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CDK/CK1 inhibitors roscovitine and CR8 down-regulate amplified MYCN in neuroblastoma cells

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Abstract

To understand the mechanisms of action of (R)-roscovitine and (S)-CR8, two related pharmacological inhibitors of cyclin-dependent kinases (CDKs), we applied a variety of '-omics' techniques to the human neuroblastoma SH-SY5Y and IMR32 cell lines: [1] kinase interaction assays, [2] affinity competition on immobilized broad-spectrum kinase inhibitors, [3] affinity chromatography on immobilized (R)-roscovitine and (S)-CR8, [4] whole genome transcriptomics analysis and specific quantitative PCR studies, [5] global quantitative proteomics approach and Western blot analysis of selected proteins. Altogether the results show that the major direct targets of these two molecules belong to the CDKs (1, 2, 5, 7, 9, 12), DYRKs, CLKs, CK1s families. By

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CONFLICT OF INTEREST

Dr. Meijer is co-inventor on the roscovitine patent. Dr. Galons, Dr. Oumata and he are co-inventors on the CR8 patent. Dr. Meijer and Dr. Galons are co-founders of ManRos Therapeutics. Dr. Meijer is President and CSO of ManRos Therapeutics.

inhibiting CDK7, CDK9 and CDK12, these inhibitors transiently reduce RNA polymerase 2 activity, which results in down-regulation of a large set of genes. Global transcriptomics and proteomics analysis converge to a central role of MYC transcription factors down-regulation. Indeed CDK inhibitors trigger rapid and massive down-regulation of MYCN expression in MYCN amplified neuroblastoma cells as well as in nude mice xenografted IMR32 cells. Inhibition of casein kinase 1 may also contribute to the antitumoral activity of (R)-roscovitrine and (S)-CR8. This dual mechanism of action may be crucial in the use of these kinase inhibitors for the treatment of MYC-dependent cancers, in particular neuroblastoma where MYCN amplification is a strong predictor factor for high-risk disease.

Keywords

cyclin-dependent kinase; CDK; casein kinase 1; CK1; kinase inhibitor; roscovitrine; CR8; c-Myc; MYCN; neuroblastoma; medulloblastoma; interactomics; transcriptomics; proteomics

INTRODUCTION

Over 258 protein kinase inhibitors (low molecular weight compounds and antibodies) are currently in clinical trials, primarily against cancers: 116 are in phase I, 82 in phase II, 37 in phase III, and 23 have reached the market (1-4). Although most were initially targeted against a specific protein kinase relevant to a specific disease, thorough selectivity studies show that kinase inhibitors target multiple kinases (5-8). This suggests that the mechanisms underlying their therapeutic effect are more likely due to a favorable combination of additive effects on different kinases rather than to a specific action on a unique kinase target. There is no strict correlation between target selectivity and therapeutic efficacy, advocating for the multi-target inhibitor pharmacologic approach, as illustrated by selectivity studies performed on marketed clinical kinase inhibitors (6-7, 9-10).

Among the 518 human kinases, cyclin-dependent kinases (CDKs) have received considerable attention because of their fundamental involvement in many physiological processes and in many diseases (11). Consequently, a large variety of pharmacological inhibitors of CDKs have been identified, optimized and characterized (see reviews in (12-18). Sixteen CDK inhibitors are currently in clinical trials. Among the first CDK inhibitors are the purines olomoucine, roscovitrine, purvalanol, and their analogues (reviews in 14, 19). The most recent analogues include (S)-CR8 (20-22), N-&-N1 (23, 24), and others (25-28). (R)-roscovitrine (CYC202 or Seliciclib) is currently in late phase 2 clinical trial against NSC lung cancer, breast and nasopharyngeal cancer (29-33). Roscovitrine is frequently used as a 'selective' inhibitor of CDKs in fundamental biological studies. Despite its initial identification as a CDK inhibitor and its rather good selectivity compared to many other kinase inhibitors also acting by competition with ATP binding at the catalytic site, roscovitrine also binds other protein targets (34, 35).

CR8 was developed recently as a more potent, 'second generation' analogue of roscovitrine (Figure 1) (20-22). Although it is slightly more potent (2-5 fold) at inhibiting the kinase targets of roscovitrine, it was found to be much more potent than roscovitrine (50-100 fold) at inducing apoptotic cell death in a variety of cell lines (20, 21). This suggests that the direct

targets of CR8 and roscovitine may differ, and that their mechanism of action might also be different.

To understand and compare the cellular effects of roscovitine and CR8, we used a variety of 'omics' approaches to examine the effects of these drugs on the human neuroblastoma SHSY5Y and IMR32 cell lines which were chosen as cellular models. Results show that both molecules have a complex pattern of primary targets, that they mostly down-regulate the expression of key genes involved in cell cycle regulation, apoptosis, NF κ B pathway, etc... Nevertheless, global analysis points out towards a central role of c-MYC and MYCN down-regulation in the action of these drugs. Both CDK inhibitors trigger rapid and massive down-regulation of MYCN in MYCN amplified neuroblastoma cell lines either in culture and when xenografted in nude mice. This data confirm and extend previous observations showing the links between CDKs and MYC, as well as the favorable effects of CDK inhibition on MYC-dependent cells (36-45), suggesting that MYC dependence may constitute a selection criterion of cancer subgroups for treatment by CDK inhibitors. This is of particular importance for neuroblastoma where MYCN amplification is invariably associated with poor prognosis (review in 46-48). In addition roscovitine and CR8 inhibit CK1 ϵ , a target recently validated in MYC overexpressed context (49).

RESULTS

CR8 is a potent cell death inducer derived from roscovitine

CR8 (Supplementary Figure S1) has been optimized as a second-generation derivative of roscovitine (20-22). Although slightly more potent than roscovitine as an inhibitor of various kinase targets, CR8 was found to be much more potent than its parent molecule at inducing cell death in a variety of cells (average 71.2 fold higher potency on 6 cell lines in ref. 20; average 58.5 fold higher potency on 9 neuroblastoma cell lines in ref. 22). To further explore the differences in their potency the two compounds were tested in the NCI 60 tumor cell lines panel. Results revealed an average 102, 31 and 15 fold higher potency of CR8 over roscovitine as measured by GI₅₀, TGI and LC₅₀, respectively (Table 1; Supplementary Figure S2). No specific tumor type was found to display hyper- or hypo-sensitivity to CR8. In order to investigate and compare the intracellular mechanisms of action of both CDK inhibitors in detail, we selected the human neuroblastoma SH-SY5Y (non-amplified MYCN) and IMR32 (amplified MYCN) cell lines as representative tumor cell lines. To investigate the effects of CR8 on cell cycle progression we exposed IMR32 cells to 5 μ M CR8 for 24 hr and analyzed the cell cycle phase distribution by FACS. The expected, yet modest, increase in G2/M at the expense of G1 and S phase was observed (Figure 1A). We also ran cell synchronization experiments and compared the effects of CR8 on G1 (obtained by serum starvation), G2/M (obtained by nocodazole treatment) and asynchronous, Log phase IMR32 cells. Figure 1A confirmed the expected synchronization and displays the effects of CR8. Dose-response curves showed that CR8 acts with equal potency at all cell cycle stages in terms of cell death induction (Figure 1B).

Interactomics study of roscovitine and CR8. 1. Kinase interaction screen

The molecular targets of roscovitine have been quite extensively studied by various methods including screening on various panels of purified kinases (29, 34, 35, 50, 20), yeast three hybrid screens (51), interaction assays (6) and affinity chromatography (34). Various methods have shown that CR8 and roscovitine display an apparently similar specificity, with slightly enhanced activity of CR8 compared to roscovitine on CDKs (2-4 fold) and CK1 (7-10 fold) (20). To investigate and compare the molecular targets of roscovitine and CR8 using global approaches, we first made use of the DiscoverX KinomeScan® assay (402 kinases) (7) (Supplementary Figure S3; Table 2; Supplementary Table S1). Results show that the main targets of both compounds belong to the CK1 (casein kinase 1), CDK, CLK (cdc2 like kinase) and DYRK (dual specificity, tyrosine phosphorylation regulated kinase) families of kinases (Table 2). Interestingly, CK1 ϵ (CSNK1 ϵ) appeared as the first interaction target for both compounds among the 402 kinases.

