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SUBJECT AREAS:
BIOCATALYSIS
DNA METABOLISMReceived
28 April 2014Accepted
16 June 2014Published
2 July 2014Correspondence and
requests for materials
should be addressed to
A.C. (andrei.chabes@
medchem.umu.se)

Phosphines are ribonucleotide reductase reductants that act via C-terminal cysteines similar to thioredoxins and glutaredoxins

Vladimir Domkin¹ & Andrei Chabes^{1,2}¹Department of Medical Biochemistry and Biophysics, Umeå University, SE-901 87 Umeå, Sweden, ²Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå University, SE 90187 Umeå, Sweden.

Ribonucleotide reductases (RNRs) catalyze the formation of 2'-deoxyribonucleotides. Each polypeptide of the large subunit of eukaryotic RNRs contains two redox-active cysteine pairs, one in the active site and the other at the C-terminus. In each catalytic cycle, the active-site disulfide is reduced by the C-terminal cysteine pair, which in turn is reduced by thioredoxins or glutaredoxins. Dithiols such as DTT are used in RNR studies instead of the thioredoxin or glutaredoxin systems. DTT can directly reduce the disulfide in the active site and does not require the C-terminal cysteines for RNR activity. Here we demonstrate that the phosphines tris(2-carboxyethyl)phosphine (TCEP) and tris(3-hydroxypropyl)phosphine (THP) are efficient non-thiol RNR reductants, but in contrast to the dithiols DTT, bis(2-mercaptoethyl)sulfone (BMS), and (S)-(1,4-dithiobutyl)-2-amine (DTBA) they act specifically via the C-terminal disulfide in a manner similar to thioredoxin and glutaredoxin. The simultaneous use of phosphines and dithiols results in ~3-fold higher activity compared to what is achieved when either type of reductant is used alone. This surprising effect can be explained by the concerted action of dithiols on the active-site cysteines and phosphines on the C-terminal cysteines. As non-thiol and non-protein reductants, phosphines can be used to differentiate between the redox-active cysteine pairs in RNRs.

RNRs catalyze a key reaction in the *de novo* synthesis of deoxyribonucleoside triphosphates (dNTPs), the building blocks of DNA. RNRs are divided into three classes and several subclasses that differ in terms of subunit composition, free radical chemistry, dependence on metals and oxygen, and allosteric regulation¹⁻³. The majority of eukaryotic RNRs belong to Class I. The minimal architecture of Class I RNRs is an R1-R2 heterodimer, where R1 is the large subunit consisting of two polypeptides (encoded by RRM1 in mammals) and R2 is the small subunit also consisting of two polypeptides (encoded by RRM2 or RRM2b in mammals). Each R1 polypeptide contains one active site, one allosteric specificity site, and one allosteric activity site, and each R2 polypeptide contains a free tyrosyl radical necessary for catalysis. During the conversion of a ribonucleotide into a 2'-deoxyribonucleotide, the catalytically active cysteines in the active site form a disulfide bond⁴. Before the next round of catalysis, this bond has to be reduced by the second pair of cysteines located in the flexible C-terminus of the second R1 polypeptide of the large subunit⁵⁻¹⁶. The C-terminal cysteine pair is in turn reduced by thioredoxins^{17,18} or glutaredoxins¹⁹. Glutaredoxin system can reduce the C-terminal thiols of RNR by the glutathionylation mechanism²⁰. Oxidative stress and diseases that change the reduced-oxidized glutathion ratio may affect the reduction mechanism of RNR *in vivo*. Thioredoxins and glutaredoxins cannot directly reduce the disulfide bond in the active site due to steric hindrance³. Detailed models illustrating the participation of the R1 polypeptides can be found in^{15,16,20}.

The original reductant for determining RNR activity was dihydrolipoic acid²¹, but since the mid-1960s the artificial dithiol reductants DTT (1,4-dithiothreitol, Cleland's reagent) and, to a lesser extent, the DTT isomer 1,4-dithioerythritol have been the most commonly used^{22,23}. DTT is a much less efficient reductant of bacterial and phage RNRs than thioredoxins and glutaredoxins^{10,23,24}, but the mammalian RNRs are reduced by DTT with approximately the same efficiency as thioredoxin or glutaredoxin^{20,25}. At higher concentrations, DTT inhibits RNR activity through an unknown mechanism²⁶⁻²⁸. Interestingly, the C-terminal cysteines are not required for RNR activity in the presence of DTT because a mutant RNR lacking the C-terminal cysteines shows normal

