR spectra suggests that the decomposed product might be a polymer. Accordingly, a decomposition sample was subjected to ultrafiltration (cut-off 3.5 kDa) and it turned out that the active component was of high MW. Finally the pure polymeric compound derived from **1**, named **P1** was isolated.

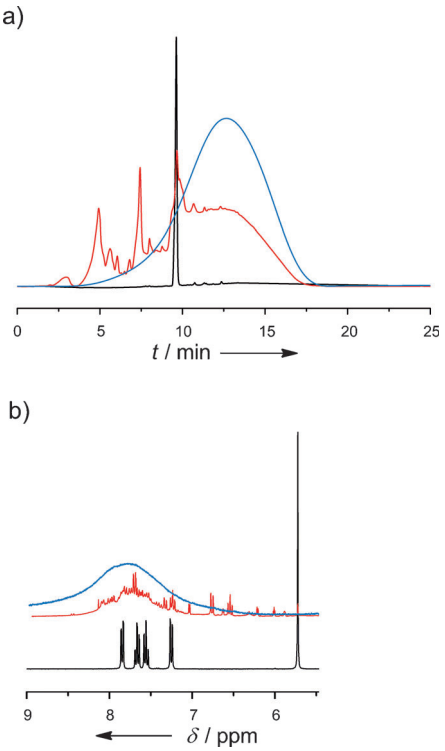


Figure 2. a) HPLC analysis, UV detection at 254 nm, b) ^{1}H NMR spectra. Black line: **1**; red line: **1** decomposed after 10 days heating at 50 °C; blue line: **P1**.

By gel permeation chromatography (GPC) it was shown that **P1** exhibits a broad molecular distribution with a weight-average molecular weight (M_W) of around 40 kDa (Figure S3 in the Supporting Information). UV/Vis spectroscopy of **P1** shows an absorption peak at 498 nm (2.49 eV) associated with the π–π* transition, indicating a well conjugated π electron system in the backbone. Absorption at 498 nm could also be observed in the commercial compound **1** (Figure S4 in the Supporting Information). Interestingly, for the related compounds **2**–**5** polymer formation was also observed. Subsequent isolation resulted in compounds **P2**–**P5** (Figure S5 in the Supporting Information). In the IR spectra of **P1**–**P4** the signal of the carboxylic acid group is still present (Figure S6 and Table S1 in the Supporting Information). This is in accordance to the observation that compounds **P1**–**P4** can be easily dissolved in water at basic pH in contrast to **P5**. In a gel electrophoresis experiment, the "polyanionic character" of **P1** was confirmed. At pH 7.8 **P1** migrated to the anode. At this pH, **P5** and at pH 3.6, both compounds remained at the starting point (Figure S7 in the Supporting Information). Based on our findings that in the polymeric compounds **P1**–**P4** the β-C–H on the pyrrole ring signal is missing, the benzoic acid group is still present and a conjugated system of double bonds has been demonstrated, the only plausible structure, to the best of our knowledge, is shown in Figure 3 with **P1** as an example.

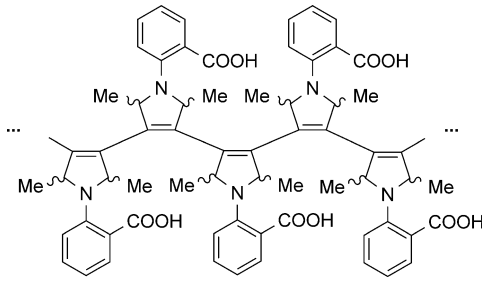


Figure 3. Supposed structure of the polymeric compound **P1**.

The ability of **1**–**5** and **P1**–**P5** to inhibit *E. coli* RNAP was determined in an in vitro transcription assay (Table 1). None of the monomeric compounds affected transcription, whereas the polymers bearing a carboxylic acid group (**P1**–**P4**) inhibited *E. coli* RNAP in a concentration-dependent manner with IC_{50} values between 0.6 and 2.5 μg mL^{-1}. **P5** lacking the carboxylic acid group was inactive indicating that the carboxylic acid group is essential for inhibitory activity.