Interactomics study of roscovitine and CR8. 2. Affinity chromatography

To evaluate the scope and nature of the direct targets of roscovitine and CR8, we used an affinity chromatography method successfully exploited previously with other CDK inhibitors (52, 53, 34; Oumata et al., in preparation). Each compound was immobilized on agarose beads through a linker. The position of the linker on the inhibitor was selected on the basis of the inhibitors' interactions and orientation in the CDK2 (34, 20, 54) and CDK9 (22) ATP-binding sites, as revealed by kinase/inhibitor co-crystal structures. Extracts of human neuroblastoma SH-SY5Y and IMR-32 cells were run on the immobilized inhibitors, the beads were extensively washed and the bound proteins were resolved by SDS-PAGE followed by silver staining (Figures 2A, 2B). Individual protein bands were cut from the stained gels and proteins were identified following mass spectrometry of tryptic fragments (34). Results are provided in Figures 2A, 2B (kinases) and in Supplementary Table S2 (all protein targets). Although many targets were common for both drugs in these two cell lines (and in other tissues and cell types not reported here), CR8 appeared to bind more proteins than roscovitine.

Interactomics study of roscovitine and CR8. 3. Competition affinity chromatography

A third approach was used to analyze the kinase targets of roscovitine and CR8. This method is based on an affinity matrix comprising a set of well-characterized broad-spectrum kinase inhibitors (KinAffinity® developed by Evotec) to enrich the subproteome of endogenously expressed kinases of cells or tissues. Kinase inhibitors are screened against these matrix-bound proteins to reveal the inhibitor's quantitative cellular kinase profile.

First, a lysate of SILAC-labeled SH-SY5Y cells was applied to KinAffinity® beads adjusted to different concentrations. These quantitative binding experiments allow determining the concentration of the immobilized compounds required to obtain 50% binding of each target protein to the matrix (BC₅₀). Second, a lysate of SILAC-labeled SH-SY5Y cells was applied to 10-fold diluted KinAffinity® beads in the presence of increasing concentrations of free roscovitine or CR8. These quantitative competition experiments determine the free compound concentration required for 50% target protein to remain bound to the matrix (CC₅₀). The final K_{d,free} values for the free compound were calculated for each target

protein using the Cheng-Prusoff equation (55). A total of 184 distinct protein kinases were enriched from SH-SY5Y cells (Supplementary Table S3A). Targets were ranked according to their affinities with K_d values ranging from 0.081 up to 30 μ M (Table 3; Figures 2C, 2D; Supplementary Tables S3B and S3C). A total of 18 and 20 target kinase proteins (i.e. K_d values <30 μ M) were identified in SH-SY5Y cells for roscovitine and CR8, respectively (Table 3). Of these, 4 and 6 were protein targets specific to roscovitine and CR8, respectively, and 14 were shared by both drugs (Supplementary Tables S3B and S3C).

Whereas roscovitine displayed the highest affinities (five best K_d values) for PAK4, CDK7, CDK12 (CRK7), CDK9 and CK1 δ , CR8 displayed the highest affinity for CDK12, CDK9, MSK2, CK1 δ and CDK10. Furthermore, several other protein kinases involved in regulation of the cell cycle, cell proliferation, alternative splicing and neuronal functions (CK1 ϵ , CLK2, DYRK1A or CDK5) were also identified as roscovitine and CR8 targets. Other proteins detected on the matrix, such as cyclin T1, T2, K, H and B2, are likely to bind indirectly, through their CDK7, CDK9, CDK1 partners. This is illustrated by the very similar K_d values of partner proteins (CDK7/cyclin H/MAT1 or CDK9/cyclin T1). Finally, several phosphatidylinositol-5-phosphate 4 kinases were identified as CR8 targets. The previously identified roscovitine target pyridoxal kinase (PDXK) (35) is expressed in SH-SY5Y cells and bound to the KinAffinity® matrix, yet it was not competed by roscovitine or CR8. This might be explained by the characteristic composition of the KinAffinity® matrix, which exclusively contains broad spectrum kinase inhibitors that bind their target kinases via the ATP-binding site. Since roscovitine is not directly binding at the PDXK ATP site, but at the pyridoxal site, displacement of PDXK from a matrix comprising ATP-competitive compounds might not occur. Alternatively, PDXK may bind very poorly to the KinAffinity® matrix.

Transcriptomics study of roscovitine and CR8

As a global approach to comparatively investigate the effects of roscovitine and CR8, we next performed a parallel transcriptomics study for both compounds. SH-SY5Y cells were exposed for 4 hrs to 50 μ M roscovitine, 5 μ M CR8 or the corresponding level of DMSO vehicle. mRNAs were extracted, purified, reverse-transcribed and the products hybridized on 44K Human Whole-Genome Agilent Technologies chips. The transcriptome was analyzed using the GeneSpring and DAVID software. Results show that, after 4 hrs, roscovitine and CR8 altered (>2 fold) the expression of 1,523 and 1,138 genes, respectively (Figure 3A). Most altered genes were down-regulated (1,321 and 1,037 for roscovitine and CR8, respectively) (Figure 3B), although a few were up-regulated (202 and 101, respectively) (Figure 3C). All down-regulated and up-regulated genes are listed in Supplementary Tables S4 and S5. An EASE analysis of these down-regulated and up-regulated genes is shown in Supplementary Tables S6 and S7, respectively. Table S8 shows down-regulated genes which are direct roscovitine/CR8 targets, cell cycle, apoptosis or NF κ B regulators. Genes showing a >10 fold down-regulation, following exposure to roscovitine or CR8 are listed in Table 4. To confirm the validity of this study, we verified the effects of the two drugs on the transcription of a selection of genes using straight PCR (Figure 3D) or quantitative PCR (Figure 3E): CDK7, GSG2 (haspin), c-MYC, p27^{Kip1}, ING1, SIAH1, NEDD9, and actin or GAPDH as controls. Starting from the same number of

cells, and measuring expression after a short exposure time, probably allows the use of housekeeping genes to normalize the data; this might not have been the case for longer exposure times and in situations where total expression of a large number of mRNAs is altered, and when higher levels of mRNAs might be present as seen in MYC overexpressing cells (56). In our case SH-SY5Y do not show overexpression of MYCN and thus mRNA quantification may reflect the reality. This might not have been the case with IMR32 cells which overexpress MYCN. Results confirmed those obtained in the Agilent global approach, notably the massive down-regulation of c-MYC expression. The potential importance of the effects of the two compounds on Myc expression was highlighted by global analysis of the expression data which showed a convergence on Myc (Supplementary Figure S4).

Proteomics study of roscovitine and CR8

We next used a global quantitative proteomics approach to investigate the effects of roscovitine and CR8. SH-SY5Y cells were exposed for 8 hrs to 50 μ M roscovitine, 5 μ M CR8, the corresponding level of DMSO vehicle, or left untreated. Cells were pelleted and stored frozen until further processing. Proteins were extracted and subjected to trypsin proteolysis. The resulting tryptic peptides were then labeled with iTRAQ, and all samples were pooled prior to LC-MS analysis. A total of 18,193 peptides corresponding to 1,320 proteins were identified and quantified (Figure 4A-C; Supplementary Tables S9, S10). Roscovitine triggered down-regulation of substantially more proteins than CR8 (226 versus 41). Expression of 25 proteins was altered by both compounds. Once again, global analysis of the protein expression data showed a convergence on MYC (Supplementary Figure S4).

