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SOD1 dimerisation assay development

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Abstract	<p>Monomerization and aggregation of the homodimeric enzyme Cu/Zn-superoxide dismutase 1 (SOD1) are events involved in the familial variant of the degenerative neuromuscular disorder Amyotrophic lateral sclerosis (ALS). To prevent monomerization and aggregation, we search for stabilising compounds that can bind to a pocket between the monomers. The objective of this work was to test published SOD1 stabilisers (Ray <i>et al.</i>) for binding using STD-NMR and to use the verified binders as positive controls when developing an HTS-assay based on SOD1 activity. The result of the work showed that the published compounds do not bind to SOD1, and that SOD1 activity is not a good marker for SOD1 stability. To continue this work, new positive controls have to be identified, and a new HTS-assay has to be developed.</p>	
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SOD1 dimerisation assay development

Sara Olsson

Sammanfattning

Att utveckla nya läkemedel är dyrt. För att minska kostnaderna och öka chanserna att lyckas utvecklar läkemedelsföretagen tidigt i processen effektiva analysmetoder, där man snabbt och enkelt kan testa effekten av olika molekyler på det potentiella målet för läkemedlet (t.ex. ett enzym eller en receptor). En hög kapacitet är viktig då de flesta läkemedelsföretag har molekyllbibliotek med flera miljoner molekyler som ska testas. Det är också viktigt att metoden hittar molekyler som har effekt och filtrerar bort alla andra.

Syftet med examensarbetet var att utveckla en effektiv filtreringsmetod för att identifiera molekyler som binder till, och stabiliserar, proteinet SOD1. SOD1 är inblandat i den degenerativa nervsjukdomen amyotrofisk lateralskleros (ALS), för vilken det idag saknas verksamma läkemedel. Som utgångspunkt användes tidigare publicerade molekyler med en positiv effekt på stabiliteten hos SOD1.

Idén till filtreringsmetoden var att man skulle mäta proteinets aktivitet, med och utan molekyler närvarande, för att se om molekylerna hade effekt på stabiliteten. Tyvärr visade det sig att aktiviteten inte var ett bra mått på stabiliteten. Det visade sig också under arbetets gång att de publicerade molekylerna inte binder till SOD1, därmed bör det diskuteras om de verkligen kan ha den påstådda effekten.

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Table of contents

Table of contents.....	2
1. Introduction.....	3
1.1 Preclinical drug development	3
2. Project background	4
2.1 Amyotrophic lateral sclerosis (ALS)	4
2.2 SOD1 and ALS	5
3. Methods.....	6
3.1 Nuclear magnetic resonance (NMR)	6
3.1.1 Introduction.....	6
3.1.2 Theory	6
3.1.3 The NMR signal.....	8
3.1.4 NMR in practice.....	10
3.2 Saturation transfer difference-NMR (STD-NMR).....	10
3.3 Relaxation filter NMR	11
3.4 Assay development	12
3.4.1 Background	13
3.4.2 Developing an assay	13
3.4.3 Statistical analysis	14
3.4.4 Performing a screen	14
3.5 Analytical gel filtration	14
4. Materials and experimental procedures	15
4.1 SOD1.....	15
4.2 Compounds	15
4.3 NMR	16
4.4 Activity Assay development.....	17
3.4.1 SOD Assay Kit-WST	17
4.5 Gel filtration.....	19
4.5.1 SDS PAGE.....	19
5. Results.....	20
5.1 NMR	20
5.2 Activity assay development	23
5.3 Gel filtration.....	26
5.4 ESI-MS	31
6. Discussion	33
6.1 Future work.....	34
7. Conclusions.....	35
8. Acknowledgements.....	35
9. References.....	36

1. Introduction

1.1 Preclinical drug development

In the pharmaceutical industry the search for new drugs never ends. It is an expensive business, and only few of the starting points reach the market. There are many different strategies about the drug discovery process; how to go from a compound library to hit molecules, on to lead molecules and lead series, further on to candidate drugs, which enter clinical trials, and at last to commercially available drugs. These strategies are constantly evolving, side by side with the technology development in the area.

The lead generation strategy described below is one widely used by pharmaceutical companies and in academic research today [1] (figure 1). One prerequisite for the use of this method is that a compound library is available. Most pharmaceutical companies have internal compound libraries, but there are also some public databases available.

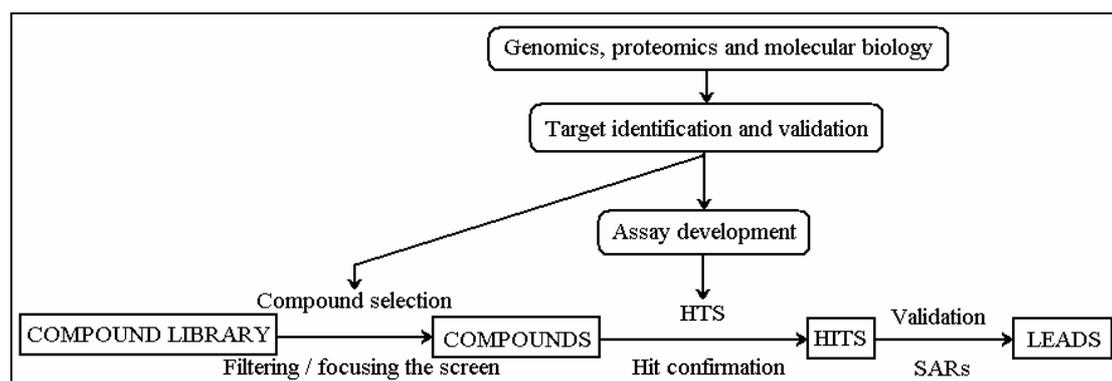


Figure 1. The process of generating leads from a compound library.

To begin with, the purpose of the project must be clear, in other words; what disease or condition are we trying to cure? Before a drug target can be designated, extensive knowledge of the disease has to be collected. Basic research in the areas of genomics, proteomics and molecular biology comes in handy in the process of understanding the disease and identifying a possible drug target. When the target protein has been identified, a way of selecting active compound has to be developed. The compounds are usually tested for their effect on the target in a high throughput screening (HTS) assay. The high throughput is especially important if a large and diverse set of target proteins are to be tested. The demands on the design of an HTS assay are many; it has to be fast, cheap, robust and preferably simple. There are many different kinds of assay formats to choose from, and it should be noted that the quality of the resulting data depends greatly on the choice of both assay and detection method.

Before the developed assay can be used, there is the question of which compounds to run through it. There are different ways to go when limiting the screening set, two of

them are filtering and focusing the screen [2]. To filter a screen is to identify compounds that should be avoided. Reasons to avoid compounds can be for example the suspicion of toxicity or reactivity. Physical parameters can also be used as filtering criteria; Lipinski's "rule of five" is a well-known filtering approach that removes compounds with number of hydrogen-bond donors >5, hydrogen-bond acceptors >10, molecular mass >500 Da and logP >5. This filter should remove compounds that are likely to have poor bioavailability, typically through low solubility or cell permeability. To focus a screen is to identify compounds with high probability of being hits in the assay. One approach is to screen libraries that have been targeted to a specific gene family. Another way to go is to use a computerized model of the target and create screening libraries by docking sets of compounds to that model. Several different software applications, designed for this purpose, are commercially available. Both filtering and focusing a screen have their limitations and an experienced chemist should always control the results before running the assay.

When the screening sets have been chosen and the screening campaign has been completed, hopefully hits have been generated. A hit is a molecule that has the desired biological and pharmacological effects *in vitro*. It has to be chemically pure and stable under assay conditions. It has to be soluble and permeable but not electrophilic. In order to reduce the number of false positives, the hits have to be confirmed by at least one independent screening method [3]. In the process of validation, the structure activity relationship (SAR) has to be established. A SAR is a kind of framework for the chemical properties of the desired molecule, for example where hydrophobic groups are situated or where there are opportunities for hydrogen bond formation.

At the end of this process, a series of lead molecules with a well-defined SAR should have been generated. This series will be developed further by chemists, and hopefully in about 9 to 12 years [1], result in a drug that can cure the disease intended.

2. Project background

2.1 Amyotrophic lateral sclerosis (ALS)

ALS is a progressive and degenerative neuromuscular disorder that is inevitably fatal. The motor neurons of the brainstem and spinal cord are degenerated, which leads to a gradual weakening of muscles, increased muscle spasticity, muscle atrophy and eventually the fatal event; paralysis of lung muscles. The disease affects 2-6 people per 100 000 world wide, and about 200 people each year in Sweden. There are two types of ALS: sporadic and familial. The more common sporadic ALS has no obvious genetic component, whereas familial ALS (FALS) is inherited. Approximately 5-10% of all ALS-cases are FALS. The age of onset is usually 50-60, although FALS generally has an earlier onset. Half of those diagnosed with the disease die within three years, one fourth dies after five years and only one tenth still lives ten years after the diagnose is set. As of today, there is no effective treatment to stop or reverse the course of the disease [4].

2.2 SOD1 and ALS

The relationship between FALS and Cu/Zn-superoxide dismutase (SOD1), a homodimeric metalloenzyme (figure 2), was established in the beginning of the 1990's [5]. Since then, mutational studies have gained some insights to the underlying mechanisms of the disease. The mutated SOD1, involved in FALS, has an increased propensity to monomerize and aggregate [6, 7]. Although the mechanism of neural death in ALS is not known, it is believed that the aggregates can be directly responsible [8, 9]. The catalytic activity of the enzyme on the other hand, is evidently not connected to the disease.

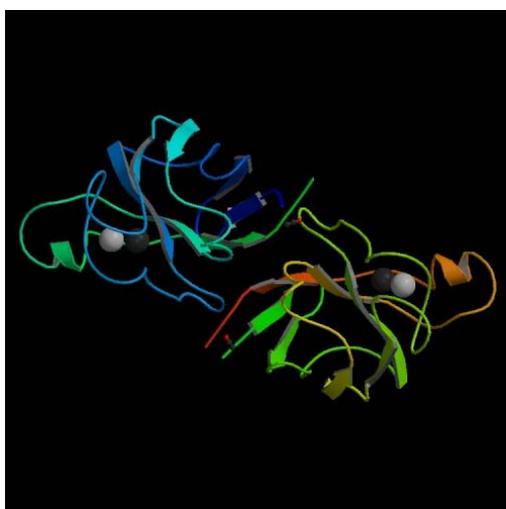


Figure 2. Cu/Zn-superoxide dismutase 1 (SOD1). PDB code 1SPD

The SOD1 homodimer has been thoroughly studied with X-ray crystallography (e.g. Protein Data Bank ID code 1SPD), and a cleft between the monomers where a small stabilizing compound could fit has been identified [10]. Such a compound could prevent the dimer to monomerize and aggregate, and thereby maybe slow the progression of both FALS and sporadic ALS. Work has already been done along these lines by Ray *et al.* [10], through a virtual screen and low throughput screening, resulting in the identification of stabilizing, low molecular weight compounds. These are some of the first small steps when developing a drug, but more extensive studies have to be performed, and questions have to be answered:

Do the compounds really bind in the cleft between the monomers?

Does the binding really prevent monomerization and aggregation?

Are any of the compounds viable starting points for developing drug candidates?

If a pharmaceutical company is going to be able to investigate these and other properties concerning SOD1 and ALS, high throughput screening methods have to be developed.

Thus, the objective of this project was to test the compounds identified by Ray *et al.*, as well as compounds from a virtual screen performed at Biovitrum, for direct binding

using STD-NMR. Verified binders would then be used to develop an assay for high throughput screening, investigating the stabilizing effect of the compound on the dimer.

3. Methods

3.1 Nuclear magnetic resonance (NMR)

NMR is a spectroscopic method, in which absorption of radio frequency energy by atomic nuclei in a magnetic field provides information of their molecular environment.

3.1.1 Introduction

Magnetic nuclei possess an intrinsic angular momentum known as spin. When an atom is placed in an external magnetic field, its spin adopts a direction, a polarization; this polarization is fixed in relation to the magnetic field. All possible directions in space occur, but the spins pointing in the same direction as the magnetic field (parallel to the field) have the lowest energy, while the spins pointing in the opposite direction of the field (antiparallel to the field) have the highest energy. This results in a net spin magnetization parallel to the field. The difference in energy between the highest and lowest energy level is very small and gives rise to a smaller signal than many other spectroscopic techniques do.