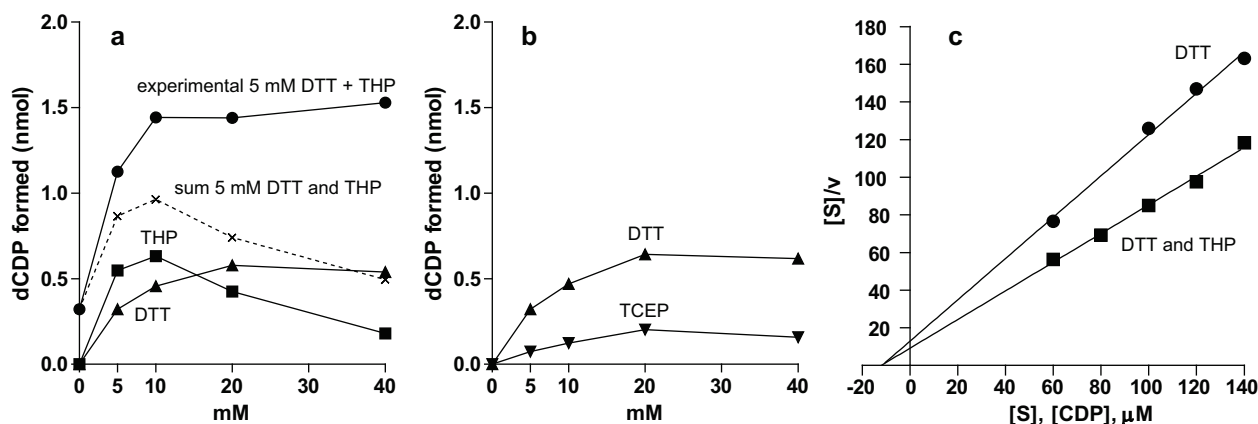


Figure 1 | Activity of wild-type mouse RNR in the presence of different reducing agents. RNR activity is measured in nmols of dCDP formed after 5 min of incubation. (a). THP alone (■), DTT alone (▲), 5 mM DTT together with THP at given concentrations (●), the summed values for 5 mM DTT added to values for THP at given concentrations (×). (b). TCEP alone (▼), DTT alone (▲). (c). Hanes-Woolf plot demonstrating the kinetic effects of adding THP to reaction mixtures containing DTT. The term ‘*v*’ is the RNR activity in nmols of dCDP formed over 5 min of incubation in the presence of 20 mM DTT (●) or 20 mM DTT with 20 mM THP (■). THP does not change the K_m of substrate binding (~ 12 mM) but increases V_{max} .

activity^{5–8,10}. To what extent DTT reduces the C-terminal cysteine pair during an assay with a wild-type RNR is not known, but the results presented below suggest that DTT might be a less effective reductant of the C-terminal disulfide than of the active-site disulfide.

Here we have investigated the efficiency of several powerful reducing agents that have not been tested in any published RNR activity assays. These agents include the dithiols BMS (bis(2-mercaptoethyl)-sulfone)²⁹ and DTBA (dithiobutylamine)³⁰ and the phosphines TCEP (tris(2-carboxyethyl)phosphine)^{31,32} and THP (tris(3-hydroxypropyl)phosphine). The main drawback of DTT (with a pKa 9.2) compared to the dithiols BMS and DTBA (with a pKa 7.9 and 8.2, respectively) is DTT’s low activity in neutral solutions where the concentration of DTT’s active thiolate form ($R-S^-$) is low. Another drawback of DTT, as well as the other dithiols, is their instability in the presence of oxygen; dithiols are easily oxidized in solution, especially in the presence of heavy metals. Phosphines are non-thiol reductants that were first used for the reduction of disulfide bonds in proteins in 1969 when the cleavage of disulfide bonds in gamma globulin by TCEP was reported³³. Another phosphine used for the reduction of disulfide bonds in proteins is THP. Compared to TCEP, THP has a simpler structure, has no charged carboxyl groups, and is more efficient in reducing oxidized glutathione. TCEP and THP are odorless and water-soluble, unreactive towards other functional groups in proteins, and are capable of reducing very stable disulfides (e.g. oxidized DTT) rapidly and completely. Unlike DTT, phosphines are stable in solution and retain their reducing power over a broad pH range. Both TCEP and THP are commercially available and cost approximately the same as DTT.

In this work we show that DTT is a more efficient RNR reductant than DTBA but is significantly less efficient than BMS. Interestingly, phosphines are also efficient RNR reductants, but in contrast to DTT

they have almost no effect on the activity of the mutant RNR lacking the C-terminal cysteines. This indicates that phosphines are unable to directly reduce the active-site disulfides. Surprisingly, simultaneous use of dithiols and phosphines results in up to 3-fold higher RNR activity compared to the highest RNR activities that can be achieved when each reducing agent is used alone. This effect can be explained by the concerted actions of dithiols on the active-site cysteines and phosphines on the C-terminal cysteines and by the ability of phosphines to maintain dithiols in the reduced state.