We next analyzed the effects of the two drugs on the levels of a selection of proteins (encoded by genes which showed down-regulation in the transcriptomics study) by Western blotting with appropriate antibodies (Figure 4D, 4E). SH-SY5Y cells were exposed to various concentrations (Figure 4D) or different durations (Figure 4E) to roscovitine or CR8. Cells were extracted and proteins analyzed by SDS-PAGE and Western blotting. Results confirmed dose- and time-dependent down-regulation of proteins like c-MYC and p27^{KIP1}, while a few proteins, like GSG2 (haspin), which showed down-regulation at the mRNA level (Figure 4D, 4E), did not show any significant down-regulation at the protein level, illustrating the only partial coupling between mRNA and protein levels.

CDK inhibitors down-regulate MYCN expression

Given that the data strongly support the conclusion that MYC is down-stream target of CDK inhibition, and the importance of MYC in the development of poor prognosis neuroblastoma (46-48), we decided to focus our attention to the effects of CR8 on MYCN in neuroblastoma cells. MYCN is a rapid turnover protein as demonstrated by the use of MG132, a proteasome inhibitor, and cycloheximide (CHX), a global inhibitor of protein synthesis. Inhibition of proteasome leads to rapid accumulation of MYCN while protein synthesis inhibition leads to rapid MYCN down-regulation (Figure 5A). Exposure to CR8 leads to a rapid (less than 6 hrs) down-regulation of MYCN in IMR32 cells (Figure 5B). We next tested the effects of CR8 on the stability of MYCN in nine different neuroblastoma cell lines (Figure 5C), six of which showing massive amplification of MYCN and three without

detectable MYCN expression, although these three cell lines express c-myc. Exposure to CR8 resulted in a dose-dependent and essentially complete disappearance of MYCN in all six MYCN amplified cell lines. Among the three non-amplified MYCN cell lines, CR8 triggered c-Myc down-regulation only in SH-SY5Y cells. No difference in the time-course of MYCN down-regulation induced by CR8 was seen between asynchronous, G1 synchronized (serum starvation) and G2/M synchronized (nocodazole treatment) IMR32 cells, suggesting that the cell cycle stage is not a key factor in CR8's action (data not shown).

MYCN down-regulation is associated with inhibition of CDKs rather than of other targets

MYCN down-regulation was also induced by CDK inhibitors other than CR8 and roscovitine (Figure 6): MR4 (unpublished), N- and N1 (23, 24), purvalanol A (57), SCH727965 (58), AT7519 (59), SNS-032 (60) and flavopiridol (61), but not by N6-methyl-CR8 and N6-methylroscovitine (the kinase inactive derivatives of CR8 and roscovitine, respectively) (35). Besides CDKs, roscovitine and CR8 interact with a few other kinases as shown above and earlier (20, 34, 35). To examine whether interaction with any of these targets might account for the induction of MYCN down-regulation by CDK inhibitors, we made use of a series of selective pharmacological inhibitors of these other kinases (none of these inhibit CDKs) (Figure 6). IMR32 cells were exposed to various concentrations of pharmacological inhibitors of DYRK1A (Leucettine 41 (62, 63), harmine (63), CLKs (TG003) (64), CK1 (IC261 (65), DRF053 (25), D4476 (66)), and MEK1 (UO126, PD098059 (67)). In contrast to a general kinase inhibitor (staurosporine (68)), none of these inhibitors induced down-regulation of MYCN. Despite preferential interaction of roscovitine and CR8 with CK1, none of the reported CK1 inhibitors triggered MYCN down-regulation. These results suggest that CR8 and roscovitine, and other CDK inhibitors, trigger MYCN down-regulation through inhibition of CDKs rather than by interaction with secondary targets.

CDK8 reduces IMR32 tumor growth and down-regulates tumor MYCN in xenografts

We next tested the effects of CR8 on tumor growth and MYCN expression following IMR32 engraftment in immunocompromised mice (Figure 7). IMR32 xenografts were developed by subcutaneously injecting cells, either in suspension (Figure 7A) or in a Matrigel/cell slurry (Figure 7B), into the flank of NOD scid gamma (NSG) mice. Once tumor growth was established, mice were randomly distributed in two groups and treated with either vehicle (DMSO/PEG300/water, 5/50/45) or CR8 (2 mg/mL in vehicle). Vehicle and drug were administered daily by intraperitoneal injection. Tumor growth was monitored regularly and tumor volumes, based on caliper measurements, were calculated as described in ref. 69. Results clearly show that CR8 slows down tumor growth (Figures 7A, 7B). When the CR8-resistant tumors were left out, tumor growth was clearly arrested and in fact tumor regression was observed (Figure 7B). To confirm the effect of CR8 on MYCN expression, tumors were collected at the end of the xenograft experiments, extracts were prepared and equal protein content samples were loaded on SDS-PAGE gels, followed by Western blotting (Figure 7C). Anti-actin antibodies confirmed roughly equal protein loading of all 20 samples. Anti-MYCN antibodies confirmed that all control tumors expressed high levels of MYCN. In contrast, most but not all CR8 treated tumors showed complete disappearance or

at least massive down-regulation of MYCN expression (Figure 7C). Mcl-1 and CK1 ϵ were also down-regulated as a result of exposure of mice to CR8 (data not shown), as expected from the effects of CDK inhibitors on Mcl-1 expression (22) and the correlation between MYCN overexpression and CK1 ϵ expression (49).

DISCUSSION

In vitro targets of roscovitine and CR8

In this study we have investigated in detail the selectivity of the kinase inhibitory purines roscovitine and CR8. Firstly, catalytic assays and interaction assays performed with recombinant protein kinases confirm and extend previously known data for roscovitine (6, 9, 20, 34, 50, 51) and CR8 (20). Both compounds show affinity for sub-classes of CDKs, namely CDK1, 2, 3, 5, 7, 9, but also DYRKs (1A, 1B), CLKs (1, 2, 4) and CK1s (ϵ , γ). Strikingly, CK1 ϵ appears to be one of the best targets of the two compounds.

Native targets of roscovitine and CR8

There are limitations associated with the use of recombinant kinases in selectivity assays: lack of natural post-translational modifications, lack of interacting partners, abnormal folding of an unknown proportion of the produced kinases, use of excess amounts of kinases, lack of competition between different targets, limitation to the cloned kinases, etc... Therefore, we investigated the native targets of roscovitine and CR8 in neuroblastoma cell lines using two affinity chromatography approaches (Figure 2, Table 3, Supplementary Tables S2 and S3). These approaches provide a global view of potential targets (kinases/non-kinases) of roscovitine and CR8 from a given cell line/ tissue. Results confirm previously identified targets such as the above mentioned CDKs, CK1s (CK1 ϵ in particular), CLKs, DYRKs, pyridoxal kinase. They also point towards new potential targets such as PAK4, CDK10, CDK12 and associated cyclin K (70-74), FADK1 and phosphatidylinositol-5-phosphate 4-kinases (CR8). Inhibition of the catalytic activity of these kinases has to be confirmed in vitro. Using the same competition affinity method with mouse brain or K562 leukemia cells extracts, we also identified PAK4, CDK7, CDK9, CDK12, DYRK1A, CK1 δ , CK1 ϵ and phosphatidylinositol-5-phosphate 4-kinases (CR8) as targets (unpublished). A number of non-kinase proteins were found to bind to roscovitine- and CR8-beads (non-specific binders, direct or indirect interactors or kinase substrates?).

Down-stream targets of roscovitine and CR8

Investigating native targets from specific cell lines or tissues has limitations too: dependence on target expression which varies in different cell lines or tissues, prevalence of the most abundantly expressed proteins, preponderance of soluble targets. It is likely that cellular responses to roscovitine and CR8 are the end result of a complex pattern of interactions with the targets identified in vitro. In the context of anti-cancer activity towards c-MYC/MYCN - driven tumors, we believe that the main action of roscovitine and CR8 can be explained by a combined action on two sets of targets: the transcription regulating CDKs (CDK7/cyclin H/ MAT1, CDK9/cyclin T and CDK12/cyclin K) and the closely related CK1 δ and CK1 ϵ .