If an electromagnetic radiation pulse of a suitable duration and frequency (ν) is applied perpendicular to the external magnetic field, the net magnetization will also become perpendicular to the external field. Such a pulse is called a 90° pulse and will cause coherent superposition of the energy states. After the pulse, the response can be measured and turned into an NMR spectrum. ν , also known as the resonance frequency is specific for every type of nucleus, so it is only possible to directly detect one type of nucleus, for example ^{13}C or ^1H , in one experiment. The response to the pulse is different depending on the environment of the nucleus as the immediate surroundings affect the magnetic field at the nucleus. The local magnetic field determines the resonance frequency and thereby the response to the pulse. This property, known as chemical shift, makes it possible to distinguish between for example different ^1H -atoms within the same molecule. Compared to other spectroscopic methods, NMR is rather insensitive, but it still is a powerful technique for studying molecules at an atomic level.

3.1.2 Theory

Besides atomic number and mass number, all atomic nuclei also have a spin quantum number, denoted as I . I depends on the number of unpaired protons and neutrons of the nucleus. The value of I in the lowest nuclear energy state is called the ground state nuclear spin, or simply the nuclear spin. ^1H and ^{13}C , the nuclei most frequently used in NMR, have $I = \frac{1}{2}$. Nuclei with no net spin ($I = 0$), such as ^{12}C and ^{16}O , cannot be

used in NMR, as there is no separation of energy levels in an external magnetic field and therefore no NMR spectrum.

Spin angular momentum \mathbf{I} is a vector with quantified direction and magnitude. The direction of the vector indicates the axis of the rotational motion of the spin in a magnetic field, and is called the spin polarization axis. \mathbf{I} has $2I + 1$ projections onto any chosen axis, for example the z-axis (the z-axis points, by convention, in the direction of the external magnetic field). The z-component of \mathbf{I} is quantified:

$$I_z = m \hbar \quad (\text{eq. 1})$$

where $\hbar = h/2\pi$, h is Planck's constant, and m is the magnetic quantum number, ranging from $-I$ to $+I$ in integer steps:

$$m = -I, -I + 1, -I + 2, \dots, 0, \dots, I - 1, I \quad (\text{eq. 2})$$

Hence a nucleus with $I = 1/2$ has two quantified energy states: $I_z = \pm 1/2 \hbar$, while a nucleus with $I=1$ has three states: $I_z = 0, \pm 1 \hbar$, when a magnetic field is applied. The energy difference ΔE between the states, is equal to

$$\Delta E = h\nu \quad (\text{eq. 3})$$

The magnetic moment $\boldsymbol{\mu}$ of a nucleus is proportional to its spin angular momentum.

$$\boldsymbol{\mu} = \gamma \mathbf{I} \quad (\text{eq. 4})$$

where γ is the gyromagnetic ratio, specific for each type of nucleus. The magnetic moment is parallel to the spin polarization if γ is positive, as most often is the case for atomic nuclei, and antiparallel if γ is negative.

If no external magnetic field is present, the spin polarization axes of nuclei can point in all possible directions and the distribution is isotropic. When an external magnetic field is applied, the spin polarization moves around the field. This rotational motion is known as precession. The angular frequency of spin precession ω_0 , called the Larmor frequency, is equal to

$$\omega_0 = -\gamma B^0 \quad (\text{eq. 5})$$

where B^0 is the strength of the magnetic field at the site of the particle. A Larmor frequency of $400 \cdot 10^6 \pi \text{ rad s}^{-1}$ implies that the nuclear spin completes 200 million revolutions around its precession cone every second.

A certain time after the application of the external magnetic field the distribution of spin polarizations reaches thermal equilibrium. The time constant T_1 , called the relaxation time constant, takes in account the time required for this equilibrium to form. At thermal equilibrium, there is an anisotropic distribution of spin polarizations

with a net magnetic moment parallel to the external magnetic field, also called longitudinal or Z-magnetization.

As mentioned above, the difference in energy between the highest and the lowest energy state is very small for all nuclei; as a consequence, the populations of its energy levels are almost the same. The small difference in population between the energy levels is what makes the NMR signal weak compared to other spectroscopic methods, as it is the energy difference that gives rise to the signal. The difference in energy between the states is also known as the resonance condition:

$$\Delta E = \hbar \gamma B \quad (\text{eq. 6})$$

As seen in equation 6, the resonance condition depends on the size of the external magnetic field. A higher external field gives a higher energy difference and therefore a larger signal (higher sensitivity).

3.1.3 The NMR signal

To detect an NMR-signal a 90° pulse is applied. This is a radio frequency pulse that rotates the net magnetization 90° to the transverse plane (x-y plane). This net magnetic moment perpendicular to the magnetic field is called transverse magnetization. The transverse magnetic moment decays with time. This homogenous decay is taken account for in T_2 , a time constant known as the transverse relaxation constant. Due to the difference in tumbling rates, T_2 is short for large molecules, and long for small molecules, this means that the decay of the transverse magnetic moment in general is faster for large molecules than it is for small molecules. A rotating magnetic moment gives rise to a rotating magnetic field. A changing magnetic field induces an electric field. The electric field causes an oscillating current in a wire coil if it is placed in the field (figure 3).

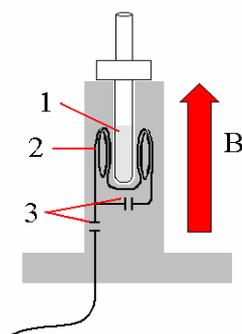


Figure 3. A single-channel NMR-probe. (1) The sample is inserted into the probe in a glass tube. (2) Wire coils are placed to generate the RF pulse and then to detect the rotating transverse magnetisation. The external magnetic field (B) is longitudinal to the sample. The two capacitors (3) are used to “tune the probe” into giving an as high signal as possible.

The NMR detected signal or free-induction decay (FID) is an oscillating current due to the precessing transverse magnetization. Figure 4 shows the pulse sequence and the signal of an ordinary 1D ^1H NMR experiment.

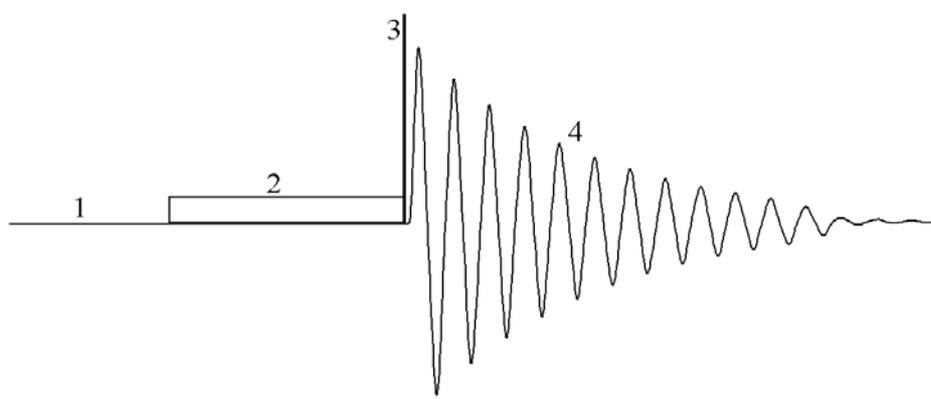


Figure 4. The set up of an ordinary 1D ^1H NMR experiment. (1) Nuclear magnetic spins in the sample reach thermal equilibrium in the external magnetic field. (2) A low level of radiation at the exact frequency of the solvent, aimed at suppressing the signal of it, is sometimes used (In a ^1H NMR experiment the suppression pulse is used if the sample contains H_2O). (3) A 90° pulse is applied on the sample to rotate the net magnetization to the x-y plane. (4) Instantly after the pulse, the spectrometer detects and amplifies the FID.

The FID, detected by the coils (figure 3), describes the NMR spectrum as a function of time. To achieve a spectrum in the frequency domain, a Fourier transform (eq. 7) is applied to the FID.

$$S(\Omega) = \int_0^{\infty} s(t) \cdot e^{-i\Omega t} dt \quad (\text{eq. 7})$$

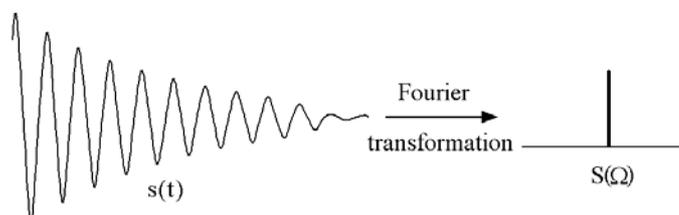


Figure 5. A simplified figure of the Fourier transformation of a FID ($s(t)$) into an NMR spectrum in the frequency domain ($S(\Omega)$).

As mentioned earlier, the resonance frequency depends on both the nature of the nucleus and its surrounding environment. If the surroundings are electron rich, the electrons shield the nucleus from the magnetic field, consequently the field strength that the nucleus experience is lower than B^0 . This lowers the resonance frequency according to equation 5. The lowered resonance frequency is what lies behind the chemical shift δ , seen in an NMR spectrum.

$$\delta = 10^6 \left(\frac{\omega_0 - \omega_{0,TMS}}{\omega_{0,TMS}} \right) \quad (\text{eq. 8})$$

where the Larmor frequency of TMS, $\omega_{0,TMS}$ is used as a reference. δ is measured in ppm.

3.1.4 NMR in practice

When performing an NMR experiment there are several important parameters that have to be adjusted, to get as high signal as possible. The buffer in which to run the experiment has to be considered. The hydrogen content of the buffer should be as low as possible as the hydrogen give rise to signals in the spectra (in ^1H NMR). This is the reason why most ^1H NMR buffers are based on deuterium instead of hydrogen, but even so, the peak from the hydrogen of the solvent will always be present at some level. When the sample has been prepared, the temperature at which to run the sample should be decided and fixed, as it affects most of the parameters below. When the sample has been inserted into the spectrometer, the homogeneity of the external magnetic field across the sample has to be optimised. This is done by adjusting a set of auxiliary room temperature electromagnets to compensate for the inhomogeneity of the main static field. This process, called “shimming the magnet”, is semi-automatic in the instruments used today. When the “shimming” has been performed, the probe has to be “tuned”. This is often done manually by turning the capacitors. The “tuning” is performed in order to efficiently deliver radio frequency energy into the sample volume and to achieve the highest sensitivity of detection. Next the parameters of the pulse sequence have to be investigated. There are ready-made standard pulse sequences to use in different NMR experiments, but a few parameters might need to be changed: The pulse width (pw) of the 90° pulse has to be adjusted to obtain as high signal as possible. The centre of the NMR spectrum, at the frequency of H_2O , called the carrier frequency (tof) depends for example on the temperature of the sample and has to be adjusted. The number of times the spectrometer applies the pulse or the number of transients (nt), is the last parameter to be decided. nt is always a multiple of four, as the spectrometer applies the pulse from four different directions. If nt is increased by a factor of two, the signal to noise ratio only increases by a factor of $\sqrt{2}$, while the experiment takes twice as long to finish. Even so this time is often well spent, if a readable spectrum is to be the outcome.

3.2 Saturation transfer difference-NMR (STD-NMR)

STD-NMR is a technique that detects ligand-protein interactions by saturation transfer [11, 12]: At one ^1H resonance frequency of a specific part of the protein (e.g. 0.7 ppm), a weak radio frequency (RF) field saturates the protein for a certain time period (usually a couple of seconds). Perfect saturation is accomplished when the thermal equilibrium in the external magnetic field is broken, the populations of the different energy states are equalized and there is no net magnetization. The saturation is then transferred to the rest of the protein, as well as to ligand molecules bound to it, by spin diffusion. When the saturated molecules dissociate from the protein, and transfer into solution, the saturation leads to an attenuation of their signal. The achieved spectrum is subtracted from a reference spectrum, where the RF field has been applied at a frequency far away from both protein and ligand (e.g. 30 ppm), resulting in an STD spectrum showing only the signals from compounds interacting with the protein (figure 6).

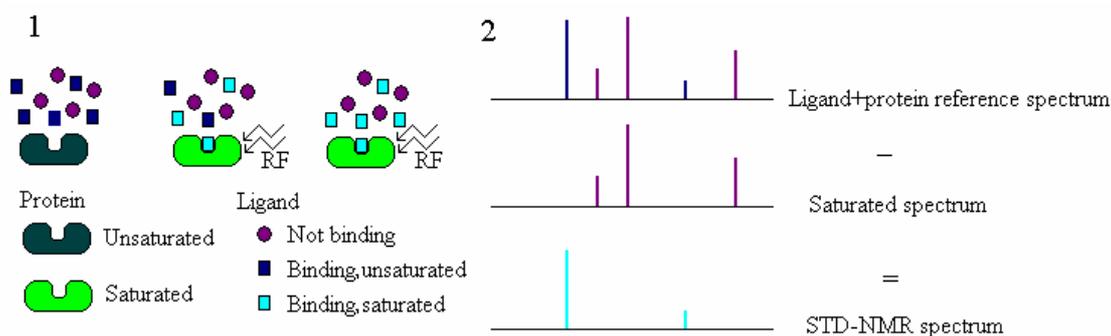


Figure 6. (1) The protein becomes saturated by the means of a train of weak RF pulses. The saturation is transferred, by spin diffusion, to any ligand molecule bound to the protein. The saturated ligand is exchanged into solution, where its NMR signal is attenuated. (2) The saturated spectrum is subtracted from the reference spectrum, resulting in a spectrum where the only signals are those from the saturated ligand.

