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Inhibition of yeast ribonucleotide reductase by Sml1 depends on the allosteric state of the enzyme

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Sml1 is an intrinsically disordered protein inhibitor of *Saccharomyces cerevisiae* ribonucleotide reductase (ScRR1), but its inhibition mechanism is poorly understood. RR reduces ribonucleoside diphosphates to their deoxy forms, and balances the nucleotide pool. Multiple turnover kinetics show that Sml1 inhibition of dGTP/ADP- and ATP/CDP-bound ScRR follows a mixed inhibition mechanism. However, Sml1 cooperatively binds to the ES complex in the dGTP/ADP form, whereas with ATP/CDP, Sml1 binds weakly and noncooperatively. Gel filtration and mutagenesis studies indicate that Sml1 does not alter the oligomerization equilibrium and the CXXC motif is not involved in the inhibition. The data suggest that Sml1 is an allosteric inhibitor.

Keywords: enzyme kinetics; intrinsically disordered protein; mixed inhibition; nucleotides; oligomerization

Ribonucleotide reductase (RR) catalyzes the conversion of ribonucleotides to 2′-deoxyribonucleotides, which is the rate determining step of dNTP synthesis. Allosteric regulation of RR substrate specificity and overall activity is essential for maintaining balanced and adequate deoxynucleotide pools during S-phase [1]. In other phases of the cell cycle, RR activity is also regulated at the level of transcription [2] and subunit localization [3], and in the case of *Saccharomyces cerevisiae* RR (ScRR) and recently discovered in human RR (hRR), activity is limited by small protein inhibitors, Sml1 (suppressor of mec-1 lethality) and IRBIT, respectively [4–6]. Sml1 is believed to down-regulate ScRR activity through interactions with the catalytic subunit, but the mechanism by which it functions to block activity is unknown [4,5,7–10]. Here, we report multiple turnover inhibition kinetics and biochemical analyses of Sml1 that demonstrate a pattern of inhibition that depends on the identity of the allosteric regulator bound to the enzyme. Together the results suggest a mechanism in which effector binding modulates the affinity and cooperativity of Sml1, which may act to modulate down-regulation of ScRR activity during the cell cycle.

ScRR is representative of eukaryotic RR and consists of a multimeric catalytic α-subunit [$\alpha = n$ where $n = 2$, 4 or 6] and a heterodimeric complex comprised of two β-subunits ($\beta\beta'$ heterodimers [$\beta\beta' = m$, $m = 1$, 2 or 3], where the β′-subunit lacks the key residues required for generating the catalytic free radical) [11]. The α-subunit contains

Abbreviations
A-site, activity site; C-site, catalytic site; CXXC, C-terminal motif; dNDPs, deoxyribonucleoside 5′-diphosphates; dNTPs, deoxyribonucleotide triphosphates; NDPs, ribonucleoside 5′-diphosphates; RR, ribonucleotide reductase; ScRR, *Saccharomyces cerevisiae* ribonucleotide reductase; ScRR1, subunit of *Saccharomyces cerevisiae* ribonucleotide reductase; SEC, size exclusion chromatography; S-site, specificity site; TR, thioredoxin.

two different nucleotide binding sites that allosterically regulate substrate specificity and overall activity (Fig. 1A) [1,12]. Binding of ATP, dATP, dGTP, or dTTP to the allosteric specificity site (S-site) induces α-subunit dimers and determines the preference for NDP substrates binding to the catalytic site (C-site) where reduction of the 2′OH occurs (Fig. 1A) [1,12–17]. A second allosteric site, termed the activity site (A-site), regulates the overall activity of the enzyme by binding either the allosteric activator ATP or the allosteric inhibitor dATP, eliciting the formation of either active or inactive RR α-subunit hexamers, respectively (Fig. 1A) [1,12,14–16,18–21]. Both modes of regulation of RR are central to the maintenance of a balanced pool of dNTPs during DNA synthesis [18,22].

ScRR activity is also under tight regulation by the small protein inhibitor Sml1. Sml1 is a 104 amino acid protein which is intrinsically disordered; and levels of Sml1 fluctuate throughout the cell cycle, diminishing when DNA synthesis or repair is needed [5,7,23,24]. The cellular concentration of Sml1 is regulated by phosphorylation which leads to ubiquination-dependent degradation [8,23–29]. Sml1 is known to bind to the α-subunit, but little else is known regarding the mechanism by which it inhibits ScRR [4,5,7–10]. The ability of RR to exist in multiple allosterically regulated forms raises the question of whether these states have differential sensitivity to inhibition by Sml1.