Results

The phosphines THP and TCEP are efficient RNR reductants that function cooperatively with DTT. The activity of recombinant mouse RNR was determined in the presence of DTT or THP (Fig. 1a). The RNR activity was slightly higher in the presence of THP than DTT, and the concentration of the reducing agent required to achieve the highest RNR activity was lower for THP than for DTT (10 mM versus 20–40 mM, respectively). RNR activity was also assayed in the presence of a constant concentration of DTT (5 mM) and increasing concentrations of THP. To our surprise, the RNR activity in all samples with both reducing agents (upper line in Fig. 1a) was significantly higher and was greater than the sum of the activities obtained with 5 mM DTT alone and increasing concentrations of THP alone (dotted line in Fig. 1a).

To extend this observation, we determined RNR activity in the presence of both DTT and THP at concentrations of 0 mM, 5 mM, 10 mM, 20 mM, and 40 mM in all possible combinations (Table 1). In some combinations, the activity in the presence of both DTT and THP was higher than the sum of the activities obtained with each reducing agent alone (i.e., higher than additive) and this additional

Table 1 | Activities of wild-type mouse RNR with different combinations of DTT and THP

THP, mM \ DTT, mM	0	5	10	20	40
0		0.55	0.63	0.43	0.18
5	0.32	1.13 (+0.26)	1.44 (+0.49)	1.44 (+0.69)	1.53 (+1.03)
10	0.46	1.05 (+0.04)	1.24 (+0.15)	1.42 (+0.54)	1.41 (+0.77)
20	0.58	1.06 (−0.07)	1.00 (−0.21)	1.20 (+0.19)	1.12 (+0.36)
40	0.54	0.74 (−0.35)	0.81 (−0.36)	0.88 (−0.09)	0.79 (+0.07)

Values are RNR activity measured in nmols of dCDP formed after 5 min of incubation. Values in parenthesis show the difference between the actual activity in reactions with both DTT and THP at given concentrations and the sum of the activities obtained with DTT and THP at the same concentrations in separate reactions.

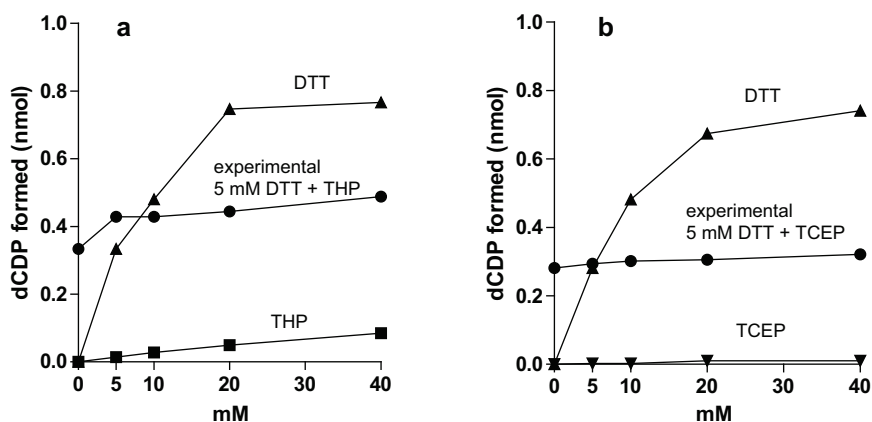


Figure 2 | Activity of mutant mouse RNR lacking the redox-active cysteines in the C-terminus of the large subunit in the presence of different reducing agents. RNR activity is measured in nmols of dCDP formed after 5 min of incubation. (a). THP alone (■), DTT alone (▲), 5 mM DTT together with THP at given concentrations (●). (b). TCEP alone (▼), DTT alone (▲), 5 mM DTT together with TCEP at given concentrations (●).

effect is shown by positive numbers in parenthesis in Table 1. In other combinations, the activity was lower than the sum of each reagent alone (i.e., lower than additive) and this difference is shown by negative numbers in parenthesis in Table 1. In all cases, though, simultaneous use of DTT and THP invariably resulted in higher RNR activity compared to what could be achieved using either reducing agent alone at the same concentration. The most pronounced positive cooperative effects (higher than additive) were observed at low DTT concentrations (5 mM and 10 mM) and high THP concentration (20 mM and 40 mM), which resulted in activities nearly 3-fold higher than the highest activities obtained with the individual reducing agents. Another phosphine commonly used in biochemical assays, TCEP, was a weaker RNR reductant than DTT (Fig. 1b), but it also demonstrated a cooperative effect with DTT (Supplementary Table 1). The addition of THP to the reaction mixtures containing DTT did not change the K_m for CDP substrate binding ($\sim 12 \mu\text{M}$) but did increase the V_{max} (Fig. 1c).