Some of the factors that affect the outcome of STD-NMR are: The dissociation rate of the ligand, the ratio ligand/protein, the irradiation time and frequency of the RF field and the strength of the magnetic field.

If the dissociation rate (off rate) is very slow, only a small fraction of ligand molecules will be saturated during the saturation time, resulting in a very weak NMR signal. If the dissociation rate is fast, a greater number of molecules will bind to the protein, hence increasing the signal, at least to a certain point. If the affinity is very low, the probability of the binding event can be too small to even give a signal detectable over the noise. STD-NMR can be used on ligands binding to proteins with dissociation constant, K_D , between 10^{-8} and 10^{-3} M assuming diffusion-controlled on-rates.

If a large excess of ligand is present, one protein-binding site can be used to saturate many ligand molecules in a few seconds. From the high ligand/protein ratio generally used in STD-NMR it is clear that only a relatively small amount of protein is needed for the measurements.

If the ligand has a peak near the saturation frequency, direct saturation can occur. This means that the ligand can give rise to a signal even though it does not bind to the protein. To avoid this, the protein should be irradiated at a frequency where there are no ligand peaks. There is an asymptotic relation between irradiation time and strength of signal, hence, the longer the time, the stronger the signal, to a maximum value. The strength of the external magnetic field affects both the sensitivity and the spin diffusion rate. A stronger external field gives a more sensitive and efficient method.

3.3 Relaxation filter NMR

Relaxation filter NMR is another method that detects binding [12]. As opposed to STD-NMR the outcome of this method is independent of the kinetics of the binding,

weak as well as very high affinity ($K_D < \text{ca } 10 \text{ nM}$) or covalent binders can be detected.

The difference between relaxation filter- and regular NMR experiments lies in the proportion between ligand and protein concentrations (excess protein or equimolar amounts of protein and ligand is used) and the manner of detection. In relaxation filter NMR, after the 90° pulse; a spin lock filter is applied for a certain time, before the FID is collected (figure 7).

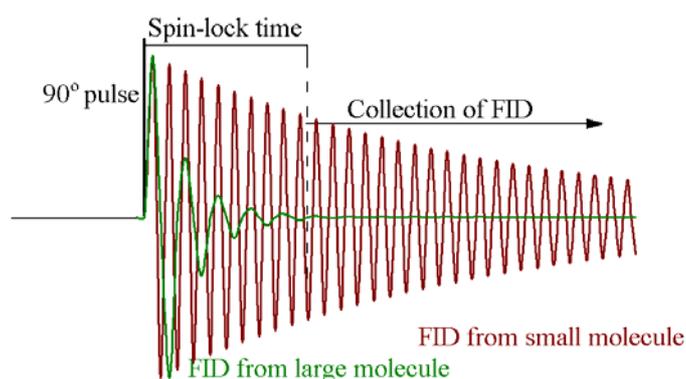


Figure 7. In relaxation filter NMR, the only signal collected is the FID of small molecules (red line). Large molecules lose their transverse polarization before their FID (green line) is collected.

During the spin lock time, large molecules lose their transverse magnetization; hence their signals are lost before the FID is collected. Small molecules, on the other hand keep their magnetization for a longer period of time. If a small molecule (ligand) binds to a large molecule (protein) the ligand will lose its magnetization at the same rate as the protein does, and a decrease in ligand signal will be the result. If the binding is relatively strong the ligand signal will disappear completely, if the binding is relatively weak the signal will decrease to a smaller extent compared to a reference spectrum where the protein is absent. To get an idea of the relative affinities, different spin lock times can be used. If the ligand signal disappears when the spin lock time is short, the affinity is relatively high. One disadvantage of this method is that it is quite protein consuming, as the ratio ligand/protein should be close to 1 and a concentration of at least a few μM is needed for detection. This makes relaxation filter NMR less suitable as a screening technique.

3.4 Assay development

As mentioned earlier, HTS assays are important tools in the search for new drug candidates. The purpose of an HTS assay is to extract interesting compounds from large compound libraries, at a low cost and as fast as possible, without losing quality in terms of assay performance.

3.4.1 Background

In the recent decades, new technologies and automated systems have led to lowered costs and improvements in speed and capacity of HTS systems. The number of data points is no longer the main problem, but the actual quality of them becomes more important, demanding higher information content of the generated leads [1].

There are many different types of assays. The majority of them can be divided into one of two general types, cell-based or biochemical. The two types have different advantages and disadvantages, for example: Cell-based assays can provide more biological information about if and especially how a compound affects a receptor or ion channel. One problem is that several potential targets within a pathway are screened simultaneously and some of the generated hits may not modulate the desired target, but another protein in the same pathway. Biochemical assays on the other hand have advantages when screening intracellular targets, and if the biochemical assay is well optimised, it will have less data scatter than cell-based approaches.

Biochemical assays can be divided further into separation-based assays and homogeneous assays. The difference between these types is that the reaction product is separated from the starting material before it is detected in separation-based assays, while no separation is needed in the homogeneous assays. This makes homogeneous assays better suited for automation and HTS, as robots simply have to add reagents to the sample before measuring the result. Separation based assays have an advantage in the fact that most compounds cannot interfere with the signal, thereby resulting in a larger signal window than most homogeneous assays have.

3.4.2 Developing an assay

When developing an assay, the first thing to consider is the goal of the screen and what type of assay is best suited for that purpose. Almost as important as the choice of assay format is the choice of detection method. There are many different methods to choose from; absorbance, fluorescence, luminescence and methods based on radioactive isotopes are just some of them. When the type of assay and detection method has been chosen, the intensive work to evaluate and optimise the assay begins. The assay should be sensitive enough for the proposed function, but it also has to be robust and reproducible. Parameters such as the linear range of target concentration, sample volumes, running temperature, pH, ionic strength and incubation times all have to be settled. These first developing steps are often performed in larger volumes than the finished assay will use, so the next coming step is to miniaturize the assay. The miniaturization process reduces sample volumes and makes use of high-density plates. This often leads to a reduction of assay performance as the surface-to-volume interaction and the exposure to oxygen increases. The reduced volumes also increase liquid handling and detection errors, including for example the impact of evaporation that becomes apparent when volumes are reduced below the 100 μ l scale. Even so, the miniaturization is still considered worthwhile as it leads to increased throughput and reduced costs. A miniaturized assay also set higher demands on efficient data handling as multiple data points are retrieved in each experiment. Automation of the assay requires programming of robots and detection apparatus.

3.4.3 Statistical analysis

Statistical analysis of retrieved data is very important during both assay development and the screening process, even though the type of analysis differ between the two stages. During assay development, the quality of the assay is what needs to be evaluated. Later in the screening process, quality assurance and the evaluation of data to find compounds with desired activities dominates the analysis.

In the assay development process, it is important to look into if the assay format and detection method has any chance of a real application; is there a possible screening window and is the sensitivity of the assay high enough for its purpose? Different metrics are used to evaluate these parameters. The modified screening window coefficient Z' (equation 9) is the most widely used factor for measuring assay quality:

$$Z' = 1 - \left(\frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|} \right) \quad (\text{eq. 9})$$

where σ_{c+} is the standard deviation for the positive control of the assay and σ_{c-} is the standard deviation for the negative control. $\mu_{c+} - \mu_{c-}$ defines the difference in mean values for the positive and negative controls. A Z' value of 0.5 or higher indicates that the positive and negative control differ significantly. In other words, a screening window exists, and the assay has acceptable characteristics for HTS. The reproducibility and stability of the assay are evaluated by comparing the divergence in signals from identical samples within one plate, between plates and from day to day.

3.4.4 Performing a screen

The first screening of compounds is performed at a relatively high compound concentration. Dose-response experiments are then performed on the compounds that show a positive activity. A positive activity is defined differently in various laboratories, but unless the hit rate is unexpectedly high, the average of the positive controls plus three standard deviations is often used to significantly establish a hit.

3.5 Analytical gel filtration

Analytical gel filtration (GF) is a size exclusion chromatography method. It consists of a column packed with gel particles, a UV-detector and a fraction collector. As the sample runs through the column, large molecules move through the column faster than small molecules do. As a result, the largest molecules reach the end of the column and thereby the UV-detector first, while the smallest molecules reach the end of the column just before the solvent does. When proteins are separated, absorbance at 280 nm is often preferred as detection wavelength. The size of the molecules can be estimated by comparison to a standard K_{av} curve, where K_{av} (equation 10) is plotted against $\log(\text{MW})$ for known proteins.

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad (\text{eq. 10})$$

where V_e is the elution volume of the protein, V_0 is the void volume of the column and V_t is the total column volume. A peak of UV absorbance in the chromatogram represents the analyte, and the area of that peak is proportional to the amount of it. V_e for a protein depends not only on the size of the protein but also on the structure of it. If the protein structure has an extended rather than globular form, it can appear larger than it is. This makes this method qualitative rather than quantitative.

4. Materials and experimental procedures

4.1 SOD1

Cu/Zn-SOD1 consists of two identical monomers. Each monomer is built up by two α -helices, two β -sheets, and is stabilized by one internal disulphide bond. The monomers also contain one copper and one zinc ion each. The monomers are joined together mainly by hydrophobic interactions. The metal ions are very important for both the stability and the function of the protein, which is to catalyze the dismutation of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SOD1 is one of the most important antioxidative enzymes and is present in most eukaryotic cells exposed to oxygen. Cu/Zn SOD1 has a molecular weight of approximately 32.5 kDa.

Mikael Oliveberg's group at the Stockholm University kindly supplied the protein material. The proteins were expressed and purified according to reference 4. A number of mutant proteins are routinely used in Oliveberg's laboratory. The rationale is both the possibility to avoid reducing agents in the buffer solution as well as the modulation of protein stability and tendency to aggregate upon unfolding. The received mutants of SOD1 were C6A C111A, L144F/C6A C111A and G93A/C6A C111A.

4.2 Compounds

The compounds from Ray *et al.* were a mixture of possible starting points for drugs, electrophiles and compounds too reactive or insoluble to be considered as good hits. Six of the compounds were purchased from Sigma-Aldrich. Close analogues to four of the compounds were ordered from Biovitrum Compound Collection (BCC) (Table 1). The remaining five of the compounds were considered too reactive to be of any interest. The compounds purchased from Sigma-Aldrich were given an internal reference number (BVT identity number).

Table 1: Compounds used by Ray *et al.* Obtained from Sigma-Aldrich (where noted) and BCC

Vial Label	BVT identity	MW (g/mol)	Concentration (mM)	Solved in	Sigma-Aldrich Reference number
1	BVT149366	240.35	50	DMSO-d6	R944831
2	BVT149371	206.25	50	DMSO-d6	R746665
3	BVT149367	169.16	50	DMSO-d6	R258172
4	BVT149370	220.30	50	DMSO-d6	R263125
5	BVT149369	178.18	50	DMSO-d6	R699055
6	BVT149368	242.28	50	DMSO-d6	R593761
7	BVT10456	253.26	50	DMSO-d6	
8	BVT12180	218.26	50	DMSO-d6	
9	BVT27656	282.10	50	DMSO-d6	
10	BVT27837	251.33	50	DMSO-d6	

Micael Jacobsson at Biovitrum performed a virtual screen, where compounds from BCC were docked to SOD1 using the software GLIDE. The resulting compounds (Table 2) were ordered from BCC, ten as solids and 19 as 10 mM solutions in DMSO. 50 mM stock solutions of the solid compounds were prepared in DMSO-d6.