Considering the mode of inhibition of the polymers, it is worth mentioning that there are positively charged amino acid residues on the surface of RNAP, including the DNA binding channel (Figure 4), serving as a counterpart for the negatively charged DNA. Therefore, it can be rationalized that the inhibitory mechanism of **P1** is based on multiple

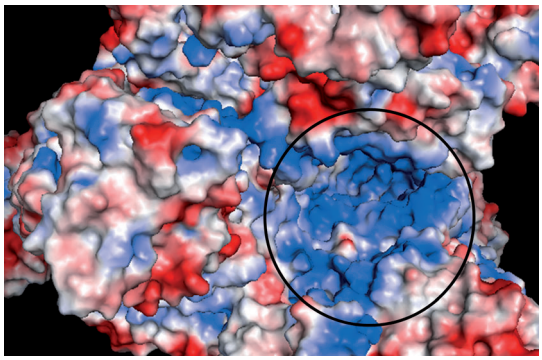


Figure 4. Electrostatic surface of *E. coli* RNAP core enzyme visualized using PyMOL (PDB ID: 3LU0).[18] The circle highlights the DNA binding channel. Red shows the negatively charged and blue positively charged electrostatic potential surface.

electrostatic interactions between the negatively charged carboxylate groups and the positively charged protein surface. In this context it is of interest that some other polyanions including polysulfated heparins have already been reported to inhibit RNAP.[15–17]

The electrostatic interaction of **P1** with RNAP was demonstrated by gel electrophoresis. In the presence of RNAP the mobility of **P1** was reduced (Figure S7 in the Supporting Information). Considering the averaged molecular weight of around 40 kDa, the IC_{50} value of **P1** against *E. coli* RNAP can be calculated to be approximately 15 nM. Since the concentration of enzyme in the assay is 36 nM, it can be concluded that **P1** binds to the protein at a molar ratio of about 1:1 and is a very potent inhibitor. Under identical experimental conditions a 70-fold excess of heparin is required to obtain the same inhibitory effect.

On closer examination it is obvious that the bound polymer should sterically block the loading of the DNA template to RNAP and thus inhibit transcription at its earliest step. To corroborate this hypothesis, the influence of **P1** on DNA binding to the RNAP main channel was investigated. We expected that **P1** should be less effective if the DNA template already occupied the main channel. Indeed, our results revealed a significantly reduced RNAP inhibition when the DNA template was added prior to **P1** compared to a vice versa chronology of addition. For rifampicin, which affects the transcription process by binding to an allosteric site close to the active site within the DNA binding channel,[19] a similar trend was observed (Figure 5). As a negative control, CBR703, was used as it is described to bind to a surface-exposed groove distant from the main channel, which should not influence the binding of the DNA template to its channel.[20] As expected, CBR703 inhibited RNAP independently of whether the DNA channel was already occupied by the DNA template or not. In summary, this experiment supports our hypothesized mechanism of **P1** RNAP inhibition.

To find out whether these inhibitory profiles can also be observed for RNA polymerases from other species, RNAP from bacteriophage T7 was employed. **P1** showed a strong inhibitory effect ($IC_{50}=0.06\ \mu g\,mL^{-1}$) whereas compound **1** was inactive at 200 μM (43.3 $\mu g\,mL^{-1}$). In contrast to these findings, **P1** showed no or only moderate activity against seven other enzymes (Table S2 in the Supporting Information). In a further experiment, **P1** did not inhibit growth of *E. coli* and *Pseudomonas aeruginosa* (at 20 $\mu g\,mL^{-1}$). The lack of in vivo activity was expected as **P1** is too hydrophilic for passive diffusion and too large to permeate the porins and enter the bacterial cell.

Nevertheless, it is feasible that the knowledge gained in this study could be exploited for an antibacterial therapy. Biological in vitro effects of polyanions including inhibition of RNAP have indeed already been described.[15–17] However, the polymers were devoid of antibacterial activity. It seems viable that in vivo activity could be achieved for our polymers by strongly reducing their size, thus enabling hydrophilic substances to penetrate the porins. On the other hand polyanions of such a molecular weight should not be capable of passing the membranes of mammalian cells, and therefore they could be potential therapeutics for local infections of the skin and lung.