CDK7, CDK9 and CDK12 regulate RNA polymerase II phosphorylation and activity (70-77). We therefore focused our work towards analysis of down-stream targets, namely transcription and protein expression products. Global transcriptomics and proteomics approaches showed that the two kinase inhibitors inhibit transcription and protein expression, especially that of short-lived mRNAs and proteins, respectively, extending earlier results obtained with other cell lines (74-76). One example is the cell survival factor Mcl-1, which is rapidly down-regulated (22). Another key example investigated here are members of the MYC transcription factors, and in particular MYCN. MYCN is frequently amplified in neuroblastoma (46-48) and this event, in association with ALK mutations (81-85), is associated with poor prognosis. MYCN has a short-lived RNA and is a short-lived protein (Figure 5). We here show that roscovitine and CR8 induce rapid and essentially complete down-regulation of MYCN expression (Figure 5-7). We believe that roscovitine and CR8, by inhibiting, even transiently, CDK7/cyclin H, CDK9/cyclin T and CDK12/cyclin K, reduce RNA polymerase II activity, leading to an arrest of MYCN synthesis under continued degradation conditions. This results in rapid clearance of the MYCN protein and down-regulation of MYCN downstream targets. This fast and complete MYCN down-regulation has been shown at the protein level in various neuroblastoma cell lines (Figure 5, 6) as well as in tumors grown in animal models (Figure 7). CDK2 inhibition has also been reported as synthetically lethal to MYCN (37-39, 41, 43-45). One possibility is that CDK2 acts by phosphorylating and activating CDK9 (86). Another recently described mechanism is the CDK-mediated phosphorylation and activation of the SCF^{FBXO28} ubiquitin ligase which promotes MYC-driven transcription and tumour development (45). Besides the CDK inhibitors described here, two other types of drugs have been described to down-regulate MYC /MYCN: the Aurora kinase inhibitor CCT137690 (87) - Aurora has been reported to stabilize MYCN (40) - and BET bromodomain inhibitors (88, 89). These pharmacological treatments might be additive with CDK inhibitors.

Expression of CK1 ϵ correlates with MYCN amplification as well as c-MYC expression in neuroblastoma (49). CK1 ϵ inhibition is synthetically lethal with c-MYC and MYCN (49). Pharmacological inhibitor (IC261) and siRNAs of CK1 ϵ prevent growth of MYCN-amplified xenografts (49). CK1 inhibitors (IC261, DRF053 and D4476) clearly do not down-regulate MYCN expression (Figure 6). However CR8 was found to down-regulate the expression of CK1 ϵ (data not shown), suggesting that this effect could be down-stream of MYCN down-regulation. The fact that one of the main targets of roscovitine and CR8 is CK1 ϵ (and the related CK1 δ) suggests that this mechanism of action probably combines with CDK inhibition to generate anti-tumor activity of roscovitine and CR8 in MYCN-amplified tumors.

Altogether these results suggest that roscovitine and CR8, and probably other related purine inhibitors, display anticancer activity thanks to a combination of two different and additive effects: inhibition of CDK2, CDK7, CDK9, CDK12 and inhibition of CK1 δ/ϵ . CDK inhibition but not CK1 inhibition leads to the down-regulation of MYC and Mcl-1. The molecular events downstream to CK1 inhibition remain to be identified. MYCN overexpressing tumors are particularly sensitive to these CDK/CK1 inhibitors that might be favorably combined with ALK inhibitors for the treatment of poor prognosis neuroblastoma. These results might be expanded to the most common malignant pediatric brain tumor,

medulloblastoma, and other tumors where expression of MYC is also indicative of very poor prognosis.

MATERIAL AND METHODS

DRUGS - REAGENTS – ANTIBODIES – BUFFERS – CELL CULTURE

Synthesis of roscovitine and CR8 without and with linker—Roscovitine and CR8 were synthesized as previously described (21, 50). Compounds were stored as a dry powder, but diluted as 10 mM stock solution in dimethylsulfoxide (DMSO). Roscovitine beads were obtained as previously described (34). CR8 + linker was synthesized as described (Oumata et al., in preparation) and stored at -20°C at 100 mM in DMSO.

Reagents—The protease inhibitor cocktail was from Roche (Penzberg, Germany). Unless otherwise stated, the non-listed reagents were from Sigma (St Quentin Fallavier, France).

Antibodies—Monoclonal antibody against c-Myc was from Santa Cruz Biotechnology (CA, USA). Polyclonal antibodies against SIAH1, Haspin and DUSP1 were from Abcam (Cambridge, UK). Monoclonal antibodies against actin were from Merck (Germany). Polyclonal antibody against p27^{Kip1} was from Santa Cruz Biotechnology (CA, USA). Monoclonal antibody against MYCN was from Calbiochem (EMD Millipore, MA, USA).

Homogenization buffer—60 mM β -glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM MgCl₂, 2 mM dithiothreitol, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM phenylphosphate, 0.1% Nonidet P-40 and protease inhibitor cocktail.

Bead Buffer—50 mM Tris A (pH 7.4), 5 mM sodium fluoride, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40 and protease inhibitor cocktail.

Cell culture conditions—IMR32, LAN5 and LAN1 human cells (provided by Dr. J. Bénard, Villejuif) were grown in RPMI 1640 medium from Invitrogen (Cergy Pontoise, France) and SH-SY5Y human cells and the other neuroblastoma cell lines (respectively provided by Dr J. Boix, Lleida and Dr. J. Bénard, Villejuif) were grown in DMEM medium (Invitrogen). Medium were supplemented with 2 mM L-glutamine (Lonza, Verviers, Belgium), with antibiotics (penicillin-streptomycin) (Lonza) and 10% volume of fetal calf serum (FCS) (Invitrogen). Cells were cultured at 37°C with 5% CO₂. Drug treatments were performed on exponentially growing cultures at the indicated concentrations and during indicated times. Control experiments were carried using appropriate dilutions of DMSO.

INTERACTOMICS – AFFINITY CHROMATOGRAPHY ASSAYS

Kinase interaction panel (DiscovRx KinomeScan)—Assays were performed essentially as reported previously (6, 7) and are described in detail in the Supplementary Material section.

Affinity chromatography on immobilized roscovitine and CR8—SH-SY5Y and IMR32 cells were cultured as described, extracted with Bead Buffer and loaded on CR8 and

roscovitine sepharose beads as previously described (34) and described in more details in the Supplementary Material section.

Affinity competition assay (Evotec) - KinAffinity[®] kinase target profiling—For SILAC, SH-SY5Y neuroblastoma cells were cultivated as described previously (90, 91). *In vitro* association experiments were performed essentially as described in (91, 92). KinAffinity[®] beads (Evotec) representing a set of different broad-spectrum kinase inhibitors immobilized on Sepharose beads were applied for affinity chromatography. For competition experiments, SILAC-encoded cell extracts were added to 10-fold diluted KinAffinity[®] beads and treated simultaneously with different concentrations of roscovitine or CR8. Identification and quantification are described in full in the Supplementary Material section.

TRANSCRIPTOMICS & PROTEOMICS

Transcriptomics and Proteomics experiments were performed with SH-SY5Y cells using standard protocols described in details in the Supplementary Material section.

ELECTROPHORESIS - WESTERN BLOTTING

Following heat denaturation for 3 min, proteins were separated on a mini gel electrophoresis system (Invitrogen) using NuPage 10% Bis-Tris, 10 or 12 wells polyacrylamide gels. Electrophoresis and transfer were performed in XCell SureLock Mini-Cell system and XCell II Blot module from Invitrogen. The 0.45 µm nitrocellulose membrane was from Fisher Bioblock. These were blocked for 1 h with 5% low fat milk in Tris-Buffered Saline - Tween-20, incubated overnight at 4°C (anti-actin (1:2000), c-Myc (1:1000), MYCN (1:50), SIAH1 (1:1000), Haspin (1:500), p27^{Kip1} (1:500)) and analyzed by Enhanced Chemiluminescence. The silver staining kit was purchased from GE Healthcare.