Table 2: Compounds from virtual screen obtained from BCC

Vial Label	BVT identity	MW (g/mol)	Conc. (mM)	Solved in	Vial Label	BVT identity	MW (g/mol)	Conc. (mM)	Solved in
1	BVT491C	352.48	50	DMSO-d6	16	BVT64455	354.37	10	DMSO
2	BVT1057T	352.41	50	DMSO-d6	17	BVT64795	341.37	10	DMSO
3	BVT1080T	360.38	50	DMSO-d6	18	BVT65033	383.41	10	DMSO
4	BVT2457C	352.48	50	DMSO-d6	19	BVT65034	390.40	10	DMSO
5	BVT3221T	227.31	50	DMSO-d6	20	BVT68771	374.85	10	DMSO
6	BVT5910G	339.48	25	DMSO-d6	21	BVT69345	320.42	10	DMSO
7	BVT16244	275.35	50	DMSO-d6	22	BVT70536	246.27	10	DMSO
8	BVT39125	339.44	50	DMSO-d6	23	BVT98036	397.41	10	DMSO
9	BVT46217C	375.51	50	DMSO-d6	24	BVT98272	312.32	10	DMSO
10	BVT46286C	375.47	50	DMSO-d6	25	BVT98469	322.82	10	DMSO
11	BVT1625T	324.38	10	DMSO	26	BVT104430	259.27	10	DMSO
12	BVT49782C	352.48	10	DMSO	27	BVT107341	267.71	10	DMSO
13	BVT49784C	380.53	10	DMSO	28	BVT107415	356.21	10	DMSO
14	BVT64403	394.48	10	DMSO	29	BVT112420	391.45	10	DMSO
15	BVT64404	346.39	10	DMSO					

4.3 NMR

A 500 MHz Varian Unity spectrometer was used for the first NMR experiments. Standard 5 mm glass tubes were used, and the volume of the samples was 600 μ l. The buffer used was 20 mM TRIS-d11, 150 mM NaCl in D₂O, pH= 7.4. See table 3 for more parameters.

¹H 1D spectra were used as solubility tests for the ten compounds from Ray *et al.* In addition to the 1D STD spectrum, a ¹H 1D reference spectrum was collected for each compound/protein sample. When several experiments of the same type were

performed, a carousel sample changer was used to automatically change the samples. To get rid of the DMSO peak in the ^1H 1D reference spectrum for compounds solved in regular DMSO, a pulse sequence that saturated both DMSO and water peaks was used, this experiment is called ^1H 1D Wet. The DMSO saturating pulse was not used in the STD experiment. To get higher sensitivity a 600 MHz Varian Unity spectrometer, equipped with a cold probe, was used for the relaxation filter NMR and some of the STD-NMR experiments. A flow cell was used and the sample volume was 300 μl .

Table 3: Important parameters in the different NMR experiments. SOD1 mutant 6/111 stands for SOD1 C6A C111A, wt stands for SOD1 wild type.

Parameter	Solubility test	^1H 1D reference	^1H 1D Wet	1D STD	Relaxation filter NMR
Spectrometer used (MHz)	500	500	500	500 / 600	600
Number of transients (nt)	512	512	512	2048	128 / 1024
Compound concentration (μM)	500	200	200	200	20
Protein concentration (μM)	0	4	4	4	20
Temperature ($^{\circ}\text{C}$)	20	20 / 37	20	20 / 37	20
Saturation time (s)				2,3	
Saturation frequency (ppm)				0,7	
Spinlock time (ms)					100 / 400
SOD1 mutant used	6/111	6/111 / wt	6/111	6/111 / wt	6/111

4.4 Activity Assay development

The theory behind the developed assay was to investigate whether the activity could be used as a marker for protein stability. Activity assays are commercially available and hence would serve as a marker for protein stability given that conditions can be identified in which a stable dimer dissociates into denatured monomers, with no activity. The denaturation parameters were the same (high temperature and EDTA) as those used by Ray *et al.*, although the temperature was higher and the time of incubation was shorter.

To see if the compounds have any effect on protein stability, denaturation of SOD1 in presence of the compounds will be performed. If the level of activity after the incubation is higher when a compound was present, it will be interpreted as if the compound stabilized the SOD1 dimer.

3.4.1 SOD Assay Kit-WST

A SOD Assay Kit (Sigma-Aldrich, product number: 19160-1KT-F) was used to measure the activity of SOD1 as an inhibition activity (figure 8) in the samples [13].

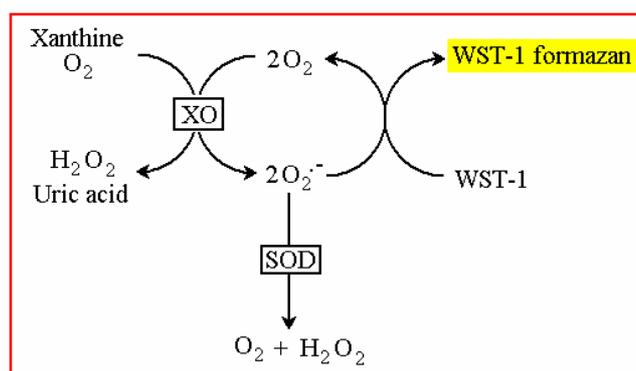


Figure 8. Principle of the SOD Assay kit WST: Xanthine oxidase (XO) reduces O₂ to O₂^{•-}. WST-1, a tetrazolium salt, produces a formazan dye upon reduction with the superoxide anion. The absorbance at 440 nm is proportional to the amount of formazan dye and thereby the amount of superoxide anion. The reduction of WST-1 is inhibited by SOD. Thus, the SOD1 activity can be measured as an inhibition activity, and can be quantified by measuring the decrease in color development at 440 nm.

SOD1 samples were diluted in 20 mM TRIS, 0.01 % BSA, pH =7.5. Samples and blanks for the activity measurements were prepared in a NUNC microwell plate according to the following table:

Table 4. Preparation of samples and blanks for the activity measurements. (All volumes from the technical manual were halved to save reagents.)

	Sample solution	ddH ₂ O	WST ws	Enzyme ws	Dilution buffer
Sample	10 µl	-	100 µl	10 µl	-
Blank 1	-	10 µl	100 µl	10 µl	-
Blank 2	10 µl	-	100 µl	-	10 µl
Blank 3	-	10 µl	100 µl	-	10µl

After the enzyme working solution was added, the samples were incubated on a plate shaker at room temperature for 60 minutes. The absorbance at 440 nm was thereafter measured using a SpectraMax plate reader (Molecular devices). The activity of SOD1 expressed as inhibition rate % could then be calculated according to:

$$SOD \text{ activity} = \left(\frac{(A_{Blank1} - A_{Blank3}) - (A_{sample} - A_{Blank2})}{A_{Blank1} - A_{Blank3}} \right) * 100 \quad (\text{eq. 11})$$

Blanks 2 and 3 were only prepared in the first run. To find the linear area of the assay for the detection method and sample format used, dilution series were made. First a logarithmic dilution was made to localize the concentration range of interest, and then a linear dilution to get a more precise understanding of the concentration dependence. When the linear area had been identified, a method of denaturation had to be developed. Different methods were attempted: 5 mM EDTA, 5 mM EDTA at 62°C, 10 mM HCl, 10 mM NaOH and incubation at 62°C. All samples were left under each of these conditions over night. After evaluation of the results, EDTA and heat was the method of choice, in agreement with the data in Ray *et al.* The denaturation process had to be optimized with regards to time and temperature for the three different SOD1

mutants. After evaluating the data it was decided that SOD1 L144F/C6A C111A had the most HTS compatible parameters, although also in this case, the required temperature is higher than optimal. After optimization for the miniaturization (96 well plate), the parameters decided for were: 0.8 μM SOD1 L144F/C6A C111A, 5 mM EDTA at 55°C for 90 minutes. The total volume in each well was 100 μl . After 90 minutes 100 μl of ice-cold buffer was added to each well to quickly quench the denaturation. Further dilution was made and the final concentration of SOD1 in the activity measurements was 4 nM.

Hence this represents an assay that consumes four orders of magnitude less material than the method used by Ray *et al.* This is an important aspect for a screening assay, since the material cost is quite high despite the miniaturization and because the common screening concentration is in the range 2 –10 μM , *i.e.* at concentrations lower than those used by Ray *et al.*

The compound screen was performed in two steps at 500 and 100 μM compound, respectively. After the screen four compounds were chosen for dose-response experiments. The compounds were tested in logarithmic series from 500 to 1.9 μM .

4.5 Gel filtration

To find out if the activity measurements from the developed assay correlates to the presence of SOD1-dimers, a gel filtration assay was performed on SOD1 C6A C111A samples treated in the same way as the activity assay samples. Because of the sensitivity of the UV detector, the concentration of SOD1 had to be changed in the gel filtration experiments, hence 50 μM SOD1 was the concentration used. The concentration of EDTA (5 mM) was kept. The gel filtration assay was performed by incubating a SOD1 solution with EDTA at 62°C for 70 minutes. Samples were removed periodically and analyzed with gel filtration. The gel filtration column used was Superdex 200 PC 3.2/30 (GE Healthcare, Uppsala), running temperature was 25°C, running buffer was 20 mM TRIS, 150 mM NaCl, pH=7.43, flow rate was 40 $\mu\text{l}/\text{min}$ and the sample volume 20 μl . The fraction volume was 80 μl . Fractions 11-20 were saved for further experiments. The result from this experiment was compared to the activity assay denaturation results from SOD1 C6A C111A.

4.5.1 SDS PAGE

SDS PAGE was run on fractions 13-15 and 16-18. Novex NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad USA) was used and SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, Carlsbad USA) was used as size marker. The experiment was performed in MES buffer.

5. Results

5.1 NMR

The solubility tests that were performed on the ten substances from Ray *et al.* showed that substances 1-8 were very soluble in water, while substances 9 and 10 were less soluble. The amount of dissolved compound is directly proportional to the peak areas, so the solubility of the compound is estimated from its peak heights (for two examples see figure 9 and 10).

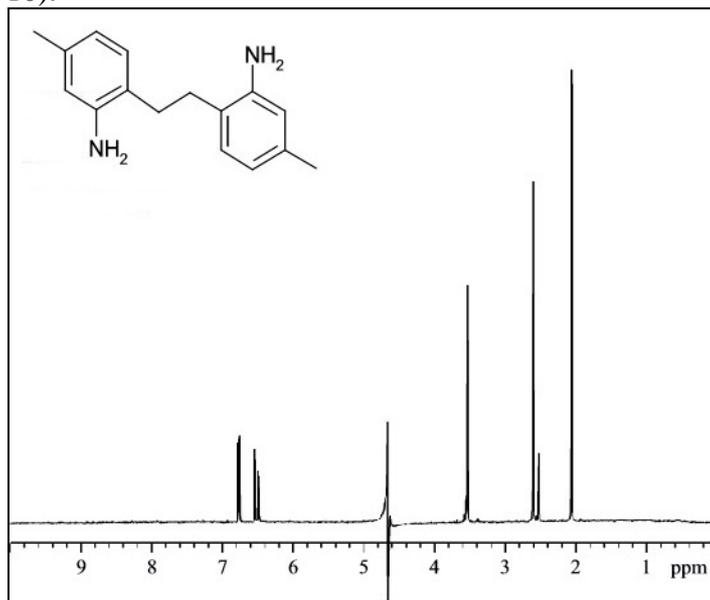


Figure 9. ^1H 1D NMR of compound 1, 500 μM compound used as a solubility test. This compound is soluble to a high degree in water. The peak at ~ 4.6 ppm originates from H_2O , the peak at ~ 3.7 ppm originates from non-denatured TRIS.

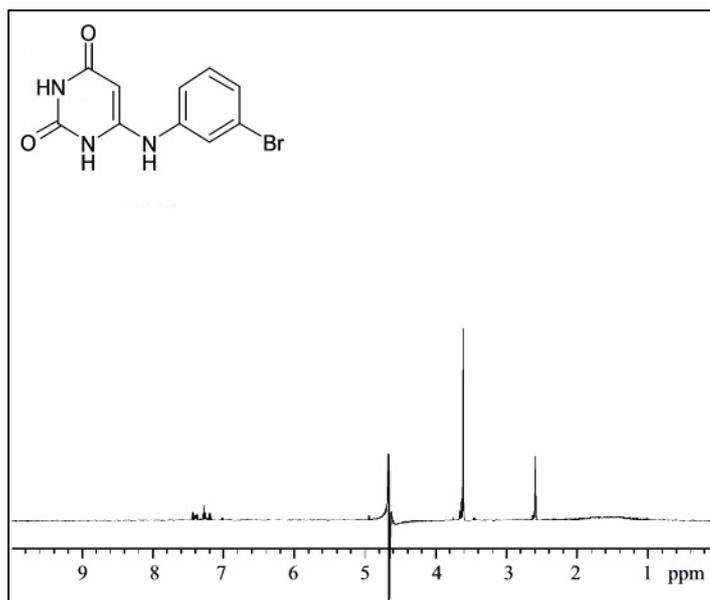


Figure 10. ^1H 1D NMR of compound 9, 500 μM compound used as a solubility test. This compound is not very soluble in water.