Phosphines and DTT do not demonstrate a significant cooperative effect in an assay with mutant RNR lacking the C-terminal cysteines. To gain insights into the mechanism behind the positive cooperative effects of combined DTT and THP, we measured the effects of these compounds on the activity of the mutant RNR lacking the C-terminal cysteines. This experiment demonstrated that THP alone is a very inefficient reductant of the mutant RNR and that THP does not significantly increase the activity of the mutant RNR

assayed in the presence of 5 mM DTT (Fig. 2a). Similarly, TCEP was unable to activate the mutant RNR (Fig. 2b). Therefore, we conclude that during the assay the phosphines THP and TCEP primarily reduce RNR via the C-terminal disulfides of the large subunit and that DTT primarily reduces the disulfides in the active site of the large subunit.

The dithiols BMS and DTBA do not require the C-terminal cysteines in an RNR assay. Having established that the phosphines TCEP and THP reduce RNR primarily via the C-terminal cysteines, we investigated whether other dithiols reduce RNR primarily via the active-site cysteines in a similar manner as DTT. BMS was a significantly more efficient RNR reductant compared to DTT and resulted in significantly higher absolute RNR activities (Fig. 3a). Due to the low solubility of BMS, we were unable to test it at concentrations above 10 mM. Similar to DTT, BMS efficiently reduced the mutant RNR lacking C-terminal cysteines (Fig. 3b). Simultaneous use of BMS and THP resulted in a higher RNR activity compared to what could be achieved using either reducing agent alone at the same concentration (Fig. 3c). Maximal RNR activity in these experiments was achieved in the presence of 5 mM BMS and 5 mM THP (Table 2).

DTBA was a less efficient RNR reductant than DTT, perhaps because DTBA has a charged amino group that can interfere with its access to the disulfide located in the active site. However, DTBA was also able to sustain the activity of mutant RNR lacking the

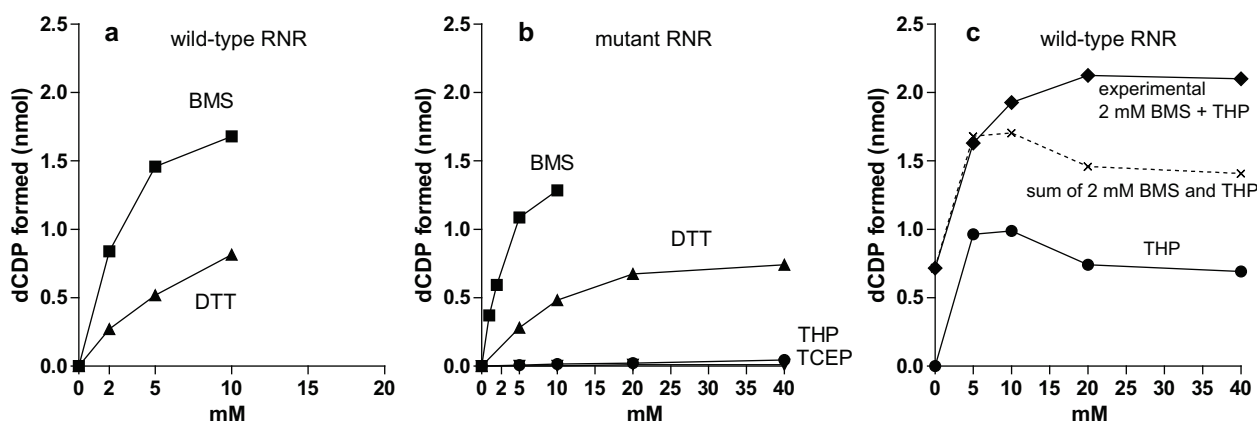


Figure 3 | Activity of RNR in the presence of different reducing agents. RNR activity is measured in nmols of dCDP formed after 5 min of incubation. (a). Wild-type mouse RNR assayed with BMS (■) or DTT (▲). (b). Mutant mouse RNR lacking redox-active cysteines in the C-terminus of the large subunit assayed with BMS (■), DTT (▲), THP (●), or TCEP (▼). (c). Wild-type mouse RNR assayed with THP alone (●) or 2 mM BMS together with THP at given concentrations (◆). The summed values for 2 mM BMS added to values for THP at given concentrations are shown as (×).