IN VIVO EXPERIMENTS

Cell lines, antibodies and reagents—IMR32 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 0.1% gentamicin. Goat polyclonal anti-actin was obtained from Santa Cruz (sc-1615) and mouse monoclonal anti-MYCN from Calbiochem (OP13). CR8 was reconstituted in DMSO at a concentration of 40 mg/mL.

Xenografts—IMR32 cells were suspended in Matrigel (BD worldwide, #354234) at a concentration of 100,000 cells/µL and kept on ice. NOD scid gamma (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were injected subcutaneously with 100 µL of chilled Matrigel/cell slurry directly into the flank and allowed to establish for two weeks prior to drug delivery. Mice were administered, by intraperitoneal injection, 100 µL of either vehicle (DMSO/PEG300/ddH₂O, 5/50/45) or CR8 (2 mg/mL), daily for up to 3 weeks. Tumor growth during the treatment was measured using digital calipers at indicated times using the formula: tumor volume = (length x width²) / 2 (69). Mice were euthanized and tumors harvested either 1 day or 3 weeks post-treatment and frozen immediately on dry ice.

Immunoblotting—Tumor samples were minced using a clean razor blade and suspended in ACK buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 1 min on ice to lyse red blood cells. Tumors were subsequently washed with PBS and suspended in RIPA buffer

(150 mM NaCl, 50 mM Tris pH 8.0, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) plus protease inhibitor (Roche #14015000). Tumor samples were maintained in RIPA buffer for 5 min on ice and sonicated until tumor dissolved. Protein concentration was determined using a protein assay dye reagent (Bio-Rad #500-0006). Proteins (30 µg) were separated using SDS-PAGE in 10% polyacrylamide gels and transferred to PVDF membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSA	bovine serum albumin
CDC2	cell division cycle 2
CDK	cyclin-dependent kinase
CHX	cycloheximide
CK1	casein kinase 1
CLK	cdc2-like kinase
CRKRS	cdc2-related protein kinase 7 (CDK12)
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
DYRK	dual specificity, tyrosine phosphorylation regulated kinase
FADK	focal adhesion kinase
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK-3	glycogen synthase kinase-3
PBS	phosphate-buffered saline
PDXK	pyridoxal kinase
RT	room temperature

REFERENCES

- Weinmann H, Metternich R. Drug discovery process for kinase inhibitors. *ChemBioChem*. 2005; 6:455–459. [PubMed: 15742380]
- Eglen RM, Reisine T. The current status of drug discovery against the human kinome. *Assay Drug Dev Technol*. 2009; 7:22–43. [PubMed: 19382888]
- Eglen R, Reisine T. Drug discovery and the human kinome: recent trends. *Pharmacol Ther*. 2011; 130:144–156. [PubMed: 21256157]
- Via, MC. Kinase-Targeted Therapeutics: Development Pipelines, Challenges, and Opportunities. Cambridge Healthtech Institute; 2011. p. 124
- Petrelli A, Giordano S. From single- to multi-target drugs in cancer therapy: when aspecificity becomes an advantage. *Curr Med Chem*. 2008; 15:422–432. [PubMed: 18288997]
- Fabian MA, Biggs WH, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, et al. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol*. 2005; 23:329–336. [PubMed: 15711537]
- Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, et al. A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol*. 2008; 26:127–132. [PubMed: 18183025]
- Goldstein DM, Gray NS, Zarrinkar PP. High-throughput kinase profiling as a platform for drug discovery. *Nat Rev Drug Discov*. 2008; 7:391–7. [PubMed: 18404149]
- Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. *Biochem J*. 2003; 371:199–204. [PubMed: 12534346]
- Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, et al. The selectivity of protein kinase inhibitors: a further update. *Biochem J*. 2007; 408:297–315. [PubMed: 17850214]
- Malumbres M, Harlow E, Hunt T, Hunter T, Lahti JM, Manning G, et al. Cyclin-dependent kinases: a family portrait. *Nat Cell Biol*. 2009; 11:1275–1276. [PubMed: 19884882]
- Knockaert M, Greengard P, Meijer L. Pharmacological inhibitors of cyclin-dependent kinases. *Trends Pharmacol Sci*. 2002; 23:417–425. [PubMed: 12237154]
- Malumbres M, Pevarello P, Barbacid M, Bischoff JR. CDK inhibitors in cancer therapy: what is next? *Trends Pharmacol Sci*. 2008; 29:16–21. [PubMed: 18054800]
- Galons H, Oumata N, Gloulou O, Meijer L. Cyclin-dependent kinase inhibitors closer to market launch? *Expert Opin. Ther. Pat*. 2013 published on-line.
- Johnson N, Shapiro GI. Cyclin-dependent kinases (cdks) and the DNA damage response: rationale for cdk inhibitor-chemotherapy combinations as an anticancer strategy for solid tumors. *Expert Opin Ther Targets*. 2010; 14:1199–1212. [PubMed: 20932174]
- Cirillo D, Pentimalli F, Giordano A. Peptides or Small Molecules? Different Approaches to Develop More Effective CDK Inhibitors. *Curr Med Chem*. 2011; 18:2854–2866. [PubMed: 21651493]
- W sierska-G dek J, Kramer MP. The impact of multi-targeted cyclin-dependent kinase inhibition in breast cancer cells: clinical implications. *Expert Opin Investig Drugs*. 2011; 20:1611–1628.
- Cicenas J, Valius M. The CDK inhibitors in cancer research and therapy. *J Cancer Res Clin Oncol*. 2011; 137:1409–1418. [PubMed: 21877198]
- Meijer L, Raymond E. Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. *Acc Chem Res*. 2003; 36:417–425. [PubMed: 12809528]
- Bettayeb K, Oumata N, Echalié A, Ferandin Y, Endicott JA, Galons H, Meijer L. CR8, a potent and selective, roscovitine-derived inhibitor of cyclin-dependent kinases. *Oncogene*. 2008; 27:5797–5807. [PubMed: 18574471]
- Oumata N, Ferandin Y, Meijer L, Galons H. Practical synthesis of roscovitine and CR8. *Organic Process Res Dev*. 2009; 13:641–644.
- Bettayeb K, Baunbæk D, Delehouzé C, Loaëc N, Hole A, Baumli S, et al. CDK inhibitors roscovitine and CR8 trigger Mcl-1 down-regulation and apoptotic cell death in neuroblastoma cell lines. *Genes & Cancer*. 2010; 1:369–380. [PubMed: 21779453]