The NMR signal from the methyl groups in SOD1 C6A C111A can be seen in figure 11, this signal looked the same in all experiments, indicating a properly folded protein that did not precipitate in the presence of the compounds. It is also clear that the methyl groups of the protein have peaks in the area of the saturation frequency, 0.7 ppm.

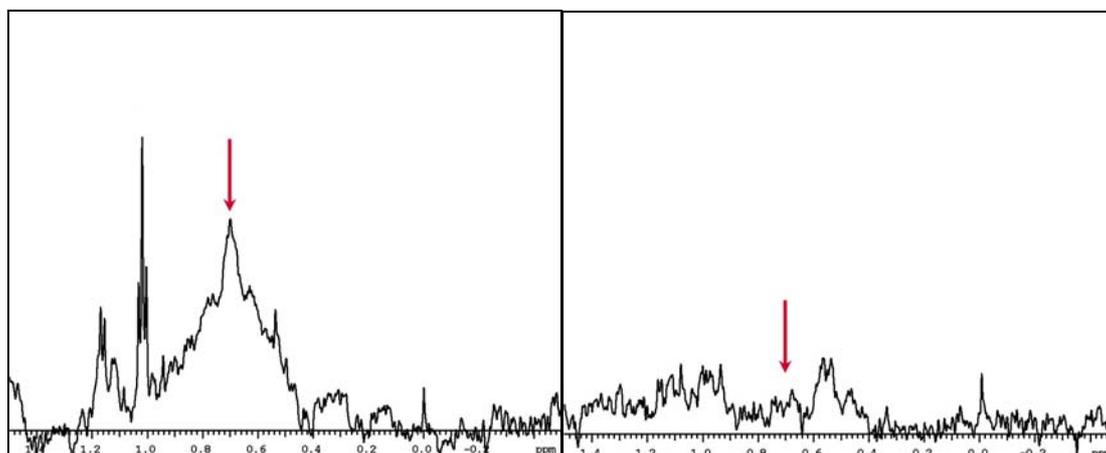


Figure 11. *Left:* SOD1 C6A C111A methyl group protein peak. The visible protein peak reaches from approximately 0.4 to 1.2 ppm. *Right:* The same part of the spectrum, but with no protein added in solution. The saturation frequency (0.7 ppm) used in the STD experiments is indicated with a red arrow

None of the STD-NMR experiments did show any clear binding, however, a very weak signal can be seen for compound 1, 9 and 10 (The STD-spectrum for compound 1 is shown in figure 12). No binding is observed for the other compounds. As an example of an STD spectrum showing no signals, the resulting STD-spectrum for compound 4 is shown in figure 13.

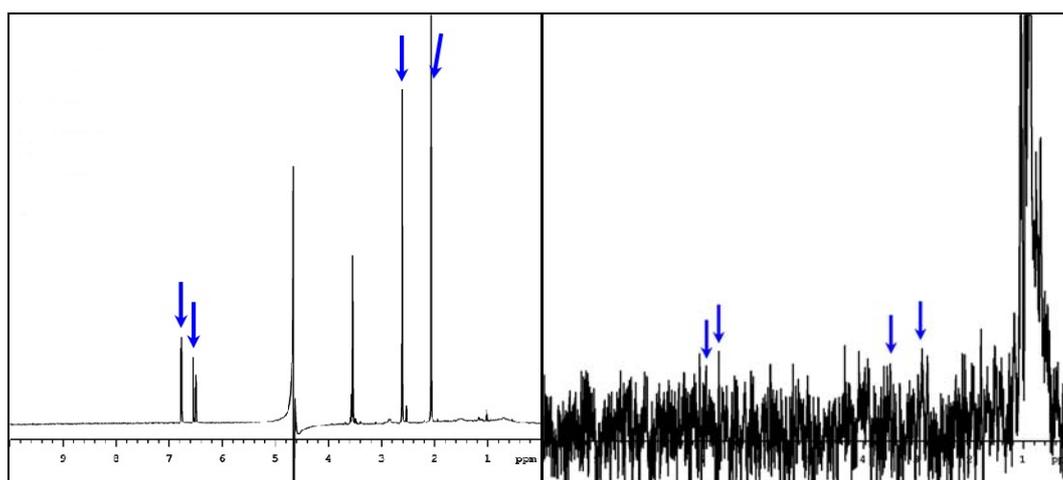


Figure 12. *Left:* 1D ^1H reference spectrum of SOD1 C6A C111A and compound 1; the peaks at the arrows are signals from the compound. The signal at approximately 3.7 ppm originates from non-denatured TRIS. *Right:* 1D STD-NMR of SOD1 C6A C111A and compound 1; the peaks indicated by the arrows could originate from saturated compound 1, but the peaks barely rise above the noise, this indicates very low affinity binding, if any. It is also possible that the off rate is very slow.

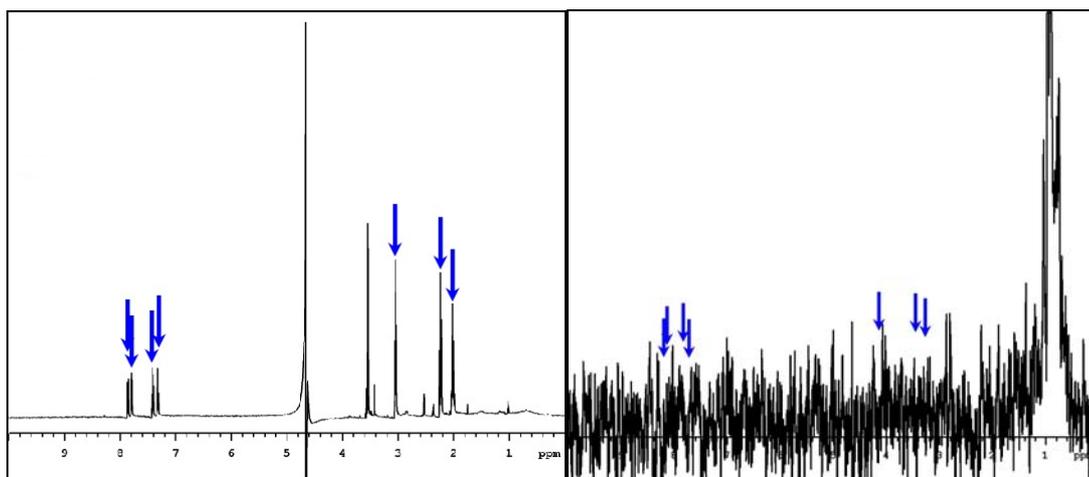


Figure 13. *Left:* 1D ^1H reference spectrum of SOD1 C6A C111A and compound 4; the peaks at the arrows are signals from the compound. *Right:* 1D STD-NMR of SOD1 C6A C111A and compound 4; there are no peaks that could originate from saturated compound 4. This would indicate either no binding or a very slow off rate.

When there was no strong sign of binding to SOD1 C6A C111A, the same experiments were performed on SOD1 wild type. An STD experiment with an increased temperature (37°C) was also performed. The results from these two experiments did not show any more sign of binding than the previous experiments (data not shown).

To see if the low STD signal was a result of a very slow off rate rather than very low affinity, relaxation filter NMR experiments were performed on equimolar amounts of compound 1 and SOD1 C6A C111A. The spin-lock times used were 400 and 100 ms, the resulting spectrum from the 100 ms experiment is shown in figure 14. The experiments revealed no significant decrease of the compound signal in the presence of the protein as compared to the compound signal in the absence of protein, thus demonstrating that the affinity of the compound to the protein is very low. A compound with such low affinity would not be considered a hit in a NMR screening operation.

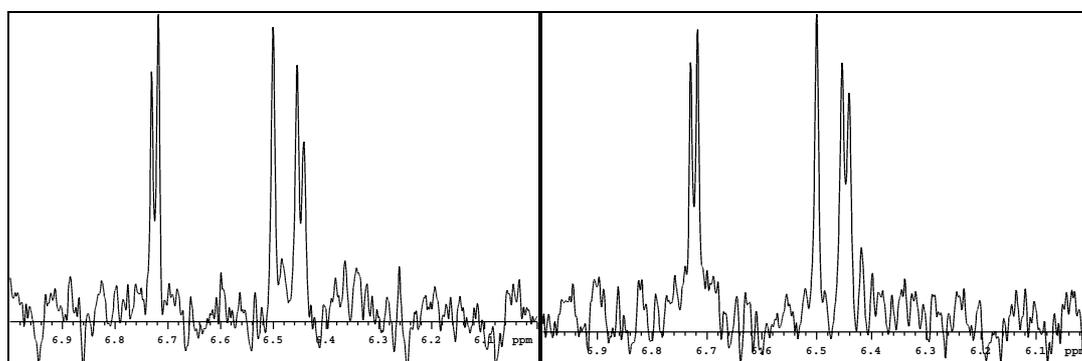


Figure 14. Relaxation filter NMR (spin-lock time=100 ms) on $20\ \mu\text{M}$ SOD1 C6A C111A and $20\ \mu\text{M}$ compound 1. *Left:* No SOD1 present. *Right:* The presence of SOD1 resulted in no significant decrease in compound peak.

Not one of the 29 compounds from the virtual screen performed at Biovitrum showed any sign of binding in the STD-NMR experiments performed on them and SOD1 C6A C111A (data not shown).

5.2 Activity assay development

The results from the logarithmic and linear titration series are shown in figure 15 and 15 respectively. The linear area for SOD1 C6A C111A reaches from 0 to 2 nM and the linear area for L144F/C6A C111A reaches from 0 to 4 nM. The results are shown as raw data from the assay in order not to implement any calculation errors. When interpreting the results a high OD (440) value indicates low SOD1 activity.

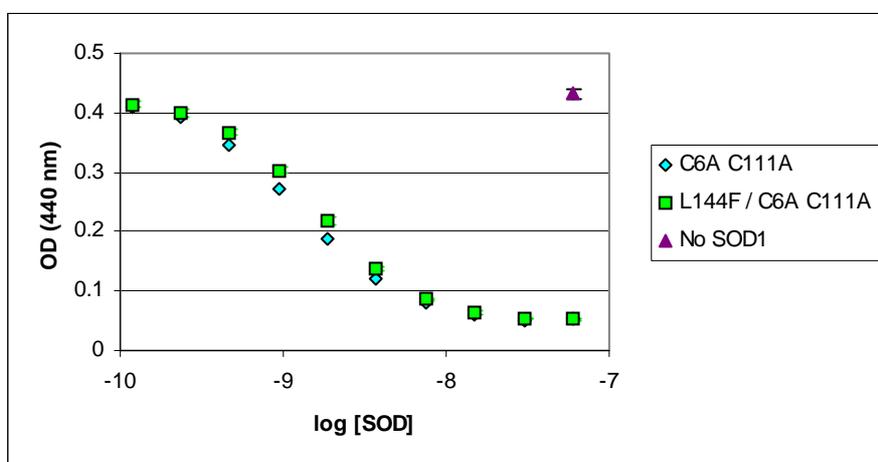


Figure 15. Logarithmic titration of SOD1 C6A C111A and SOD1 L144F/C6A C111A. A high OD(440) value indicates low SOD1 activity and reduced OD(440) values are indicative of increased SOD1 activity

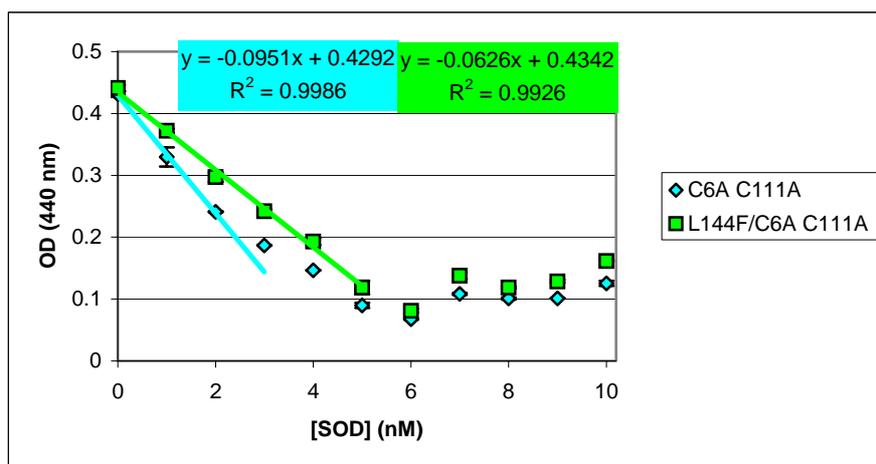


Figure 16. Linear titration of SOD1 C6A C111A and SOD1 L144F/C6A C111A. A high OD(440) value indicates low SOD1 activity and reduced OD(440) values are indicative of increased SOD1 activity. A linear regression was made in the linear area; equations and R^2 -values are at the top of the diagram (blue=C6A C111A, green= L144F/C6A C111A). The linear area reaches up to 2 nM for SOD1 C6A C111A and 4 nM for SOD1 L144F/C6A C111A.