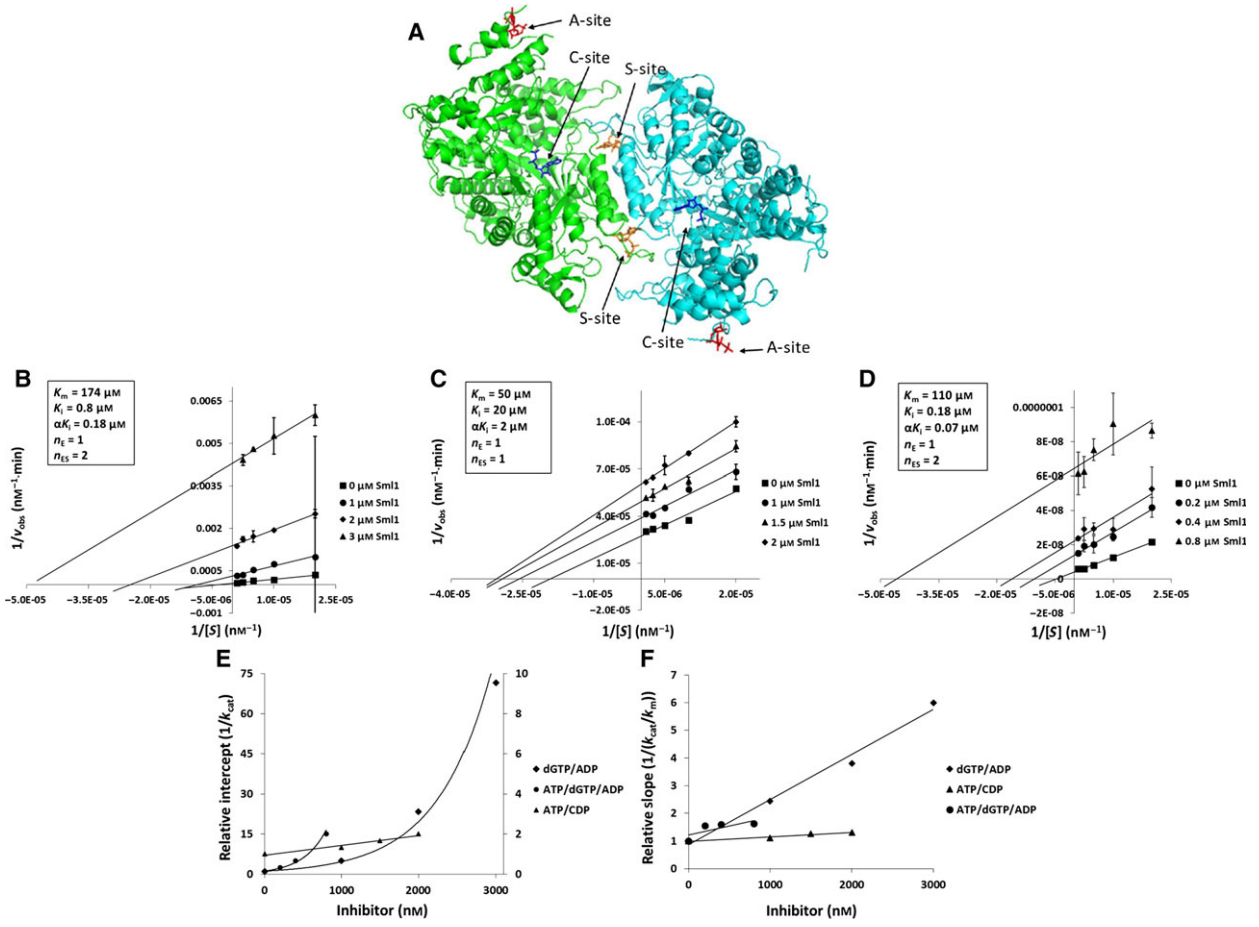


Fig. 1. Mode of inhibition of dimeric and hexameric ScRR by Sml1. (A) Structure of ScRR1 dimer with highlighted C-site, S-site, and A-site. Structure of ScRR1 dimer (PDB 3S87) was generated in Pymol. (B) Linear fit of the dGTP/ADP dimer in a double reciprocal plot, demonstrating mixed inhibition. Inset contains parameters provided from the global fit analysis. (C) Linear fit of the ATP/CDP hexamer in a double reciprocal plot, demonstrating mixed inhibition. Inset contains parameters provided from the global fit analysis. (D) Linear fit of the ATP/dGTP/ADP hexamer in a double reciprocal plot demonstrating mixed inhibition. Inset contains parameters provided from the global fit analysis. (E) Intercept replot and (F) slope replot is also shown.

Materials and methods

Expression and purification of recombinant proteins

Saccharomyces cerevisiae α-subunit (ScRR1) protein, ββ′-subunit (ScRR2•ScRR4), and Sml1 were expressed and purified as described [15,30,31]. Yeast thioredoxin (TR) and thioredoxin reductase (TRR) were a gift from Dr. Sang Won Kang (Ewha Womans University, Seoul, Korea). Yeast TR and TRR protein were expressed and purified using the method adapted from Kim *et al.* [32]. ΔCXXC deletion mutant was prepared using the forward primer- 5′-CA
GAAGCTTGAGAAATGTGTTC-3′ and reverse primer-5′-GAACACATTTCTCAAGCTTCTG-3′. The expression, purification, and biochemical characterization of ΔCXXC ScRR1 is equivalent to that of wild-type ScRR1.

Labeling of mutant S60C Sml1

Sml1 containing a fluorescence tag was prepared as described in reference [33]. All fluorescent studies were done with $Cys^{14}Ser/Ser^{60}Cys$-labeled Sml1. $Cys^{14}Ser/Ser^{60}Cys$ Sml1 was labeled with Alexa 350 C_5-maleimide.

Size exclusion chromatography

Gel filtration was performed as described in references [16,33]. All SEC work was carried out using a Superdex 200 10/300 GL column (GE Lifesciences, Piscataway, NJ, USA). ATP/dATP-induced oligomers of ScRR1 were analyzed in the presence of 3 mM ATP/50 μM dATP. The dATP hexamer–Sml1 complex was prepared by incubating 10 μM of ScRR1 and 50 μM dATP for 10 min and then by adding 40 μM of $Ser^{60}Cys$ Sml1 to a final volume of 100 μL. The resulting mixture was further incubated for 10 additional minutes on ice. For the ATP hexamer–Sml1 complex, we used 3 mM ATP but used the same order of additions and incubation times as with the 50 μM dATP hexamer complex.