23. Bettayeb K, Sallam HH, Ferandin Y, Popowycz F, Fournet G, Hassan M, et al. N-&-N, a new class of cell death-inducing, kinase inhibitors derived from the purine roscovitine. *Mol Cancer Ther.* 2008; 7:2713–2724. [PubMed: 18790752]
24. Popowycz F, Fournet G, Schneider C, Bettayeb K, Ferandin Y, Lamigeon C, et al. Pyrazolo[1,5-a]-1,3,5-triazine as a purine bioisostere: access to potent CDK inhibitor (R)-roscovitine analogue. *J Med Chem.* 2009; 52:655–663. [PubMed: 19128055]
25. Oumata N, Bettayeb K, Ferandin Y, Demange L, Lopez-Giral A, Goddard M-L, et al. Roscovitine-derived, dual-specificity inhibitors of cyclin-dependent kinases (CDKs) and casein kinase 1 (CK1). *J Med Chem.* 2008; 51:5229–5242. [PubMed: 18698753]
26. Trova MP, Barnes KD, Barford C, Benanti T, Bielaska M, Burry L, et al. Biaryl purine derivatives as potent antiproliferative agents: inhibitors of cyclin dependent kinases. Part I. *Bioorg Med Chem Lett.* 2009; 19:6608–6612. [PubMed: 19846305]
27. Trova MP, Barnes KD, Alicea L, Benanti T, Bielaska M, Bilotta J, et al. Heterobiaryl purine derivatives as potent antiproliferative agents: inhibitors of cyclin dependent kinases. Part II. *Bioorg Med Chem Lett.* 2009; 19:6613–6617. [PubMed: 19854650]
28. Jorda R, Paruch K, Krystof V. Cyclin-dependent kinase inhibitors inspired by roscovitine: purine bioisosteres. *Curr Pharm Des.* 2012; 18:2974–80. [PubMed: 22571665]
29. Meijer L, Borgne A, Mulner O, Chong JPJ, Blow JJ, Inagaki N, et al. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur J Biochem.* 1997; 243:527–536. [PubMed: 9030781]
30. Aldoss IT, Tashi T, Ganti AK. Seliciclib in malignancies. *Expert Opin Investig Drugs.* 2009; 18:1957–1965.
31. Benson C, White J, De Bono J, O'Donnell A, Raynaud F, Cruickshank C, et al. A phase I trial of the selective oral cyclin-dependent kinase inhibitor seliciclib (CYC202; R-Roscovitine), administered twice daily for 7 days every 21 days. *Br J Cancer.* 2007; 96:29–37. [PubMed: 17179992]
32. Hsieh WS, Soo R, Peh BK, Loh T, Dong D, Soh D, et al. Pharmacodynamic effects of seliciclib an orally administered cell cycle modulator in undifferentiated nasopharyngeal cancer. *Clin Cancer Res.* 2009; 15:1435–1442. [PubMed: 19228744]
33. Hui AB, Yue S, Shi W, Alajez NM, Ito E, Green SR, et al. Therapeutic efficacy of seliciclib in combination with ionizing radiation for human nasopharyngeal carcinoma. *Clin Cancer Res.* 2009; 15:3716–3724. [PubMed: 19470731]
34. Bach S, Knockaert M, Reinhardt J, Lozach O, Schmitt S, Baratte B, et al. Roscovitine targets, protein kinases and pyridoxal kinase. *J Biol Chem.* 2005; 280:31208–31219. [PubMed: 15975926]
35. Tang L, Li MH, Cao P, Wang F, Chang WR, Bach S, et al. Crystal structure of pyridoxal kinase in complex with roscovitine and derivatives. *J Biol Chem.* 2005; 280:31220–31229. [PubMed: 15985434]
36. Sjostrom SK, Finn G, Hahn WC, Rowitch DH, Kenney AM. The Cdk1 complex plays a prime role in regulating N-myc phosphorylation and turnover in neural precursors. *Dev Cell.* 2005; 9:327–338. [PubMed: 16139224]
37. Goga A, Yang D, Tward AD, Morgan DO, Bishop JM. Inhibition of CDK1 as a potential therapy for tumors over-expressing MYC. *Nat Med.* 2007; 13:820–827. [PubMed: 17589519]
38. Hydbring P, Bahram F, Su Y, Tronnersjö S, Högstrand K, von der Lehr N, et al. Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation. *Proc Natl Acad Sci USA.* 2009; 107:58–63. [PubMed: 19966300]
39. Molenaar JJ, Ebus ME, Geerts D, Koster J, Lamers F, Valentijn LJ, et al. Inactivation of CDK2 is synthetically lethal to MYCN over-expressing cancer cells. *Proc Natl Acad Sci USA.* 2009; 106:12968–12973. [PubMed: 19525400]
40. Otto T, Horn S, Brockmann M, Eilers U, Schüttrumpf L, Popov N, et al. Stabilization of NMyC is a critical function of Aurora A in human neuroblastoma. *Cancer Cell.* 2009; 15:67–78. [PubMed: 19111882]
41. Campaner S, Doni M, Hydbring P, Verrecchia A, Bianchi L, Sardella D, et al. Cdk2 suppresses cellular senescence induced by the c-myc oncogene. *Nat Cell Biol.* 2010; 12:54–59. [PubMed: 20010815]

42. Wang X, Cunningham M, Zhang X, Tokarz S, Laraway B, Troxell M, et al. Phosphorylation regulates c-Myc's oncogenic activity in the mammary gland. *Cancer Res.* 2011; 71:925–36. [PubMed: 21266350]
43. Horiuchi D, Kusdra L, Huskey NE, Chandriani S, Lenburg ME, Gonzalez-Angulo AM, et al. MYC pathway activation in triple-negative breast cancer is synthetic lethal with CDK inhibition. *J Exp Med.* 2012; 209:679–96. [PubMed: 22430491]
44. Chen Y, Tsai YH, Tseng SH. Inhibition of cyclin-dependent kinase 1-induced cell death in neuroblastoma cells through the microRNA-34a-MYC-survivin pathway. *Surgery.* 2013; 153:4–16. [PubMed: 22703967]
45. Cepeda D, Ng HF, Sharifi HR, Mahmoudi S, Cerrato VS, Fredlund E, et al. CDK-mediated activation of the SCF^{FBXO28} ubiquitin ligase promotes MYC-driven transcription and tumorigenesis and predicts poor survival in breast cancer. *EMBO Mol Med.* 2013 online.
46. Grotzer MA, Castelletti D, Fiaschetti G, Shalaby T, Arcaro A. Targeting Myc in pediatric malignancies of the central and peripheral nervous system. *Curr Cancer Drug Targets.* 2009; 9:176–88. [PubMed: 19275758]
47. Gustafson WC, Weiss WA. Myc proteins as therapeutic targets. *Oncogene.* 2010; 29:1249–1255. [PubMed: 20101214]
48. Albiñ A, Johnsen JI, Henriksson MA. MYC in oncogenesis and as a target for cancer therapies. *Adv Cancer Res.* 2010; 107:163–224. [PubMed: 20399964]
49. Toyoshima M, Howie HL, Imakura M, Walsh RM, Annis JE, Chang AN, et al. Functional genomics identifies therapeutic targets for MYC-driven cancer. *Proc Natl Acad Sci USA.* 2012; 109:9545–950. [PubMed: 22623531]
50. Meijer, L.; Bettayeb, K.; Galons, H. Roscovitine (CYC202, Seliciclib). In *Monographs on enzyme inhibitors.* In: Smith, PJ.; Yue, E., editors. *CDK inhibitors and their potential as anti-tumor agents.* Vol. 2. CRC Press; Taylor & Francis: Boca Raton, FL.: 2006. p. 187-226.chapter 9
51. Becker F, Murthi K, Smith C, Come J, Costa-Roldan N, Kaufmann C, et al. A three-hybrid approach to scanning the proteome for targets of small molecule kinase inhibitors. *Chem Biol.* 2004; 11:211–223. [PubMed: 15123283]
52. Knockaert M, Gray N, Damiens E, Chang YT, Grellier P, Grant K, et al. Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immobilised inhibitors. *Chem & Biol.* 2000; 7:411–422. [PubMed: 10873834]
53. Knockaert M, Viking K, Schmitt S, Leost M, Mottram J, Kunick C, et al. Intracellular targets of paullones: identification by affinity chromatography using immobilized inhibitor. *J Biol Chem.* 2002; 277:25493–25501. [PubMed: 11964410]
54. Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. *Eur J Biochem.* 1997; 243:518–526. [PubMed: 9030780]
55. Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol.* 1973; 22:3099–3108. [PubMed: 4202581]
56. Lovén J, Orlando DA, Sigova AA, Lin CY, Rahl PB, Burge CB, Levens DL, Lee TI, Young RA. Revisiting global gene expression analysis. *Cell.* Oct 26. 2012; 151(3):476–82. [PubMed: 23101621]
57. Gray N, Wodicka L, Thunnissen AM, Norman T, Kwon S, Espinoza FH, et al. Exploiting chemical libraries, structure, and genomics in the search for new kinase inhibitors. *Science.* 1998; 281:533–538. [PubMed: 9677190]
58. Parry D, Guzi T, Shanahan F, Davis N, Prabhavalkar D, Wiswell D, Seghezzi W, et al. Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. *Mol Cancer Ther.* 2010; 9:2344–2353. [PubMed: 20663931]
59. Squires MS, Cooke L, Lock V, Qi W, Lewis EJ, Thompson NT, et al. AT7519, a cyclin-dependent kinase inhibitor, exerts its effects by transcriptional inhibition in leukemia cell lines and patient samples. *Mol Cancer Ther.* 2010; 9:920–928. [PubMed: 20354122]
60. Misra RN, Xiao HY, Kim KS, Lu S, Han WC, Barbosa SA, et al. N-(cycloalkylamino)acyl-2-aminothiazole inhibitors of cyclin-dependent kinase 2. N-[5-[[[5-(1,1-dimethylethyl)-2-