In summary, we could demonstrate that the activity of the hit compound **1** found in an experimental screening approach can be attributed to a highly active polymeric impurity formed by decomposition of **1**. Remarkably, the closely related compound **4** is also described as a RNAP inhibitor.[7] In our assay "synthesized" **4** was inactive, whereas the polymerized compound strongly inhibited the bacterial enzyme. Furthermore, **2** was also reported to be active against poliovirus RNAP,[21] which seems doubtful with respect to our findings. It is worth mentioning that compounds **1**–**4** are reported to inhibit HIV-1 gp41,[22] anthrax lethal factor[23] and Eph receptors.[24] Remarkably around 500 related derivatives of *N*-phenyl-2,5-dimethyl pyrrole are reported in numerous patents and publications as biologically active compounds;[25] nevertheless, they do not have a clean slate and have been propagated as pan assay interference compounds (PAINS).[26] However, the molecular mechanism of the false positive results remained "mysterious" until this investigation.

In conclusion, researchers should be cautious with compounds that tend to polymerize, as even traces of polymeric impurities might cause tremendous effects in biological assays.

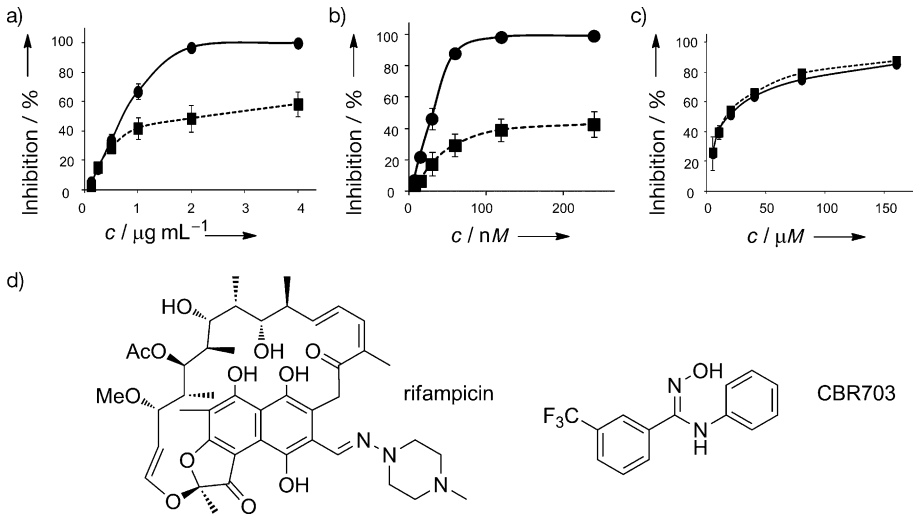


Figure 5. *E. coli* RNAP transcription assay with different chronology of addition of DNA template and inhibitor. Solid line: normal test procedure: inhibitor was added before the DNA template. Dashed line: reversed test procedure: DNA template was added before the inhibitor. Concentration-dependent inhibition of: a) **P1**, b) rifampicin, c) CBR703; d) structures of rifampicin and CBR703.

Acknowledgements

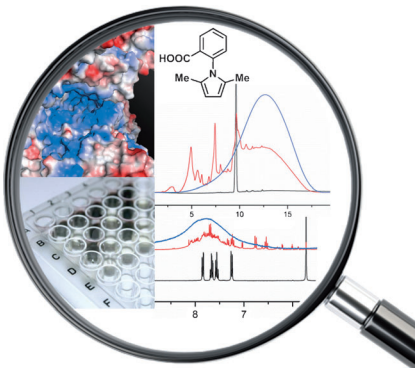
W.Z. thanks the China Scholarship Council for a PhD fellowship. The authors thank Jeannine Jung for technical assistance.

Keywords: biological screening · drug discovery · experimental artifacts · inhibitors · polyanions