Enzyme assays

The steady-state kinetics of ScRR were measured in the presence and absence of Sml1 *in vitro* using [^{3}H]CDP and [^{14}C]ADP reduction assays as described [16], using 0.5 μM ScRR1 dimer and 5 μM ScRR2•ScRR4 heterodimer. The radiolabeled dNDP products were separated from unreacted NDP substrates using borate chromatography and quantified by isotope counting [16]. When ATP/CDP was used as the effector–substrate pair, [^{3}H]CDP concentrations were varied from 0.05 to 1 mM. Similarly, when dGTP/ADP was used as the effector–substrate pair, [^{14}C]ADP concentrations were varied from 0.05 to 1 mM. ATP was omitted from dGTP/[^{14}C]ADP experiments for the examination of ScRR dimer kinetics. For inhibition studies with [^{3}H]CDP, 1, 1.5, and 2 μM of Sml1 were used while in studies with dGTP/[^{14}C]ADP, 1, 2, and 3 μM of Sml1 monomer were used, and with ATP/dGTP/[^{14}C]ADP, 0.2, 0.4, and 0.8 μM of Sml1 were used. All enzyme kinetic experiments were performed in duplicate.

Enzyme inhibition experiments performed in the presence of TR instead of DTT consisted of the same assay design as WT except for the substitution of the TR system (100 μM TR, 1 μM TRR, 2 mM NADPH) in place of DTT. Prior to activity assay experiment, a dialysis of the ScRR1 sample was performed to remove DTT, which is needed for purification and storage purposes.

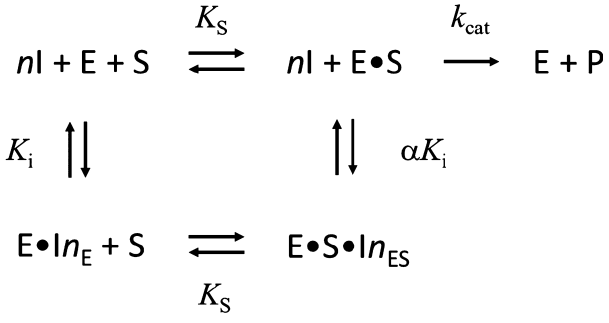
Analysis of steady-state inhibition kinetics

To investigate the mechanism of Sml1 inhibition, we the evaluated steady-state ScRR reaction kinetics measured at a range of Sml1 and NDP substrate concentrations in the context of a general equilibrium model for mixed inhibition (Scheme 1).

To characterize the inhibition mechanism and estimate the apparent inhibitor affinity for E and ES as well as the cooperativity of binding to these two enzymes forms, the steady-state inhibition data were globally fit to a general rate equation for mixed inhibition (further description of derivation in supplemental text),

$$v_{obs} = \frac{[S]k_{cat}[E]_{total}}{K_m\left(\frac{1+[I]^{n_E}}{K_i}\right) + [S]\left(1 + \frac{[I]^{n_{ES}}}{\alpha K_i}\right)},$$

in which K_i is the affinity of Sml1 to the free enzyme (E in Scheme 1) and αK_i is the affinity for the ES complex such that the value α measures the difference in affinity of Sml1 for these two enzyme forms. In Scheme 1, the parameter n is the stoichiometry of Sml1 binding to RR. In equation 1, n_E and n_{ES} are used as variables to evaluate cooperativity of binding of Sml1 to the free (E) and substrate-bound (ES) forms of the enzyme, respectively. The data were first fit to a model in which α = 1 to evaluate a purely non-competitive mechanism. However, a value less than 1



Scheme 1. General scheme for mixed inhibition of RR (E) by Sml1 (I).

provided a better fit to the data. Models in which $n_E = n_{ES} = 1$ were subsequently evaluated and as discussed in the text, this model describes the inhibition mechanism of Sml1 inhibition of the ATP-bound form of the enzyme. Integer values of n_E and n_{ES} greater than 1 were also individually tested for the dGTP-bound form of the enzyme and the goodness of fit was evaluated by comparing the magnitude of the squared differences between model and experimental data.

Results and Discussion

Steady-state kinetic analysis of the modes of ScRR1 inhibition by Sml1

Dimerization of RR is induced by binding of the effectors dGTP, dTTP, dATP, and ATP to the S-site, while binding of ATP to the A-site occurs with lower affinity and induces the formation of RR α-subunit hexamers [1,13–17]. To gain information on the interaction of Sml1 with these different allosteric states, we first analyzed inhibition of the dimeric, dGTP-bound state of ScRR, which has specificity for ADP as a substrate. Linear fit of the steady-state inhibition data on double reciprocal plots, $1/v_{obs}$ versus 1/[S], indicates that both the k_{cat}/K_m and k_{cat} are affected by the presence of Sml1, and the slope ($1/(k_{cat}/K_m)$) and *y*-intercept increase as a function of Sml1 concentration (Fig. 1B). This result demonstrates that Sml1 functionally interacts with both free enzyme (E) and the enzyme–substrate complex (ES). The double reciprocal plot also reveals that the decrease in k_{cat} (increase in $1/v_{obs}$ intercept) with increasing concentrations of Sml1 is nonlinear, suggesting that the binding of Sml1 to the ES complex is cooperative. Plots of the *y*-intercept ($1/k_{cat}$) versus Sml1 concentration illustrate the exponential dependence of the effect of inhibitor concentration on k_{cat} (Fig. 1E). In contrast, the interaction of Sml1 with free E appears to be noncooperative. A plot of the slope ($1/(k_{cat}/K_m)$) versus Sml1 concentration shows linear dependence of k_{cat}/K_m on Sml1 concentration (Fig. 1F).