Using the end-point values of the linear area from the titration (0 and 2 nM for SOD1 C6A C111A and 0 and 4 nM for SOD1 L144F/C6A C111A), the Z' factor was calculated for both mutants.

$$Z'(C6A C111A) = 0.91$$

$$Z'(L144F/C6A C111A) = 0.87$$

The denaturation study resulted in time curves (figures 17 and 18). As the C6A C111A-mutant never made it to the miniaturization process, denaturation was performed in eppendorf tubes at 62°C. Almost all activity was gone after 30 minutes of incubation under these conditions. The denaturation of L144F/C6A C111A was performed in a 96 well plate at 55°C. Almost all activity was gone after 80 minutes under these conditions. Denaturation of L144F/C6A C111A in eppendorf tubes showed an almost complete loss of activity after 70 minutes at 50°C (data not shown). Denaturation of G93A/C6A C111A was also performed (data not shown), but the mutant was not used further.

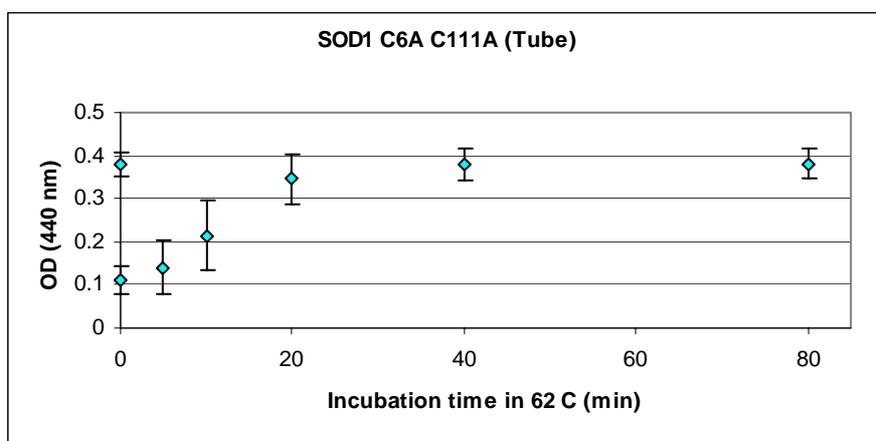


Figure 17. EDTA denaturation of SOD1 C6A C111A over time. A high OD(440) value indicates low SOD1 activity and reduced OD(440) values are indicative of increased SOD1 activity. Most of the activity is gone after approximately 20-30 minutes of incubation at 62°C

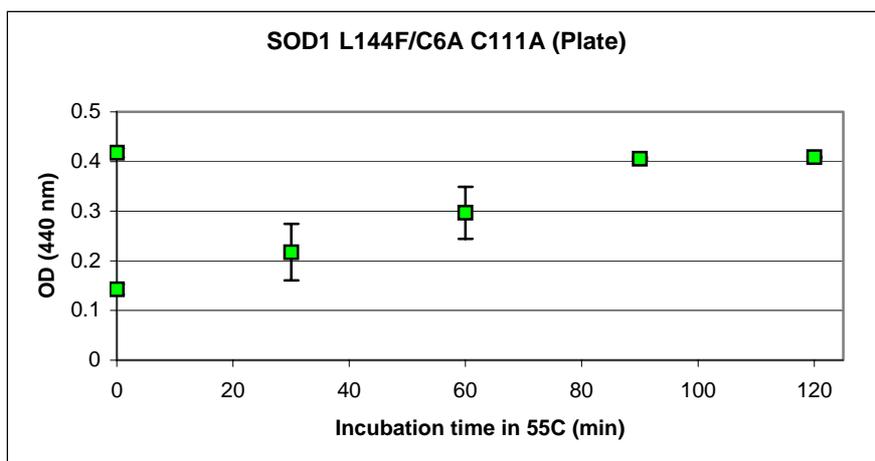


Figure 18. EDTA denaturation of SOD1 L144F/C6A C111A over time. A high OD(440) value indicates low SOD1 activity and reduced OD(440) values are indicative of increased SOD1 activity. Most of the activity is gone after approximately 70-80 minutes of incubation at 55°C.

Based on these data the parameters for substance screening were fixed to 0.8 μM SOD1 L144F/C6A C111A, 5 mM EDTA, temperature 55°C and an incubation time of 90 min. This gives a large window between signal and background, but avoids exposing the samples to incubation times far longer than those that result in complete inhibition. Results from the performed compound screen are seen in figure 19, where the compound concentrations were 100 and 500 μM respectively.

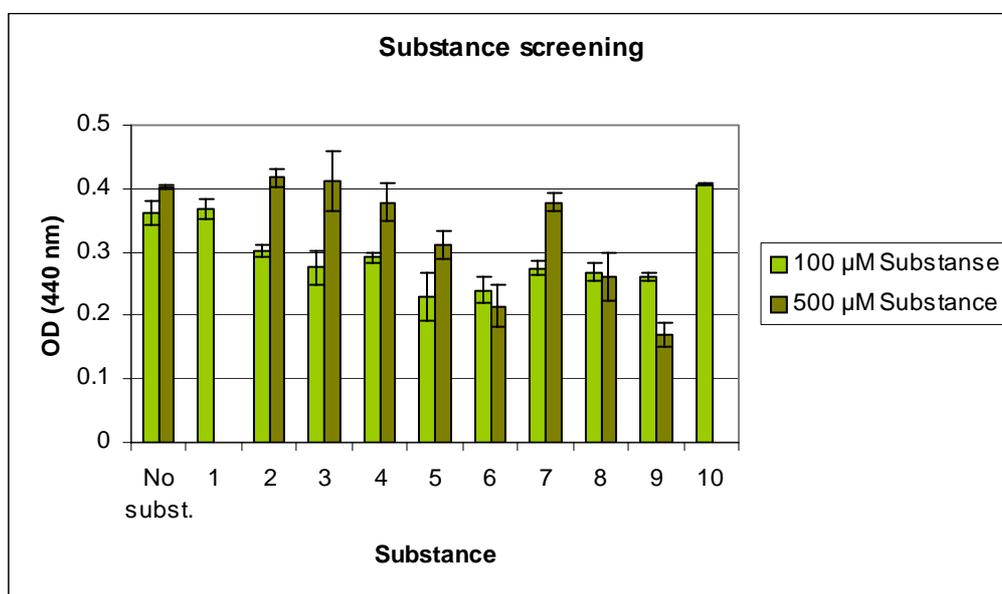


Figure 19. Screening for compound influence at 100 and 500 μM compound. The lower the height of the column bars, the higher the SOD1 activity. The fact that six of the compounds seem to have lower impact on preserving SOD1 activity at a concentration of 500 μM than at a concentration of 100 μM was not expected, and these results are probably not repeatable. The 500 μM experiment was not run on compounds 1 and 10.

Dose-response experiments were performed with compounds 5, 6, 8, and 9. Dose-response curves were achieved for compounds 5 and 6, but the result was not repeatable (data not shown).

5.3 Gel filtration

The gel filtration assay was performed under the same prerequisites as the activity assay. The results however did not show a correlation between the decrease in dimer contents and SOD1 activity, but a rather more complex picture emerged. The result of the time denaturation study is shown in figure 20.

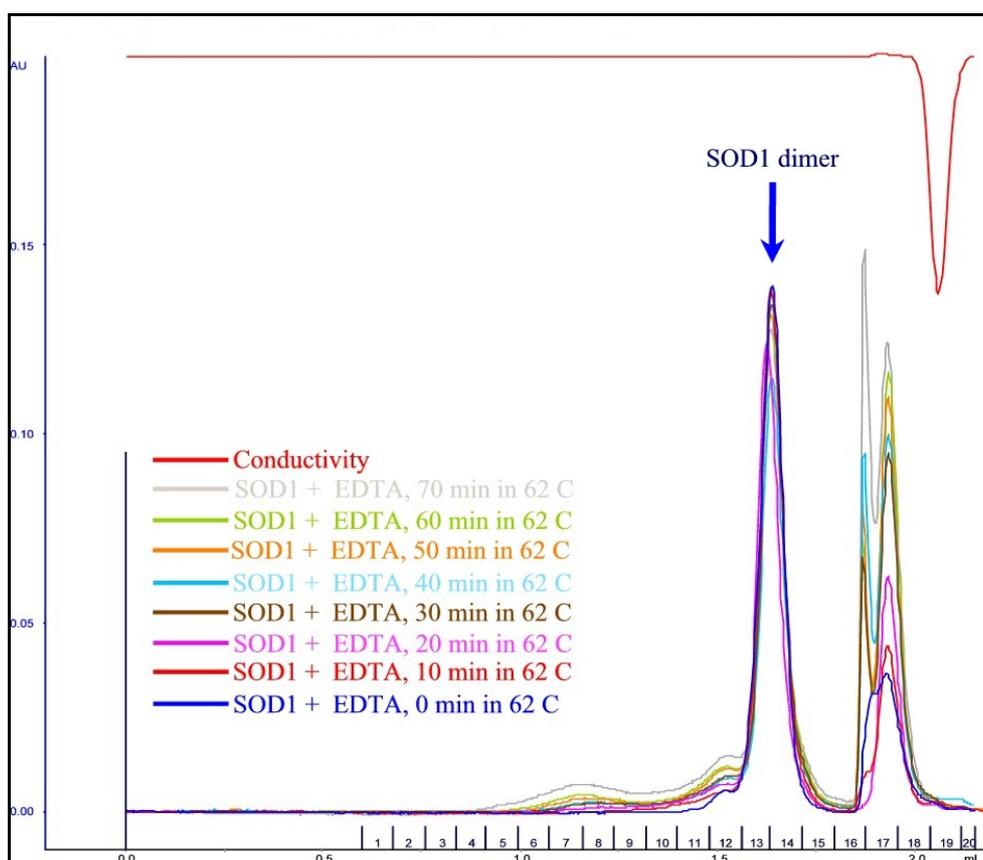


Figure 20. Denaturation of SOD1 C6A C111A in 5 mM EDTA and 62°C, the samples have been taken at the interval 0 to 70 minutes, 10 minutes apart. Three distinctive peaks appeared: $V_e=1.64$ (fractions 13, 14), $V_e=1.88$ (fractions 16, 17) and $V_e=1.93$ (fractions 17, 18). The two wide peaks in fractions 5 to 12 that increased with increasing time probably consist of aggregated SOD1.

To investigate the contents of the peaks, SDS PAGE was performed on fractions from the 40 minute-denaturation sample. The results of the gel filtration showed that the peak at $V_e=1.64$ consists of SOD1 dimer, but the peaks at 1.88 and 1.93 did not contain any protein material.

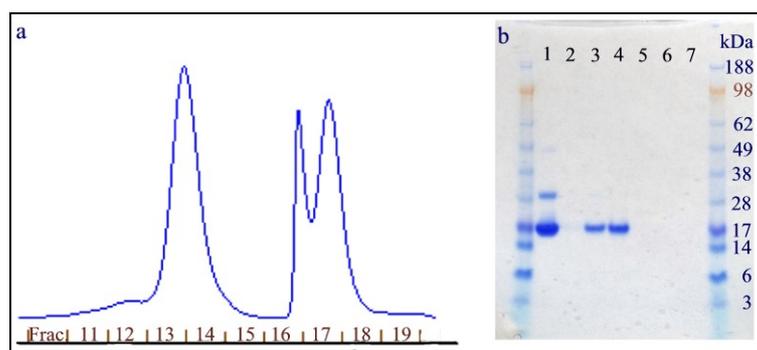


Figure 21. a) Gel filtration of 40 minute-denaturation sample. SDS PAGE was performed on material from fractions 12-14 and 16-18. b) SDS PAGE of 1. Start material; 2. Fraction 12; 3. Fraction 13; 4. Fraction 14; 5. Fraction 16; 6. Fraction 17; 7. Fraction 18. The strong band in lane 1, as well as the bands in lanes 3 and 4, corresponds to monomeric SOD1; the weaker bands in lane 1 are probably traces of dimeric SOD1 and SOD1 aggregates due to insufficient denaturation. There are no bands in lanes 5, 6 and 7; this shows that the peaks in fractions 16, 17 and 18 do not correspond to a protein.