Table 2 | Activities of wild-type mouse RNR with different combinations of BMS and THP

BMS, mM \ THP, mM	THP, mM			
	0	5	10	20
0		0.96	0.99	0.74
2	0.72	1.63 (-0.05)	1.93 (+0.22)	2.12 (+0.67)
5	1.46	2.64 (+0.27)	2.00 (-0.44)	2.03 (-0.17)
10	1.67	0.84 (-1.8)	0.35 (-2.3)	0.37 (-2.1)

Values are RNR activity measured in nmols of dCDP formed after 5 min of incubation. Values in parenthesis show the difference between the actual activity in reactions with both BMS and THP at given concentrations and the sum of the activities obtained with BMS and THP at the same concentrations in separate reactions.

C-terminal cysteines (Fig. 4a and 4b). Again, simultaneous use of DTBA and THP resulted in higher RNR activity compared to what could be achieved using the individual reducing agents alone at the same concentrations (Table 3).

Discussion

We have identified the phosphines THP and TCEP as novel RNR reductants that act primarily via the redox-active cysteines in the flexible C-terminal tails of the two polypeptides of the large subunit of RNR in a manner similar to the natural RNR reductants thioredoxin and glutaredoxin. In contrast, the dithiol DTT directly reduces the disulfides in the RNR active site and does not require the C-terminal cysteines for activity^{5–8,10}. We have extended this observation and shown that two other dithiols, BMS and DTBA, also directly reduce the active-site disulfides. Simultaneous use of dithiols and phosphines in an RNR activity assay results in up to 3-fold higher activity compared to what can be achieved when either type of reducing agent is used alone. We propose that the two electron donor systems, dithiols and phosphines, are relatively slow and rate limiting for RNR activity, and that the observed positive cooperative effects are due to the concerted actions of dithiols on the active-site cysteines and phosphines on the C-terminal tail cysteines (Fig. 5). We speculate that the reduction of the active-site disulfide by its natural reductant, the C-terminal cysteines, is more efficient than the reduction by DTT. However, after each reduction, the oxidized flexible C-terminal tail must leave the active site and become reduced (by phosphines in our assay) before returning to the active site. During this time DTT can act directly on the active-site disulfide. Our model implies that dithiols are relatively inefficient or slow reductants of the C-terminal cysteines during the RNR reaction, both because the activities of the

wild-type RNR and mutant RNR lacking the C-terminal cysteines are very similar in the presence of dithiols and because phosphines, which primarily act on the C-terminal cysteines, significantly increase wild-type RNR activity when assayed in the presence of dithiols. Interestingly, a similar differential specificity of dithiols and phosphines towards two different disulfides within the same protein has been recently reported for human quiescin-sulphydryl oxidase (QSOX). In this case, one disulfide is reduced only by DTT and glutathione while another disulfide is only reduced by TCEP³⁴. Comparison of the pKa and reduction potentials (E° , mV) for DTT (9.2 and -330), DTBA (8.2 and -320), BMS (7.9 and -310), TCEP (7.6 and n.d.)³⁵, THP (7.2 and n.d.)³⁵, Trx (6.3 and -270), and Grx (3.5 and -198) suggests that the efficiency of the different reducing agents in an RNR reaction depends not only on these values, but also on the charge of the reducing agent, pH of the reaction, and protein-protein interactions.

In some combinations of dithiols and phosphines, the observed RNR activity is significantly higher than the sum of the activities observed with each of the reducing agents used separately at the same concentrations (Fig. 1a and 3c). Both dithiols and phosphines become oxidized after they reduce RNR, and while oxidation of phosphines is essentially irreversible oxidized dithiols can be reduced by phosphines. Therefore, phosphines can maintain constant high concentrations of reduced dithiols during the RNR activity assay, which might be another explanation of the observed positive cooperative effects of phosphines and dithiols on RNR activity (Fig 5). Clearly, the electron donor system is one of the factors limiting RNR activity *in vitro*, and an interesting unanswered question is to what extent thioredoxins and glutaredoxins control RNR activity *in vivo*.

RNR is an important target for antiproliferative drugs. A few examples of drugs currently in use that target RNR include gemcitabine (for treatment of lung, bladder, and pancreatic cancers), clofarabine (for treatment of hematological cancers including pediatric leukemia), and hydroxyurea (for treatment of acute and chronic myelogenous leukemia and glioblastoma)^{15,36,37}. There is a constant search for new inhibitors that inactivate both human and microbial RNRs, the latter as targets for antimicrobial therapies. A recently developed high-throughput assay for the identification of new RNR inhibitors uses DTT as the reducing agent³⁸. The inclusion of thioredoxin and thioredoxin reductase in such an assay would complicate the interpretation of the results due to the addition of two other potential drug targets. At the same time, however, our results suggest that an RNR assay relying exclusively on DTT as an electron donor will not identify drugs that interfere with the reduction of the C-terminal cysteines. Using phosphines in such assays would elim-