Global fitting of the steady-state inhibition data to a general rate equation for mixed inhibition, as described in Materials and Methods, was used to evaluate different potential mechanistic possibilities for the dimeric, dGTP-bound state of ScRR (Fig. 1B inset, Fig. S1A). The simplest model consistent with the data involves noncooperative binding of Sml1 to the free enzyme with a K_i of 0.8 ± 0.12 μM. Fitting the data, assuming tighter or weaker binding of Sml1 to ES relative to E ($\alpha <$ or > 1), indicated that the data were best described by a mixed inhibition model in which Sml1 binds to ES with the tighter affinity ($\alpha K_i = 0.18 \pm 0.03$ μM) than E. Interestingly, the data are best described by a mechanism assuming cooperativity of Sml1 binding to the ES complex ($n_{ES} = 2$). This observation is consistent with interpretations from inspection of the double reciprocal plot.

Next, we compared the mode of inhibition of the hexameric, ATP-bound form of ScRR by Sml1, which has specificity for CDP as a substrate. As shown by double reciprocal plots of the linear fit (Fig. 1C), Sml1 has a smaller effect on the k_{cat}/K_m and k_{cat} of this ATP-bound allosteric state over the range of CDP concentrations tested, in comparison to the dimeric dGTP state. Unlike the dimeric dGTP-bound state of ScRR, a plot of the observed intercepts and slopes [$1/k_{cat}$ and $1/(k_{cat}/K_m)$] versus inhibitor concentration are both linear (Fig. 1E–F). This result is consistent with noncooperative binding of Sml1 to both E and ES. Global fitting of these data to general rate equation reveals a 10-fold difference in binding affinity of Sml1 to E and ES, 20 ± 5.6 μM and 2 ± 0.59 μM, respectively (Fig. 1C inset, Fig. S1B). These binding affinities are significantly lower relative to the dimeric dGTP-bound ScRR. The substantial difference in the binding affinity to E and ES, with the apparent preference for ES indicates that Sml1 binding to the ATP/CDP-bound ScRR also follows a mixed inhibition model. Most importantly, the global fitting further illustrated that the data are consistent with a model involving noncooperative binding of Sml1 to both E and ES ($n_E = n_{ES} = 1$).

Two different patterns of ScRR inhibition by Sml1 are observed depending on if dGTP or ATP is bound in the S-site to allosterically regulate ScRR substrate specificity. However, these two reaction conditions also differ in the oligomerization state of the enzyme as ATP binding at the A-site induces hexamerization of RR α-subunit. To determine the extent to which oligomerization or the identity of the allosteric effector bound to the S-site correlate with the mode of Sml1 inhibition, we compared the inhibition of the hexameric dGTP-bound form in the presence of ATP to induce hexamer formation. Linear fit on a double reciprocal plot indicates visual similarity with the dimeric dGTP ScRR double reciprocal plot (Fig. 1D). Plot of the intercept versus Sml1 concentration shows there is an exponential dependence of the effect of inhibitor concentration on k_{cat}, indicating cooperative binding of Sml1 to ES (Fig. 1E). As also seen with both the dimeric dGTP and the hexameric ATP/CDP, interaction of Sml1 with E appears to be noncooperative (Fig. 1F). Global fitting analysis of the data for the ATP/dGTP-bound form of ScRR shows inhibition can be described by a mixed inhibition model in which

Sml1 binds to E with a K_i of 0.18 ± 0.03 μM and binds cooperatively ($n_{ES} = 2$) to ES with a αK_i of 0.07 ± 0.01 μM (Fig. 1D inset, Fig. S1C). This analysis is consistent with expectations from inspection of the double reciprocal plot (Fig. 1D).

Examination of the inhibition kinetics of ScRR by Sml1 is relatively complex as it does not easily fit with a classic mechanism of inhibition: competitive, noncompetitive, or uncompetitive, but rather fits a mixed inhibition mechanism with specific aspects of this mechanism dependent on the allosteric state of the enzyme. Visual inspection of the double reciprocal plots clearly shows that Sml1 does not exclusively act by competitively inhibiting ScRR. All allosteric states exhibit a k_{cat} change with increase in Sml1 concentration, indicating that Sml1 is not competing with the substrate, ADP or CDP, for binding at the C-site. It can also be argued that none of the allosteric states tested follow a purely noncompetitive mechanism. Classical noncompetitive mechanism exhibits equal affinity for E and ES with no cooperativity. Attempts to fit this mechanism to the inhibition data proved to be unsuccessful. For the dimeric and hexameric ADP forms, the data fit a mixed inhibition model where there is a higher affinity for the ES than the E, 4.5- and 2.5-fold difference in affinity, respectively. In the case of CDP, it can be said that it is a mixed inhibition mechanism as there is affinity for both the ES and E, but with greater affinity for ES (10-fold greater). Thus, an important shared attribute of all allosteric forms of ScRR is that there is a higher affinity for the ES than E. It is possible that the structural change in ScRR1 specifically induced by substrate binding allows for better exposure of the Sml1-binding site for a stronger binding affinity. The differences in the structure of ScRR1 when bound to either ADP or CDP might explain why different inhibition mechanisms are observed.

Sml1s effect on oligomerization

The steady-state inhibition supports functional binding of Sml1 to both E and ES in order to inhibit ScRR activity and the binding changes observed vary based on substrate and oligomerization states. So the question remains whether or not Sml1 was binding to the dimer or hexamer forms described in the kinetic assays as expected and whether inhibition by Sml1 binding can be further explained by changes in oligomerization. It is possible that the differences in binding to these different allosteric forms of the enzyme could reflect thermodynamic or mechanistic linkage between Sml1 binding and oligomerization. Therefore, size exclusion chromatography was used to test whether Sml1 forms stable complexes with dimeric and hexameric forms of ScRR α-subunit and examine its effect on oligomerization (Fig. 2). Fluorescently labeled Sml1 was incubated with ScRR hexamers formed in the presence of both dATP and ATP (Fig. 2A–B). Although it is not possible to maintain equilibrium conditions during chromatographic separation, the data clearly show that Sml1 forms stable complexes with ScRR dimers and hexamers (Fig. 2). Sml1 binds to both active (ATP bound) and inactive (dATP bound) hexamers (Fig. 2A–B) and does not appear to result in major alteration of the oligomerization equilibrium under conditions used in enzyme assays (Fig. 2C–D).