To inquire whether the peaks at $V_e=1.88$ and 1.93 consisted of EDTA-complexes; gel filtration was performed on a solution of 5 mM EDTA and $100 \mu\text{M Cu}^{2+}$ and/or $100 \mu\text{M Zn}^{2+}$ (figure 22). This experiment proved that the peaks at $V_e= 1.88$ and 1.93 consist of complexes of EDTA and the metal ions EDTA had snatched from the protein.

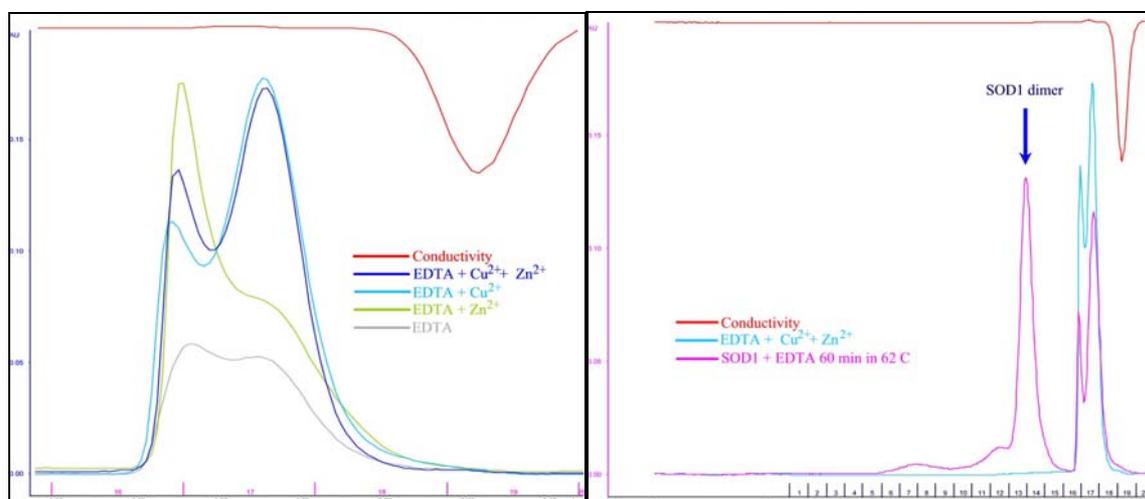


Figure 22. Left: Investigation of the peaks in fractions 16-18. 5 mM EDTA + $100 \mu\text{M Cu}^{2+}$ and/or $100 \mu\text{M Zn}^{2+}$. Right: The experiment proves that the peaks in fractions 16, 17 and 18 consist of EDTA and EDTA-metal complexes.

Enlargement of the SOD1 dimer and EDTA + metal peaks are shown in figure 23. The dimer peak does not diminish to such a degree as was predicted by the activity assay. On the other hand, the EDTA + metal peaks increase as the activity drops.

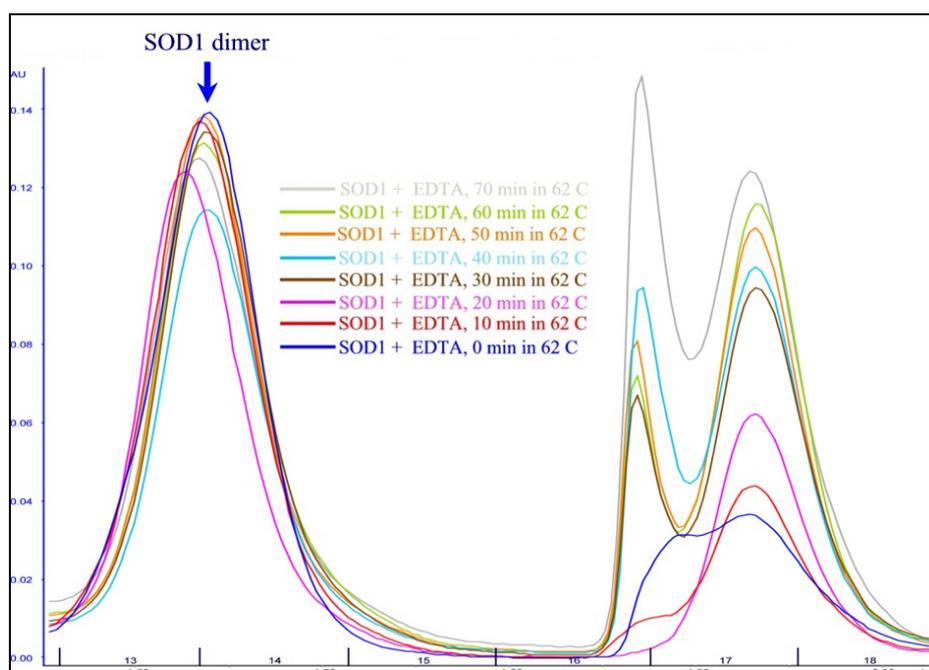


Figure 23. Enlargement of the peaks from figure 20. The peak to the left consists of SOD1 dimers, the two partially overlapping peaks to the right consists of EDTA and EDTA+metal-complexes.

The relation between SOD1 activity and the different peak areas are shown in figures 24 and 25.

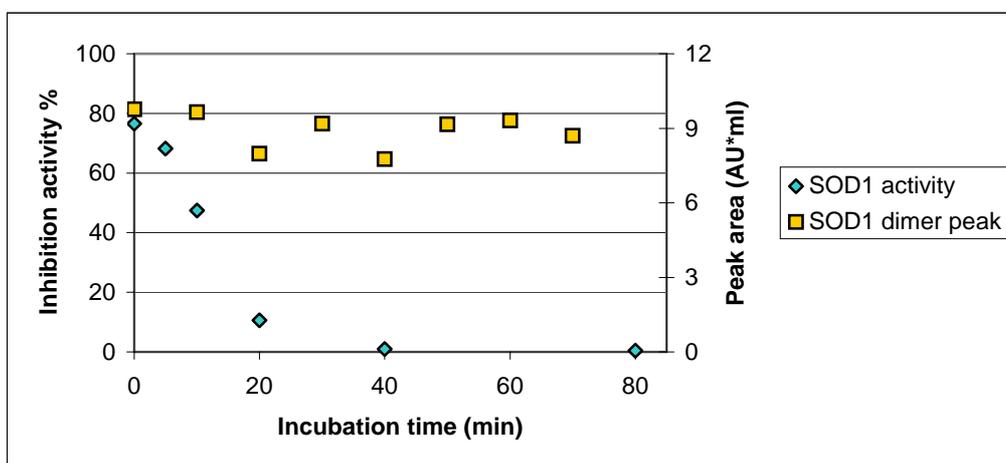


Figure 24. SOD1 inhibition activity has been calculated (equation 11) from the OD (440 nm) measurements (figure 17), and is compared with the area under each SOD1 dimer peak, which has been calculated from the gel filtration experiment. The activity and the peak area are shown, plotted against incubation time. There seems to be no correlation between SOD1 activity and area of SOD1 dimer peak.

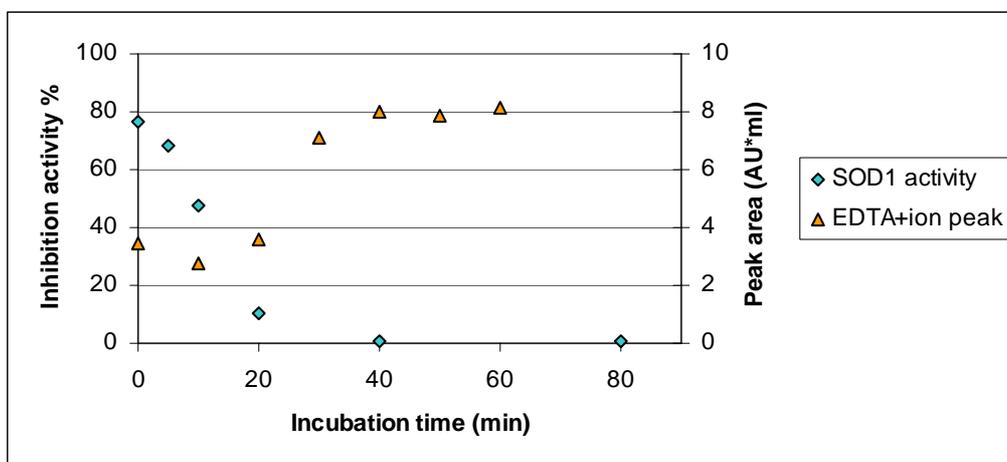


Figure 25. SOD1 inhibition activity has been calculated (equation 11) from the OD (440 nm) measurements (figure 17). The area under the EDTA + metal peaks have been calculated for each time sample. The activity and the peak area are shown, plotted against incubation time. The activity of SOD1 decreases as the peak of EDTA+ metal ions increases.

The loss of activity in the activity assay cannot be explained by the loss of SOD1 dimers under these conditions. However, it is obvious that the dimers present after 70 minutes of denaturation are not active. The correlation between the increasing EDTA+ metal peaks and the decreasing activity shows that when the metal ions are snatched from SOD1, the SOD1 activity is lost. This result is in line with previous data. The fact that SOD1 dimer is present even after metal depletion shows that the metal depleted dimers are more stable than previously thought. The conclusion is that SOD1 activity is not a good marker for the presence of dimer under the conditions used in this assay.

As 70 minutes of denaturation did not significantly decrease the presence of SOD1 dimer, a new denaturation experiment had to be performed. Samples were retrieved and analysed at 0, 3 and 5 hours (figure 26). It was shown that after 5 hours of incubation with 5 mM EDTA at 62°C, the dimer concentration had dropped to approximately 50 %. This was enough for the purpose of screening compounds for their stabilising effect.

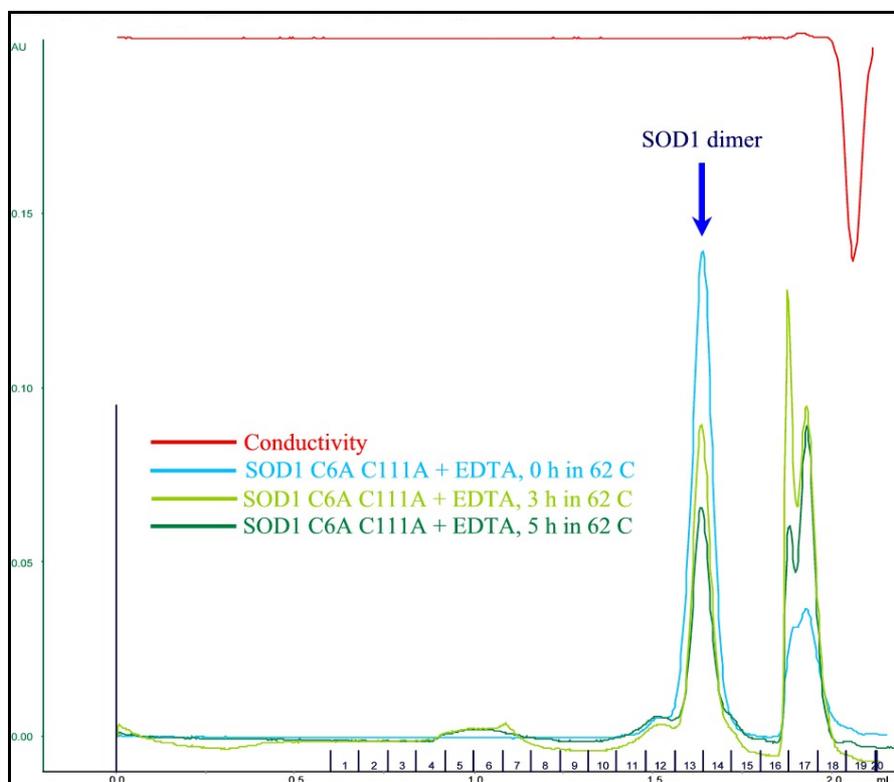


Figure 26. EDTA denaturation of SOD1 C6A C111A, samples from 0, 3 and 5 hours of denaturation at 62°C. The presence of SOD1 dimer decreases with time to approximately 50 % after 5 h.

The reduction of SOD1 dimer to approximately 50 % after 5 hours is an appropriate condition for the screening of compounds using this methodology. If the presence of dimer is reduced to a lesser extent in the presence of a compound, that compound has the desired effect. Six of the compounds from Ray *et al.* were tested. The result of the screening implied that the compounds have no or little effect on SOD1 stability. These results are not statistically guaranteed, as the experiment was only performed once.