- oxazolyl]methyl]thio]-2-thiazolyl]-4-piperidinecarboxamide (BMS-387032), a highly efficacious and selective antitumor agent. *J Med Chem.* 2004; 47:1719–1728. [PubMed: 15027863]
61. Wang LM, Ren DM. Flavopiridol, the first cyclin-dependent kinase inhibitor: recent advances in combination chemotherapy. *Mini Rev Med Chem.* 2010; 10:1058–1070. [PubMed: 21047304]
 62. Debda M, Carreaux F, Renault S, Soundararajan M, Fedorov O, Filippakopoulos P, et al. Leucettines, a class of potent inhibitors of cdc2-like kinases and dual specificity, tyrosine phosphorylation regulated kinases derived from the marine sponge leucettamine B. Modulation of alternative pre-RNA splicing. *J Med Chem.* 2011; 54:4172–4186. [PubMed: 21615147]
 63. Tahtouh T, Elkins JM, Filippakopoulos P, Soundararajan M, Burgy G, Durieu E, et al. Selectivity, cocrystal structures and neuroprotective properties of leucettines, a family of protein kinase inhibitors derived from the marine sponge alkaloid leucettamine B. *J Med Chem.* 2012; 55:9312–9330. [PubMed: 22998443]
 64. Muraki M, Ohkawara B, Hosoya T, Onogi H, Koizumi J, Koizumi T, et al. Manipulation of alternative splicing by a newly developed inhibitor of Clks. *J Biol Chem.* 2004; 279:24246–24254. [PubMed: 15010457]
 65. Behrend L, Milne DM, Stoter M, Deppert W, Campbell LE, Meek DW, et al. IC261 a specific inhibitor of the protein kinases casein kinase 1-delta and -epsilon triggers the mitotic checkpoint and induces p53-dependent postmitotic effects. *Oncogene.* 2000; 19:5303–5313. [PubMed: 11103931]
 66. Rena G, Bain J, Elliott M, Cohen P. D4476 a cell-permeant inhibitor of CK1 suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. *EMBO Rep.* 2004; 5:60–65. [PubMed: 14710188]
 67. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J.* 2000; 351:95–105. [PubMed: 10998351]
 68. Tanramluk D, Schreyer A, Pitt WR, Blundell TL. On the origins of enzyme inhibitor selectivity and promiscuity: a case study of protein kinase binding to staurosporine. *Chem Biol Drug Des.* 2009; 74:16–24. [PubMed: 19519740]
 69. Grunwald V, DeGraffenried L, Russel D, Friedrichs WE, Ray RB, Hidalgo M. Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells. *Cancer Res.* 2002; 62:6141–6145. [PubMed: 12414639]
 70. Cheng SW, Kuzyk MA, Moradian A, Ichu TA, Chang VC, Tien JF, et al. Interaction of cyclin-dependent kinase 12/CrkRS with cyclin K1 is required for the phosphorylation of the C-terminal domain of RNA polymerase II. *Mol Cell Biol.* 2012; 32:4691–4704. [PubMed: 22988298]
 71. Dai Q, Lei T, Zhao C, Zhong J, Tang YZ, Chen B, et al. Cyclin K-containing kinase complexes maintain self-renewal in murine embryonic stem cells. *J Biol Chem.* 2012; 287:25344–25352. [PubMed: 22547058]
 72. Blazek D. The cyclin K/Cdk12 complex: an emerging new player in the maintenance of genome stability. *Cell Cycle.* 2012; 11:1049–1050. [PubMed: 22391210]
 73. Kohoutek J, Blazek D. Cyclin K goes with Cdk12 and Cdk13. *Cell Div.* 2012; 7:12. [PubMed: 22512864]
 74. Blazek D, Kohoutek J, Bartholomeeusen K, Johansen E, Hulinkova P, Luo Z, et al. The Cyclin K/Cdk12 complex maintains genomic stability via regulation of expression of DNA damage response genes. *Genes Dev.* 2011; 25:2158–2172. [PubMed: 22012619]
 75. Krystof V, Baumli S, Fürst R. Perspective of cyclin-dependent kinase 9 (CDK9) as a drug target. *Curr Pharm Des.* 2012; 18:2883–2890. [PubMed: 22571657]
 76. Larochelle S, Amat R, Glover-Cutter K, Sansó M, Zhang C, Allen JJ, et al. Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II. *Nat Struct Mol Biol.* 2012; 19:1108–1115. [PubMed: 23064645]
 77. Baumli S, Hole AJ, Wang LZ, Noble ME, Endicott JA. The CDK9 tail determines the reaction pathway of positive transcription elongation factor b. *Structure.* 2012; 20:1788–1795. [PubMed: 22959624]
 78. Whittaker SR, Te Poele RH, Chan F, Linardopoulos S, Walton MI, Garrett MD, et al. The cyclin-dependent kinase inhibitor seliciclib (R-roscovitine; CYC202) decreases the expression of mitotic

- control genes and prevents entry into mitosis. *Cell Cycle*. 2007; 6:3114–3131. [PubMed: 18075315]
79. Garrofé-Ochoa X, Cosiàlls AM, Ribas J, Gil J, Boix J. Transcriptional modulation of apoptosis regulators by roscovitine and related compounds. *Apoptosis*. 2011; 16:660–670. [PubMed: 21505869]
 80. Lee JH, Min C, Kwak SJ, Yeon-Sun Seong YS. Genomewide transcription profiles altered by BMI-1026 and Roscovitine and its implication in cellular senescence. *Biochip J*. 2012; 6:362–371.
 81. Mossé YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, et al. Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature*. 2008; 455:930–935. [PubMed: 18724359]
 82. Janoueix-Lerosey I, Lequin D, Brugières L, Ribeiro A, de Pontual L, Combaret V, et al. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature*. 2008; 455:967–970. [PubMed: 18923523]
 83. Schulte JH, Lindner S, Bohrer A, Maurer J, De Preter K, Lefever S, et al. MYCN and ALKF1174L are sufficient to drive neuroblastoma development from neural crest progenitor cells. *Oncogene*. 2013; 32:1059–1065. [PubMed: 22484425]
 84. Zhu S, Lee JS, Guo F, Shin J, Perez-Atayde AR, Kutok JL, et al. Activated ALK collaborates with MYCN in neuroblastoma pathogenesis. *Cancer Cell*. 2012; 21:362–373. [PubMed: 22439933]
 85. Schönher C, Ruuth K, Kamaraj S, Wang CL, Yang HL, Combaret V, et al. Anaplastic Lymphoma Kinase (ALK) regulates initiation of transcription of MYCN in neuroblastoma cells. *Oncogene*. 2012; 31:5193–5200. [PubMed: 22286764]
 86. Breuer D, Kotelkin A, Ammosova T, Kumari N, Ivanov A, Ilatovskiy AV, et al. CDK2 regulates HIV-1 transcription by phosphorylation of CDK9 on serine 90. *Retrovirology*. 2012; 9:94. [PubMed: 23140174]
 87. Faisal A, Vaughan L, Bavetsias V, Sun C, Atrash B, Avery S, et al. The Aurora Kinase Inhibitor CCT137690 Downregulates MYCN and Sensitizes MYCN-Amplified Neuroblastoma In Vivo. *Mol Cancer Ther*. 2011; 10:2115–2123. [PubMed: 21885865]
 88. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, et al. BET Bromodomain Inhibition as a Therapeutic Strategy to Target c-Myc. *Cell*. 2011; 146:904–917. [PubMed: 21889194]
 89. Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA, et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc Natl Acad Sci USA*. 2011; 108:16669–16674. [PubMed: 21949397]
 90. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics*. 2002; 1:376–386. [PubMed: 12118079]
 91. Sharma K, Weber C, Baierlein M, Greff Z, Kéri G, Cox J, et al. Proteomics strategy for quantitative protein interaction profiling in cell extracts. *Nat Methods*. 2009; 6:741–744. [PubMed: 19749761]
 92. Conradt L, Godl K, Schaab C, Tebbe A, Eser S, Diersch S, et al. Disclosure of Erlotinib as a Multikinase Inhibitor in Pancreatic Ductal Adenocarcinoma. *Neoplasia*. 2011; 13:1026–1034. [PubMed: 22131878]