Biochemical data are consistent with the functional association of Sml1 with both the free (E) and substrate-bound (ES) forms of ScRR. Sml1 binding to the hexameric form of ScRR1 does not disrupt these hexamers, while Sml1 binding to the dimeric form does not shift the oligomerization equilibrium toward hexamer formation. Together this further supports the conclusions from the kinetic data that binding and inhibition of ScRR1 by Sml1 is determined by allosteric state of ScRR, but the inhibition is not due to a shift in equilibrium of ScRR1 oligomeric state. The physiological role of Sml1, as supported by its stable binding to both ATP and dATP hexamers, can be attributed to complimenting the allosteric inhibition of dATP. *In vitro* studies indicate dATP inhibits ScRR by 50% compared to ATP-bound ScRR under physiological conditions, while the addition of Sml1 leads to the complete inhibition of ScRR (Fig. S2).

Sml1 and the regeneration of the active site of ScRR1

It has been hypothesized that Sml1 might interfere with the reduction pathway in ScRR, targeting the CXXC motif located at the C terminus of the α-subunit (ScRR1), an intermediate in the thioredoxin (TR) reduction pathway [9,34]. In other words, does Sml1 inhibit ScRR by interfering with the rate limiting step of enzyme regeneration? After a catalytic cycle, the oxidized cysteines in the C-site require reduction prior to the next catalytic cycle. TR, one of the main reductants used in this process cannot directly reduce these cysteines so the intermediate, CXXC motif, is utilized. TR transfers a reducing equivalent to the CXXC motif and the flexible C-terminal tail then interacts with the cysteines in the C-site to transfer this reducing equivalent [34]. Removal of this CXXC motif renders the enzyme unable to be reduced naturally, but for experimental purposes, the synthetic two-electron reductant,

DTT, can be used as the reductant, whose small size allows for the reduction of the active site directly [35]. To investigate the possibility that Sml1 is targeting the reduction pathway, the CXXC motif was removed and DTT was utilized. RR activity assay was utilized to measure the enzymatic activity levels of ScRR as determined by the amount of product formed, [^{14}C] ADP in this study. Measurement of the activity of WT and ΔCXXC mutant in the presence of DTT were used as controls to test that the mutant was functional, and the results indicate that the ΔCXXC mutant activity was similar to WT (Fig. 3). Activity in WT ScRR1 in the presence of Sml1 and either DTT or TX indicated that Sml1 can inhibit product production of