[1] U. Poeschl, *Learn. Publ.* **2004**, *17*, 105–113.
[2] M. Baker, *Nat. Methods* **2010**, *7*, 787–792.
[3] a) S. L. McGovern, E. Caselli, N. Grigoieff, B. K. Shoichet, *J. Med. Chem.* **2002**, *45*, 1712–1722; b) K. E. Coan, B. K. Shoichet, *J. Am. Chem. Soc.* **2008**, *130*, 9606–9612.
[4] a) J. Inglese, R. L. Johnson, A. Simeonov, M. Xia, W. Zheng, C. P. Austin, D. S. Auld, *Nat. Chem. Biol.* **2007**, *3*, 466–479; b) D. S. Auld, N. Thorne, D. T. Nguyen, J. Inglese, *ACS Chem. Biol.* **2008**, *3*, 463–470; c) A. Jadhav, R. S. Ferreira, C. Klumpp, B. T. C. P. Mott, C. P. Austin, J. Inglese, C. J. Thomas, D. J. Maloney, B. K. Shoichet, A. Simeonov, *J. Med. Chem.* **2010**, *53*, 37–51.
[5] B. K. Shoichet, *Drug Discovery Today* **2006**, *11*, 607–615.
[6] J. B. Baell, *Future Med. Chem.* **2010**, *2*, 1529–1546.
[7] E. André, L. Bastide, P. Villain-Guillot, J. Latouche, J. Rouby, J. P. Leonetti, *Assay Drug Dev. Technol.* **2004**, *2*, 629–635.
[8] B. Pau, J. P. Leonetti, J. Rouby, *PCT Int. Appl.* **2002**, WO 2002044735A1 20020606.
[9] L. Freire-Moran, B. Aronsson, C. Manz, I. C. Gyssens, A. D. So, D. L. Monnet, O. Cars, *Drug Resist. Updates* **2011**, *14*, 118–124.
[10] I. Chopra, *Curr. Opin. Investig. Drugs* **2007**, *8*, 600–607.
[11] S. A. Darst, *Trends Biochem. Sci.* **2004**, *29*, 159–162.
[12] J. B. P. Villain-Guillot, L. Bastide, M. Gualtieri, J. P. Leonetti, *Drug Discovery Today* **2007**, *12*, 200–208.
[13] D. C. Rees, M. Congreve, C. W. Murray, R. Carr, *Nat. Rev. Drug Discovery* **2004**, *3*, 660–672.
[14] D. A. Erlanson, *Curr. Opin. Biotechnol.* **2006**, *17*, 643–652.
[15] C. T. Warnick, H. M. Zazarus, *Nucleic Acids Res.* **1975**, *2*, 735–744.
[16] K. Ono, H. Nakane, M. Fukushima, *Eur. J. Biochem.* **1988**, *172*, 349–353.
[17] F. de M. Dossin, S. Schenkenman, *Eukaryotic Cell* **2005**, *4*, 960–970.
[18] N. Opalka, J. Brown, W. J. Lane, K. A. F. Twist, R. Landick, F. J. Asturias, S. A. Darst, *PLoS Biol.* **2010**, *8*, e1000483.
[19] E. A. Campbell, N. Korzheva, A. Mustaev, K. Murakami, S. Nair, A. Goldfarb, S. A. Darst, *Cell* **2001**, *104*, 901–912.
[20] I. Artsimovitch, C. Chu, A. S. Lynch, R. Landick, *Science* **2003**, *302*, 650–654.
[21] G. Campagnola, P. Gong, O. B. Peersen, *Antiviral Res.* **2011**, *91*, 241–251.
[22] K. Liu, H. Lu, L. Hou, Z. Qi, C. Teixeira, F. Barbault, B. Fan, S. Liu, S. Jiang, L. Xie, *J. Med. Chem.* **2008**, *51*, 7843–7854.
[23] R. Noberini, M. Koolpe, S. Peddibhotla, R. Dahl, Y. Su, N. D. P. Cosford, G. P. Roth, E. B. Pasquale, *J. Biol. Chem.* **2008**, *283*, 29461–29472.
[24] D. J. Shaw, W. F. Wood, *J. Chem. Educ.* **1992**, *69*, A313.
[25] Number based on SciFinder literature search for compounds containing the *N*-phenyl-2,5-dimethyl pyrrole core.
[26] J. B. Baell, G. A. Holloway, *J. Med. Chem.* **2010**, *53*, 2719–2740.

Received: April 5, 2013
Published online: ■■ ■■, 0000

Chasing the active impurity: In the validation of a screening hit it was discovered that a polymeric trace impurity was responsible for the biological activity. Such a side product can be formed with similar compounds. During the investigations it was discovered that the negatively charged macromolecule interacts very efficiently with the protein surface of *E. coli* RNAP via electrostatic interactions.



Drug Discovery

*W. Zhu, M. Groh, J. Haupenthal, R. W. Hartmann** ..... ▮▮▮▮–▮▮▮▮

A Detective Story in Drug Discovery: Elucidation of a Screening Artifact Reveals Polymeric Carboxylic Acids as Potent Inhibitors of RNA Polymerase