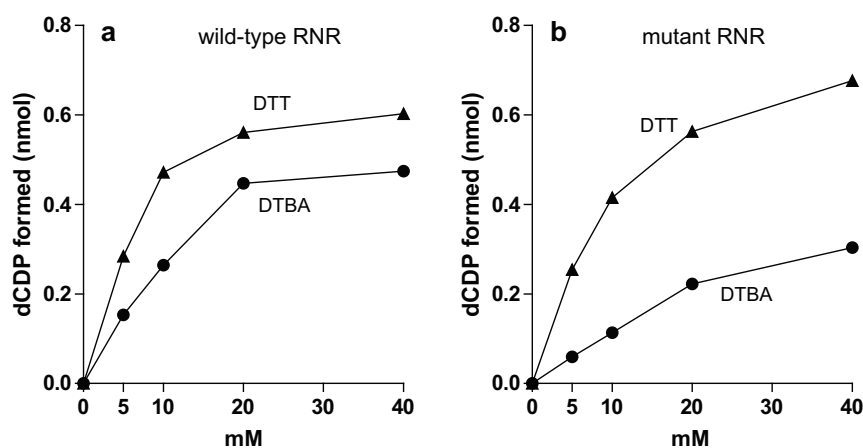


Figure 4 | Activity of RNR in the presence of different reducing agents. RNR activity is measured in nmols of dCDP formed after 5 min of incubation. (a). Wild-type mouse RNR assayed with DTBA (●) or DTT (▲). (b). Activity of mutant mouse RNR lacking the redox-active cysteines in the C-terminus of the large subunit assayed with DTBA (●) or DTT (▲).


Table 3 | Activities of wild-type mouse RNR with different combinations of DTBA and THP

		THP, mM			
		0	10	20	40
DTBA, mM	0		0.42	0.40	0.32
	5	0.10	0.57 (+0.05)	0.59 (+0.09)	0.54 (+0.02)
	10	0.27	0.47 (-0.22)	0.52 (-0.15)	0.49 (-0.10)

Values are RNR activity measured in nmols of dCDP formed after 5 min of incubation. Values in parenthesis show the difference between the actual activity in reactions with both DTBA and THP at given concentrations and the sum of the activities obtained with DTBA and THP at the same concentrations in separate reactions.

inate this problem. Phosphines could also be useful for elucidating the exact targets of several RNR inhibitors – including caracemide, cisplatin, and chlorambucil – that are known to react with sulfhydryl groups^{15,39}. Furthermore, phosphines could be used to gain a better understanding of the effects of thioredoxin and glutaredoxin on RNR subunit interactions. It has been shown that thioredoxin strengthens the R1-R2 complex of the *Escherichia coli* RNR, but whether the tighter RNR complex is solely due to the RNR-thioredoxin protein-protein interactions or is also due to the reduction of the C-terminal cysteines is not known⁴⁰. Some RNRs are not efficiently reduced by DTT. For example, the activity of the RNR from *Euglena gracilis* is strongly stimulated by *E. coli* thioredoxin but requires concentrations of DTT as high as 60 mM for efficient reduction of the substrate⁴¹. It would be interesting to investigate the efficiency of phosphines with the *E. gracilis* RNR. This enzyme belongs to the Class II RNR enzymes that directly reduce NTPs to dNTPs, and this might have practical applications for the synthesis of dNTPs *in vitro*. Finally, monitoring the accumulation of the oxidized phosphines by NMR could be a new fast and convenient way to measure RNR activity.

In summary, we have identified phosphines as a new tool that can differentiate between the redox-active cysteine pairs in the mammalian RNR without the involvement of other proteins such as thioredoxins or glutaredoxins. Together with dithiols, the phosphines can achieve very high RNR activities. These observations open new possibilities for investigating the mechanisms of the RNR reaction and RNR inhibition.