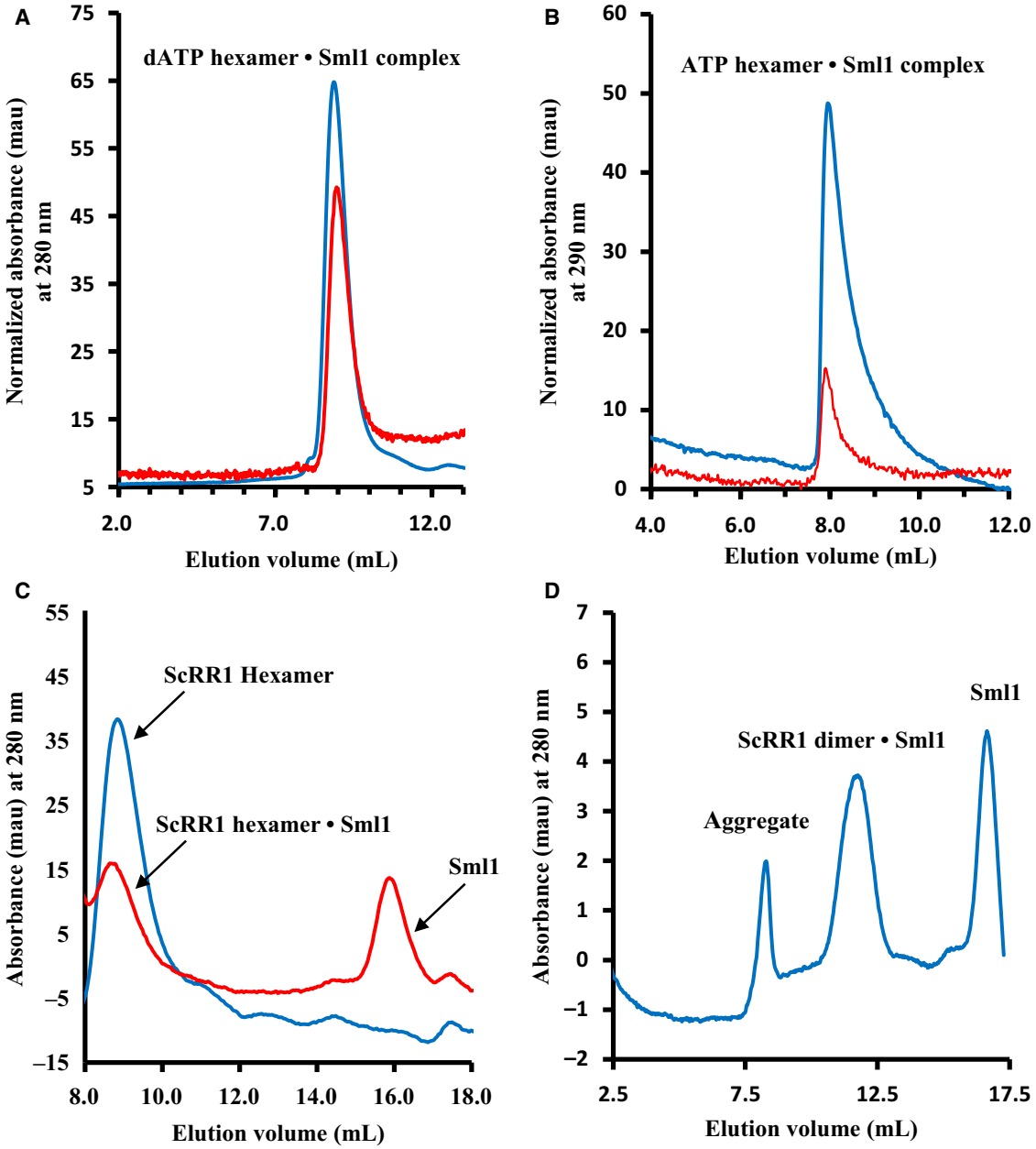


Fig. 2. SEC analysis of Sml1–ScRR1 oligomerization. (A) Alexa 350-labeled Sml1 was used to monitor interactions with ScRR1 hexamer at 344 nm. C14S S60CSml1 bound to the dATP-induced ScRR1 hexamer was monitored at 344 nm (red trace). (B) Coelution of Alexa 350-labeled Sml1 (red trace) with ATP-induced ScRR1 hexamer. (C) Injection of preformed dGTP ScRR1 dimer–Sml1 complex (red line) to gel filtration column pre-equilibrated with 50 μM dATP does not interfere with hexamer formation. Note, void volume of SEC column is 8 mL. (D) Injection of preformed dGTP ScRR1 dimer–Sml1 complex to gel filtration column does not induce hexamer formation. Note, void volume of SEC column is 8 mL.

ScRR1 despite which reductant is present. In both cases, there was essentially no product formation (Fig. 3). The key experimental condition, however, was the ΔCXXC mutant in the presence of Sml1 and DTT. Two main possibilities were anticipated in this [^{14}C]ADP reduction experiment; either (1) there would be retention of activity with DTT and the mutant in the presence of Sml1, indicating Sml1 is targeting the CXXC motif, or (2) there would be a loss of activity, indicating that Sml1 is not targeting the CXXC motif but rather another site. Results indicate that there was no [^{14}C]ADP reduction with ΔCXXC ScRR1 in the presence of DTT and 1 μM of Sml1 (Fig. 3).

Through this investigation of Sml1 targeting the CXXC motif at the C terminus of ScRR1, we have observed that Sml1 inhibition is independent of this motif. While we cannot rule out that Sml1 interferes elsewhere in the reduction pathway of ScRR1, it is important to know that Sml1 is not binding at the C terminus. It is binding elsewhere on ScRR1 which might be causing a structural conformational change to prevent DTT-mediated dNDP production. Without structural data of this complex, it is hard to distinguish precisely where Sml1 is binding on ScRR, eliciting either medium- or long-range interactions. Structural studies are currently being pursued to determine specifically how Sml1 is binding to inhibit ScRR activity.

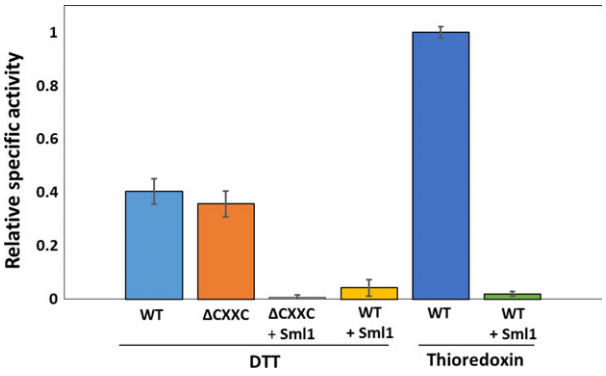


Fig. 3. Sml1 influence on the regeneration of ScRR1. Relative specific activities of wild-type ScRR and ΔCXXC ScRR in the presence and absence of Sml1 and in the presence of either DTT or the TR system. The ΔCXXC ScRR1 in the presence of DTT as the reductant and Sml1 exhibits no activity, indicating that Sml1 does not inhibit via the CXXC motif.

Conclusions

Considering the importance of RR in maintaining an adequate and balanced cellular dNTP pool for the high fidelity of DNA replication and repair, the study of RR regulation has become a focus of attention for pharmacological intervention. The inhibition of budding yeast RR (ScRR) by Sml1 is one of the few examples of a natural protein RR inhibitor, making it a great research target for the development of future pharmacological interventions against cancer and other diseases characterized by rapidly dividing cells. One of the key characteristics of Sml1 that still remains a mystery is its mechanism of inhibition, which may help us structurally understand Sml1 as an intrinsically disordered protein and how it interacts with ScRR1. The data presented here are most consistent with a model in which Sml1 acts as an allosteric inhibitor of ScRR catalysis, exhibiting different affinity in the presence and absence of effector–substrate pair and different mechanisms depending on the substrate bound (Fig. 4). It can be argued that the difference in binding between effector/substrate pairs seen here is simply because of the