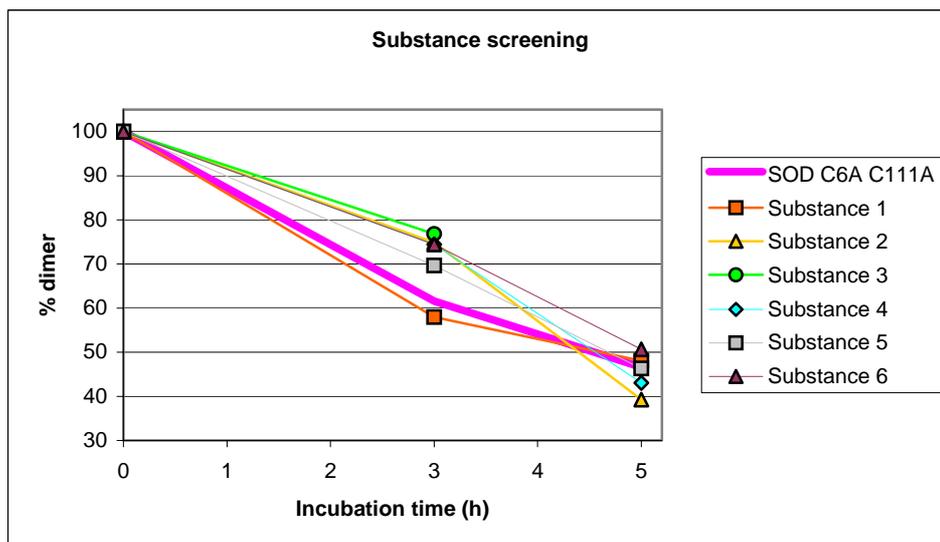


Figure 27. Compound screening result. After three hours there is a difference in % dimer, but that difference seems to disappear after 5 hours of incubation. Note that the % dimer-scale does not start at zero.

5.4 ESI-MS

As the experimental part of this diploma work had finished, one of the major questions that arose was: Why do the compounds from Ray *et al.* not bind to the protein?

One hypothesis was that the proposed binding pocket is not available to the compounds. An examination of the structure of the protein pocket did not reveal anything that supported the hypothesis, the pocket is solvent exposed, and thereby it should be possible for compounds to bind at that location. As the answer could not easily be found in the structure of the pocket, another explanation could be that it is already occupied by another ligand. To test this hypothesis a sample of SOD1 wt was given to Dr. Agneta Tjernberg at Biovitrum for ESI-MS analysis, where an exact mass determination would establish whether something is non-covalently bound to the protein or not. The results are shown in figure 28. The mass of the non-covalent binder that was found corresponds to the mass of the two metal ions that bind to each SOD1 monomer. There is also a small peak corresponding to one bound metal ion. As there is no sign of any binder except the metal ions, the hypothesis that the pocket is already occupied can be dismissed.

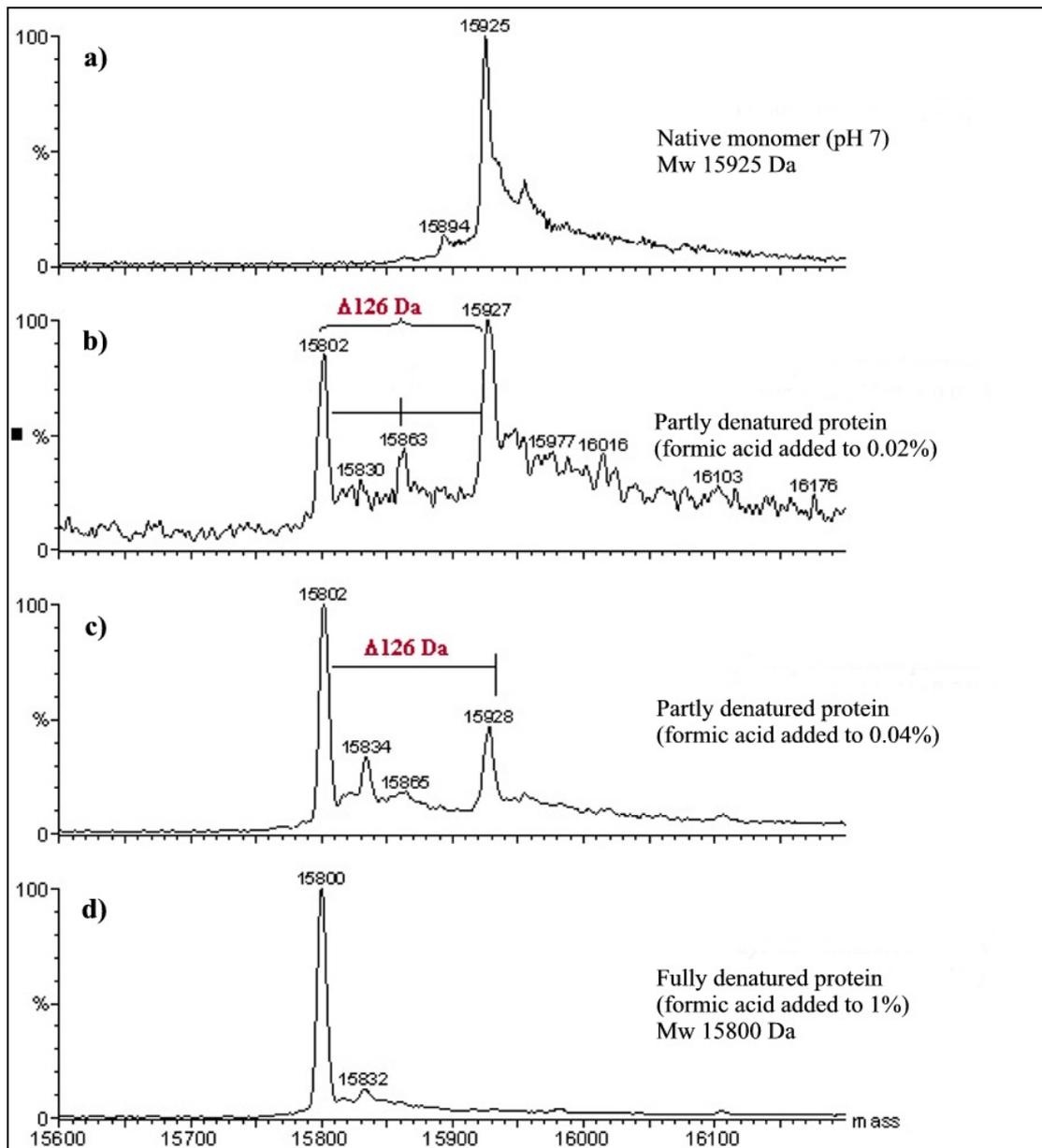


Figure 28. Results from ESI-MS on the SOD1 material used in the experiments. A mass difference of approximately 126 Da was observed between native (**a**) and fully denatured (**d**) SOD-1 monomer. Most probably this is due to the binding of the two metal ions to the monomer. The native dimer had a mass increase corresponding to four metal ions. A stepwise dissociation of the protein-metal ion complex is observed as the solution pH is gradually decreased (**b**, **c**). An additional peak 32 Da higher than the protein mass was observed both in native and denatured state; this increased mass could be a result of a partial oxidation of the protein material.

6. Discussion

The first unexpected problem encountered during this diploma work was the fact that there seemed to be no binding between SOD1 and the compounds published by Ray *et al.* If the compounds have the effect proposed by Ray *et al.*, at least some of them should have bound and the binding to the protein should have given a signal in the STD-NMR experiment. As the ESI-MS experiments showed that the pocket is not occupied, some other explanation had to be found.

One possibility is that the compounds do not bind to the fully folded protein, but to a molten globule structure of some kind. If this is the case, the reason for the absence of STD signal is that this partially denatured structure does not exist under the conditions used in the STD-NMR experiments. An interesting continuation of this problem is the attempt to design an STD-NMR experiment where this proposed molten globule structure is present so that binding to it can be detected. Such an experiment should probably be performed at a higher temperature.

Obvious differences between the study performed by Ray *et al.* and the one performed at Biovitrum are the different temperatures and incubation times used. It is not certain that time and temperature are internally exchangeable. At an increased temperature the different states of unfolding during the denaturation process can be present at shorter time as the kinetics are faster at higher temperatures. This would include the presence of the proposed molten globule structure. If the affinity of the binding is not affected to the same degree as the kinetics of the denaturation, it is possible that the state the compound would bind to is not present long enough, and thus no binding can take place.

The next problem encountered was the fact that the read out of the assay did not seem to indicate a correct presence of dimer, as was intended. This is explained by the fact that under the conditions used, the apo- (metal depleted) state of the protein was stable enough to stay folded, even though the loss of metal ions has made it inactive. The problem in this case is that the denaturation method is not strong enough to denature the apo-protein. If a higher temperature would be used, the decreased stability induced by metal depletion could be enough for the apo state to denature. Then, as there would be no SOD1 present but the fully metalated kind, the idea to use activity as a marker for amount of dimer could still be used. The problem is that the temperature used in this assay is already higher than what is considered practical for an HTS assay.

If an altogether different strategy of denaturation would be used, maybe one without the use of EDTA, then the activity could only be lost at denaturation of the protein. But the activity could be an altogether bad marker for HTS purposes, as the fact is that only relatively stable SOD1 mutants have a high enough activity level. The use of an unstabilized mutant would be better for screening purposes, but for the reason mentioned, some other marker than activity would have to be used.

One interesting property discovered in the gel filtration experiment is that the peaks from the EDTA-ion-complexes could be used to investigate the degree of metal contents in a protein solution. Maybe a method could be developed from these data that would show how many and what kind of ions the protein is binding. However these data have to be confirmed.

The lack of significant impact of the compounds from Ray *et al.* on SOD1 stability in the gel filtration assay is not statistically ensured and so the data should not be taken as a fact, before these experiments have been repeated. An interesting aspect from the gel filtration assay would be in which fractions the compounds are localized; if they would interact with the dimer they should be present in the same fraction as the dimer is. If, on the other hand, the compounds do not interact with the proteins, they should eluate at the same time as the solvent. Or the third possibility, the compounds are present in the same fractions as the SOD1 aggregates, which would indicate a binding to aggregates or other denatured states of the protein.

None of the results achieved in this work agrees with the results from the work done by Ray *et al.* One thing that increases suspicion of Ray *et al.*'s work is the fact that they had such a high rate of hits. Normally a virtual screen increases the hit rate about 10-fold, from 0.1 % to 1 %. The reported 15 hits from 100 tested compounds is far above what is usually found in both virtual and physical screening.

6.1 Future work

In accordance with the discussion above, fractions from the gel filtration should be investigated for the presence of compound by the use of ESI-MS.

To be able to confirm or disregard the work published by Ray *et al.* the exact same procedures (37°C and 50 hours of incubation) should be used in a gel filtration assay. If the results agree with the published results, more work should be put into investigating by what mechanism the compounds affect the protein stability. If the results should not agree, the compounds should be regarded as false positives, and the search for new compounds would have to go on.

By the use of STD-NMR, or some other NMR technique, a large substance screen could be made to find new compounds that bind to the protein. This screen would have to be made blindly, as there is no positive control at this point. If and when binders are identified, crystallography could be used as a control to see where the compounds bind and the complex structures achieved could be used for structure based drug design. To determine the potency of the confirmed binders, a new assay would have to be developed, this time it should be one based directly on the dimerisation of SOD1, for example a FRET based assay. Additional alternatives that should be considered is an assay that is based on the EDTA-metal complexes or on the appearance of aggregates and the quantification of those.

7. Conclusions

The compounds from Ray *et al.* do not seem to bind to SOD1; nor do the compounds from the virtual screen performed at Biovitrum. The fact that the results from this work do not agree with the results from Ray *et al.*, shows the necessity of further investigation of the compounds binding to the protein and the effect that binding might have.

An NMR screen could be performed in order to find compounds binding to SOD1. The screen should be followed by attempts to obtain complex structures of the interaction using either crystallography or NMR.

The developed activity assay is not useful in drug screening as it stands. A new assay concept based on FRET could be a way to go forward. At this time, the project has come to a “stop or go” point. Based on the results in this work and the fact that there are no positive controls makes it difficult to continue with this project, so the answer would probably be stop. But as ALS is a terrible disease with no cure and SOD1 is amongst the most interesting drug targets at this time, I know that more work will be done before SOD1 is dismissed as a drug target.

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