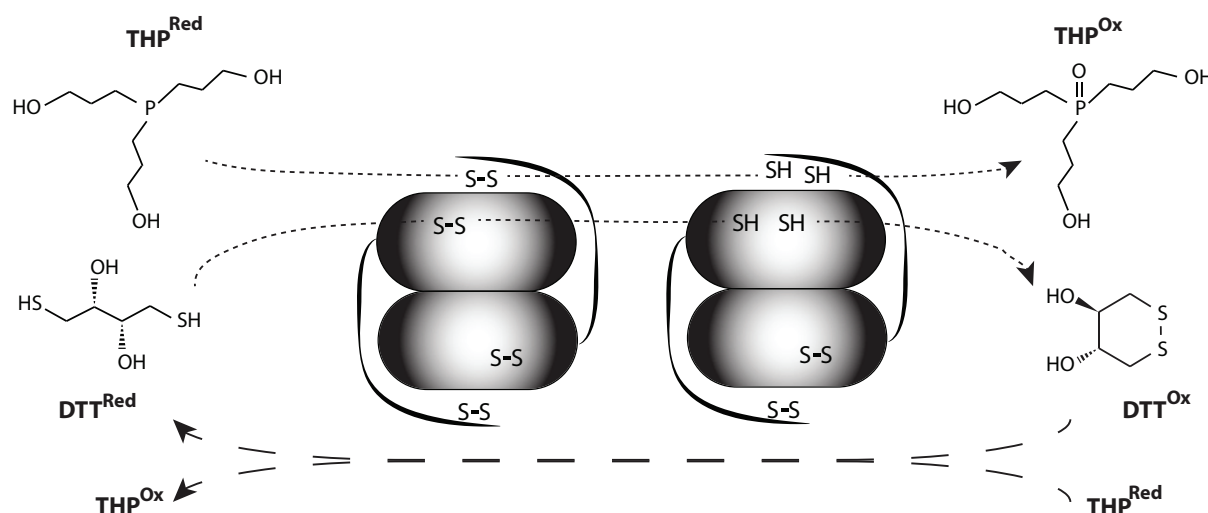


Figure 5 | A model explaining the positive cooperative effects of dithiol and phosphine reductants in RNR assays. Shaded ovals depict homodimers of the large RNR subunit. The active-site redox cysteine pairs (inside ovals) and the C-terminal tail redox cysteine pairs are shown. The positive effect between the two types of reductants can be explained by the concerted action of dithiols (DTT in the figure) on the active-site cysteines and phosphines (THP in the figure) on the C-terminal cysteines, which in turn very efficiently reduce the active-site cysteines. The action of the reductants is shown only for the upper part of the homodimer. The positive cooperative effects of dithiols and phosphines can also be explained by the recycling of DTT back to the reduced state by THP (lower arrows). Oxidation of phosphines is essentially irreversible.

Methods

Reagents. The following reagents were used: [³H]-CDP ([5-³H]cytidine 5'-diphosphate) (Amersham Biosciences, TRK338); CDP (cytidine 5'-diphosphate) (MP Biochemical, 100529); ATP (adenosine 5'-triphosphate) (Amersham Biosciences, 27-1006-03); THP (tris(hydroxypropyl)phosphine) (Novagen, 71194); DTT (dithiothreitol) (Serva, 39759.02); BMS (bis(2-thioethyl)sulphone) (Apollo Scientific Ltd., OR8251T); TCEP (tris(carboxyethyl)phosphine) (Sigma, 646547-10X1ML); HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) (Amresco, 0511-250G); dATP-Agarose, γ -aminohexyl-dATP-Agarose (Jena Bioscience, AC-122L); DEAE Sepharose FF (Pharmacia, 17-0709-01); and DTBS ((S)-(1,4-dithiobutyl)-2-amine or (S)-2-aminobutane-1,4-dithiol) (Sigma-Aldrich, 774405-1G). The dithiols DTT, BMS, and DTBA were always dissolved immediately before use.

Protein expression and purification. Wild-type mouse R1, wild-type mouse R2, and mutant mouse R1 with the Cys779Ala, Cys787Ala, and Cys790Ala substitutions were kindly provided by Prof. Lars Thelander of Umeå University and were expressed in *E. coli* essentially as described previously^{42,43}. Wild-type R1 was purified on dATP-Agarose while mutant R1 was purified on γ -aminophenyl-dATP-Sepharose prepared as described in⁴⁴ and additionally on FTLDAF-Sepharose 4B prepared as described in⁴⁵. Recombinant mouse R2 was purified on DEAE Sepharose FF as described in⁴³. Proteins were stored at -80°C in 50 mM HEPES (pH 7.3) with 100 mM KCl (R1 and mutant R1) or in 50 mM Tris-HCl (pH 7.5) (R2). The purity of the protein samples was checked by SDS-PAGE (Supplementary Fig. 1). Purified proteins were quantified by 280 nm UV light absorption using extinction coefficients of $E_{1\text{ cm}}^{1\%}$ 13.9 (for R1) and 10.2 (for R2). The specific activities of wild-type and mutant R1 measured in the presence of 20 mM DTT were both ~ 70 nmols of dCDP formed per min per mg R1. The specific activity of R2 measured with excess of R1 was ~ 150 nmols of dCDP formed per min per mg R2.