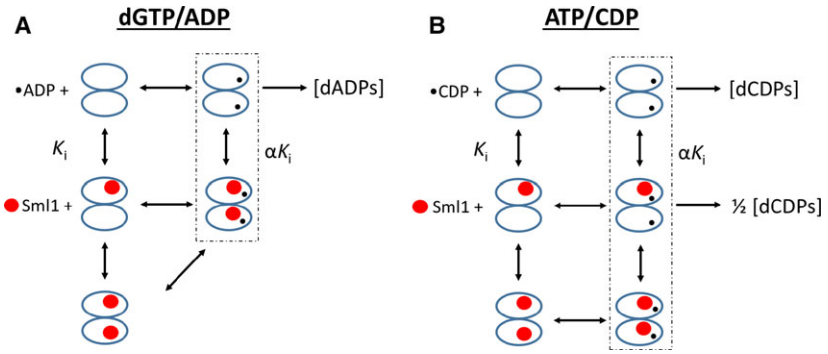


Fig. 4. Models describing CDP and ADP inhibition. (A). Binding of Sml1 to dGTP/ADP-bound ScRR1. Sml1 and substrate are indicated by (red circle) and (black dot), respectively. K_i and αK_i indicate the dissociation constant for the free enzyme and substrate-bound enzyme, respectively. States highlighted by the dotted box indicates cooperative binding of Sml1 to substrate-bound enzyme. (B). Binding of Sml1 to ATP/CDP-bound ScRR1. Sml1 and substrate are indicated by (red circle) and (black dot), respectively. K_i and αK_i indicate the dissociation constant for the free enzyme and substrate-bound enzyme, respectively. States highlighted by the dotted box indicates noncooperative binding of Sml1 to substrate-bound enzyme.

structural differences of ScRR bound to each effector/substrate.

It has been in debate since the discovery of Sml1 whether there is a human analog and whether studying Sml1 is important. Recently there was the discovery of IRBIT, a proposed human analog of Sml1 [6]. While the discovery of IRBIT is recent and there have not been extensive studies on this inhibitor, there are some similarities between IRBIT and Sml1. Both IRBIT and Sml1 further decrease the activity of RR compared to inhibition by dATP alone, with the distinction that IRBIT stabilizes only the dATP hRRM1 hexamer [6] and Sml1 has been shown to fully inhibit both the ATP and the dATP hexamer of ScRR. It was also noted that structurally, the N-terminal domain of IRBIT belongs to a class of IDPs (intrinsically disordered proteins) similar to Sml1 [6]. Similarly, p53R2 is known to make several protein–protein interactions of functional importance [36,37]. The discovery of IRBIT validates the importance of studying Sml1 for the development of antiproliferative therapeutics.

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Author contributions

TAM, SRW, CGD, AJB, TR, and MEH designed the work, and TAM and SRW performed the experimental work and analyzed the data. TR and AJB helped in the initial analysis of the kinetic data. MEH aided in the further analysis of the kinetic experiments. TAM, SRW, MEH, and CGD wrote the manuscript. MFA aided in sample preparation, intellectual discussion, and critical comments on the manuscript.

References

1 Brown NC and Reichard P (1969) Role of effector binding in allosteric control of ribonucleoside diphosphate reductase. *J Mol Biol* **46**, 39–55.
2 Huang M, Zhou Z and Elledge SJ (1998) The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell* **94**, 595–605.
3 Zhang Z, An X, Yang K, Perlstein DL, Hicks L, Kelleher N, Stubbe J and Huang M (2006) Nuclear localization of the Saccharomyces cerevisiae ribonucleotide reductase small subunit requires a karyopherin and a WD40 repeat protein. *Proc Natl Acad Sci USA* **103**, 1422–1427.
4 Chabes A, Domkin V and Thelander L (1999) Yeast Sml1, a protein inhibitor of ribonucleotide reductase. *J Biol Chem* **274**, 36679–36683.
5 Zhao X, Chabes A, Domkin V, Thelander L and Rothstein R (2001) The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *EMBO J* **20**, 3544–3553.
6 Arnaoutov A and Dasso M (2014) Enzyme regulation. IRBIT is a novel regulator of ribonucleotide reductase in higher eukaryotes. *Science* **345**, 1512–1515.
7 Zhao X, Muller EG and Rothstein R (1998) A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol Cell* **2**, 329–340.
8 Zhao X, Georgieva B, Chabes A, Domkin V, Ippel JH, Schleucher J, Wijmenga S, Thelander L and Rothstein R (2000) Mutational and structural analyses of the ribonucleotide reductase inhibitor Sml1 define its Rnr1 interaction domain whose inactivation allows suppression of mec1 and rad53 lethality. *Mol Cell Biol* **20**, 9076–9083.
9 Zhang Z, Yang K, Chen CC, Feser J and Huang M (2007) Role of the C terminus of the ribonucleotide reductase large subunit in enzyme regeneration and its inhibition by Sml1. *Proc Natl Acad Sci USA* **104**, 2217–2222.
10 Georgieva B, Zhao X and Rothstein R (2000) Damage response and dNTP regulation: the interaction between ribonucleotide reductase and its inhibitor, Sml1. *Cold Spring Harb Symp Quant Biol* **65**, 343–346.
11 Perlstein DL, Ge J, Ortigosa AD, Robblee JH, Zhang Z, Huang M and Stubbe J (2005) The active form of the Saccharomyces cerevisiae ribonucleotide reductase small subunit is a heterodimer in vitro and in vivo. *Biochemistry* **44**, 15366–15377.
12 Uhlin U and Eklund H (1994) Structure of ribonucleotide reductase protein R1. *Nature* **370**, 533–539.
13 Eriksson M, Uhlin U, Ramaswamy S, Ekberg M, Regnstrom K, Sjoberg BM and Eklund H (1997) Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure* **5**, 1077–1092.
14 Larsson KM, Jordan A, Eliasson R, Reichard P, Logan DT and Nordlund P (2004) Structural mechanism of allosteric substrate specificity regulation in a ribonucleotide reductase. *Nat Struct Mol Biol* **11**, 1142–1149.

15 Xu H, Faber C, Uchiki T, Fairman JW, Racca J and Dealwis C (2006) Structures of eukaryotic ribonucleotide reductase I provide insights into dNTP regulation. *Proc Natl Acad Sci USA* **103**, 4022–4027.
16 Fairman JW, Wijerathna SR, Ahmad MF, Xu H, Nakano R, Jha S, Prendergast J, Welin RM, Flodin S, Roos A *et al*. (2011) Structural basis for allosteric regulation of human ribonucleotide reductase by nucleotide-induced oligomerization. *Nat Struct Mol Biol* **18**, 316–322.
17 Zimanyi CM, Chen PY, Kang G, Funk MA and Drennan CL (2016) Molecular basis for allosteric specificity regulation in class Ia ribonucleotide reductase from *Escherichia coli*. *eLife* **5**, e07141.
18 Brown NC, Canellakis ZN, Lundin B, Reichard P and Thelander L (1969) Ribonucleoside diphosphate reductase. Purification of the two subunits, proteins B1 and B2. *Eur J Biochem* **9**, 561–573.
19 Rofougaran R, Vodnala M and Hofer A (2006) Enzymatically active mammalian ribonucleotide reductase exists primarily as an alpha6beta2 octamer. *J Biol Chem* **281**, 27705–27711.
20 Kashlan OB and Cooperman BS (2003) Comprehensive model for allosteric regulation of mammalian ribonucleotide reductase: refinements and consequences. *Biochemistry* **42**, 1696–1706.
21 Wang J, Lohman GJ and Stubbe J (2007) Enhanced subunit interactions with gemcitabine-5'-diphosphate inhibit ribonucleotide reductases. *Proc Natl Acad Sci USA* **104**, 14324–14329.
22 Hakansson P, Hofer A and Thelander L (2006) Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem* **281**, 7834–7841.
23 Andreson BL, Gupta A, Georgieva BP and Rothstein R (2010) The ribonucleotide reductase inhibitor, Sml1, is sequentially phosphorylated, ubiquitylated and degraded in response to DNA damage. *Nucleic Acids Res* **38**, 6490–6501.
24 Uchiki T, Dice LT, Hettich RL and Dealwis C (2004) Identification of phosphorylation sites on the yeast ribonucleotide reductase inhibitor Sml1. *J Biol Chem* **279**, 11293–11303.
25 Zhao X and Rothstein R (2002) The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. *Proc Natl Acad Sci USA* **99**, 3746–3751.
26 Uchiki T, Hettich R, Gupta V and Dealwis C (2002) Characterization of monomeric and dimeric forms of recombinant Sml1p-histag protein by electrospray mass spectrometry. *Anal Biochem* **301**, 35–48.
27 Gupta V, Peterson CB, Dice LT, Uchiki T, Racca J, Guo JT, Xu Y, Hettich R, Zhao X, Rothstein R *et al*. (2004) Sml1p is a dimer in solution: characterization of denaturation and renaturation of recombinant Sml1p. *Biochemistry* **43**, 8568–8578.
28 Sharp JS, Guo JT, Uchiki T, Xu Y, Dealwis C and Hettich RL (2005) Photochemical surface mapping of C14S-Sml1p for constrained computational modeling of protein structure. *Anal Biochem* **340**, 201–212.
29 Danielsson J, Liljedahl L, Barany-Wallje E, Sonderby P, Kristensen LH, Martinez-Yamout MA, Dyson HJ, Wright PE, Poulsen FM, Maler L *et al*. (2008) The intrinsically disordered RNR inhibitor Sml1 is a dynamic dimer. *Biochemistry* **47**, 13428–13437.
30 Chabes A, Domkin V, Larsson G, Liu A, Graslund A, Wijmenga S and Thelander L (2000) Yeast ribonucleotide reductase has a heterodimeric iron-radical-containing subunit. *Proc Natl Acad Sci USA* **97**, 2474–2479.
31 Nguyen HH, Ge J, Perlstein DL and Stubbe J (1999) Purification of ribonucleotide reductase subunits Y1, Y2, Y3, and Y4 from yeast: Y4 plays a key role in diiron cluster assembly. *Proc Natl Acad Sci USA* **96**, 12339–12344.
32 Kim JA, Park S, Kim K, Rhee SG and Kang SW (2005) Activity assay of mammalian 2-cys peroxiredoxins using yeast thioredoxin reductase system. *Anal Biochem* **338**, 216–223.
33 Wijerathna SR (2012) Structural and Biochemical Studies of Ribonucleotide Reductase Inhibition by dATP and Sml1. Dissertation Thesis, CWRU, 1–222.
34 Mao SS, Holler TP, Bollinger JM Jr, Yu GX, Johnston MI and Stubbe J (1992) Interaction of C225SR1 mutant subunit of ribonucleotide reductase with R2 and nucleoside diphosphates: tales of a suicidal enzyme. *Biochemistry* **31**, 9744–9751.
35 Domkin V and Chabes A (2014) Phosphines are ribonucleotide reductase reductants that act via C-terminal cysteines similar to thioredoxins and glutaredoxins. *Sci Rep* **4**, 5539.
36 Kuo M-L, Lee MB-E, Tang M, den Besten W, Hu S, Sweredoski MJ, Hess S, Chou C-M, Changou CA, Su M *et al*. (2016) PYCR1 and PYCR2 interact and collaborate with RRM2B to protect cells from overt oxidative stress. *Sci Rep* **6**, 18846.
37 Xie M, Yen Y, Owonikoko TK, Ramalingam SS, Khuri FR, Curran WJ, Doetsch PW and Deng X (2014) Bcl2 induces DNA replication stress by inhibiting ribonucleotide reductase. *Cancer Res* **74**, 212–223.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Fig. S1. Global fitting of inhibition kinetics.
Fig. S2. Sml1 inhibition of dATP-based holoenzyme.