Ribonucleotide Reductase Assay. The assay in this work was a shorter and simplified version of the procedure described earlier⁴⁶. All assay mixtures in a final volume of 50 μL contained 50 mM HEPES-KOH (pH 7.5), 125 mM KCl, 3 mM ATP, 8 mM MgCl_2 , 1.4 μg mouse R1 (0.3 μM as monomer), 2.8 μg mouse R2 (1.2 μM as monomer), and 0.1 mM 5-³H]-cytidine 5'-diphosphate ([³H]CDP; specific activity 25,300 cpm/nmol); the specific activity of [³H]CDP used in the assay was determined after mixing 10 mL OptiPhase HiSafe 3 scintillation liquid (Perkin Elmer) and a 1 mL solution of a known quantity of [³H]CDP in 0.2 M acetic acid. Reducing agents were used separately or in different combinations at various concentrations. Reaction mixtures were assembled in open tubes on ice because essentially no RNR reaction occurs at 0°C . Reactions were started by transferring all samples simultaneously from ice to a 25°C water bath and were incubated for 5 minutes at 25°C . Reactions were stopped by transferring all samples back to ice and quickly adding 0.5 mL of 1 M HClO_4 . After stopping the reactions, 30 μL of 16 mM dCMP was added, the tubes were closed with screw caps, and the tubes were incubated for 10 min in boiling water and cooled to room temperature. After adding ~ 60 μL of ~ 3 mM Phenol red and ~ 0.14 mL of 4 M KOH, the samples were neutralized using HClO_4 and KOH, incubated on ice for 5–10 min, and centrifuged at 4°C for 5 min. Supernatants were loaded onto pre-washed 2 mL Dowex AG 1X8, 200–400 in [H^+]-form columns prepared by replacing the Sephadex in NAP-10 columns (Amersham Bioscience)



with 2 mL of Dowex from Bio-Rad pre-packed columns. The resin was washed with 1 mL water and 42 mL 0.2 M acetic acid. The flow rate of these columns was ~1.5 mL/min and elution took 35–37 min. The [³H]dCMP fraction was eluted with 2.5 mL of 0.2 M acetic acid and collected. No [³H]CMP was present in this eluate. Absorbance at 280 nm was determined. We mixed 1 mL of eluate with 10 mL scintillation liquid, and the cpm was determined with a Liquid Scintillation System LS-6000SE (Beckman). RNR activity (nmoles of dCDP formed in 5 minutes) was calculated by multiplying cpm/A₂₈₀ by 0.000247. The coefficient 0.000247 was calculated as the amount of dCMP (30 μL of 16 mM dCMP) multiplied by the molar extinction coefficient of dCDP (13,000 M⁻¹·cm⁻¹ (E₂₈₀ at pH 2)) and divided by the specific radioactivity of [³H]CDP (25,300 cpm/nmol) (13,000 × 30 × 10⁻⁶ × 16)/25,300 = 0.000247). All assays shown in each figure panel were performed within one experiment, i.e. with the same protein batch, same buffers and simultaneous incubation of all samples. The only variables were the reducing agent or their combination. Experiments shown in each panel were performed at least twice on different occasions. Columns with Dowex were regenerated with 2–3 mL water, 16–18 mL 3 M HCl, and 10–12 mL water to remove the HCl. The regeneration procedure took about 25 min.

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Acknowledgments

We thank Lars Thelander for providing the R1 and R2 expression plasmids and for critical reading of the manuscript. This work was supported by the Swedish Foundation for Strategic Research, the Swedish Research Council, the Swedish Cancer Society, and the Medical Faculty of Umeå University.

Author contributions

V.D. and A.C. designed the study; V.D. performed the experiments; V.D. and A.C. analyzed the data and wrote the paper.



Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Domkin, V. & Chabes, A. Phosphines are ribonucleotide reductase reductants that act via C-terminal cysteines similar to thioredoxins and glutaredoxins. *Sci. Rep.* **4**, 5539; DOI:10.1038/srep05539 (2014).



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Corrigendum: Phosphines are ribonucleotide reductase reductants that act via C-terminal cysteines similar to thioredoxins and glutaredoxins

Vladimir Domkin & Andrei Chabes

Scientific Reports 4:5539; doi: 10.1038/srep05539; published online 02 July 2014; updated on 07 April 2017

This Article contains a typographical error in the Methods section under subheading ‘**Ribonucleotide Reductase Assay**’.

“Supernatants were loaded onto pre-washed 2 mL Dowex AG 1X8, 200–400 in [H⁺]-form columns prepared by replacing the Sephadex in NAP-10 columns (Amersham Bioscience) with 2 mL of Dowex from Bio-Rad pre-packed columns”.

should read:

“Supernatants were loaded onto pre-washed 2 mL Dowex AG 50W-X8, 200–400 in [H⁺]-form columns prepared by replacing the Sephadex in NAP-10 columns (Amersham Bioscience) with 2 mL of Dowex from Bio-Rad pre-packed columns”.



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