Figure 1. CR8 and IMR32 cell cycle A

IMR32 cells were kept untreated (Log phase), or exposed to 10 μ M nocodazole for 18 h or to reduced FBS (0.1%) level for 24 h. They were then exposed to 5 μ M for 24 h and their cell cycle phase distribution was analyzed by FACS. **(B)** Log Phase, G2/M or G1 synchronized IMR32 cells were exposed to various concentrations of CR8 for 24 h and their survival rate was estimated by an MTS assay.

Figure 2. Interactomics studies. A, B. Affinity chromatography purification of roscovitrine and CR8 targets from SH-SY5Y and IMR32 neuroblastoma cells

SH-SY5Y (A) and IMR32 (B) neuroblastoma cells were kept in Log phase. Extracts were prepared and loaded on immobilized roscovitrine and CR8. Beads were extensively washed and the bound proteins were resolved by SDS-PAGE followed by silver staining and identified by mass spectrometry analysis of tryptic fragments. Identified protein kinases are named in the figure. All analyzed proteins, 1-35 (roscovitrine) and 1-40 (CR8), are reported in Supplementary Table S2. C, D. Competition assay identification of roscovitrine and CR8 targets from SH-SY5Y neuroblastoma cells. Extracts of SH-SY5Y cells were loaded on affinity beads (KinAffinity®, Evotec) comprising a set of broad-spectrum kinase inhibitors designed to affinity purify endogenously expressed kinases of cells or tissues. Bound proteins were identified following exposure to increasing concentrations of roscovitrine (C) or CR8 (D). $K_{d,free}$ values were calculated and are plotted on a Log scale. Protein kinases (blue), other kinases (violet) and kinase-associated proteins (orange) are ranked from low (top) to high (bottom) $K_{d,free}$ values. Full results are reported in Supplementary Table S3.

A. altered gene probes

roscovitine -specific:

728 probes (2.31 %) (572 genes)

common to both:

1108 probes (3.52 %) (951 genes)

S-CR8 -specific:

263 probes (0.84 %) (187 genes)

B. down-regulated genes

roscovitine (1,321)

S-CR8 (1037)

C. up-regulated genes

roscovitine (202)

S-CR8 (101)

D

Figure 3. Transcriptomics studies. A-C. Overall effects of roscovitine and CR8 on gene expression in human neuroblastoma SH-SY5Y cells

Cells were exposed for 4 h to either 50 μ M roscovitine, 5 μ M CR8 or corresponding amounts of DMSO. mRNAs were extracted, purified, reverse-transcribed, amplified, labeled with cyanine-3 CTP dyes, followed by hybridization on Agilent 4x44 K human whole genome slides. Results were analyzed as described in the Material & Methods section. (A) Altered expression (>2 fold) versus non-altered expression (<2 fold) of gene probes: expression of 728 probes (corresponding to 572 genes) was changed specifically by roscovitine, expression of 263 probes (187 genes) was altered specifically by CR8 and expression of 1,108 probes (951 genes) was modified in common by both compounds. 93.33% of all probes displayed no change in expression level following treatment. Gene expression was either down-regulated (B) or up-regulated (C): numbers in parentheses indicate the total number of genes affected by roscovitine or CR8 treatment. The numbers of genes displaying modified expression only in roscovitine treated cells, only in CR8 treated cells or by both treatments are shown in blue, red and black, respectively. Percentages indicate the proportion of these genes relative to the total of genes down- or up-regulated by roscovitine or CR8 treatment. For example, roscovitine induces a >2 fold down-regulation of 1,321 genes, 429 of which (32.5%) are specific to roscovitine treatment, while 892 genes (67.5%) are also down-regulated by CR8 treatment. CR8 induces a >2 fold down-regulation of 1,037 genes, 145 of which (14%) are specific to CR8 treatment, while 892 genes (86%) are also down-regulated by roscovitine treatment. **D, E. Selected examples of down-**

regulated genes. Cells were exposed for 4 h to either 50 μ M roscovitine, 5 μ M CR8 or corresponding amount of DMSO (vehicle). **(D)** mRNAs were extracted, purified, reverse-transcribed. Fragments (base pairs number in parentheses) of CDK7, GSG2 (haspin), c-MYC, p27^{Kip1} were amplified by PCR (28 cycles) and the reaction products were resolved by electrophoresis on agarose. Expression of actin and GAPDH mRNA was used as a reference. Ctrl, no mRNA. **(E).** Quantitative PCR was also performed with p27^{Kip1}, CDK7, c-MYC, ING1, SIAH1, NEDD9. Expression is displayed relative to vehicle treated cells and normalized according to actin or GAPDH expression.

A. altered protein expression

roscovitine -specific: 197 (14.92 %)
 common to both: 29 (2.20 %)
 S-CR8 -specific: 12 (0.91 %)

B. down-regulated proteins

roscovitine (182)

S-CR8 (27)

C. up-regulated proteins

roscovitine (44)

S-CR8 (14)

Figure 4. Proteomics studies. A-C. Overall effects of roscovitine and CR8 on protein expression in human neuroblastoma SH-SY5Y cells

Cells were exposed for 4 h to either 50 μ M roscovitine, 5 μ M CR8 or corresponding amounts of DMSO. Proteins were extracted, trypsinized, iTRAQ labeled, and samples mixed prior to mass spectrometry analysis. A total of 18,193 peptides corresponding to 1,320 proteins were identified and quantified, of which 18.03% showed a >2 fold change in expression (**A**) by either one or both drugs. The level of these proteins was either down-regulated (**B**) (182 proteins by roscovitine and 27 by CR8, of which 17 were down-regulated by both) or up-regulated (**C**) (44 proteins by roscovitine and 14 by CR8, of which 8 were up-regulated by both). Numbers in parentheses represent the total number of proteins affected by roscovitine or CR8 treatment. The number of proteins displaying modified expression only in roscovitine treated cells, only in CR8 treated cells or by both treatments are shown in blue, red and black, respectively. Percentages indicate the proportion of these

proteins relative to the total of proteins down- or up-regulated by roscovitine or CR8 treatment. D, E. Selected examples of down-regulated proteins. Effects of dose (**D**) and time (**E**) of exposure to roscovitine or CR8 on the levels of GSG2 (haspin), c-MYC, p27^{Kip1} and actin. Cells were exposed (**D**) to various concentrations of roscovitine or CR8 for 24 h or (**E**) for various times to 50 μ M roscovitine, 5 μ M CR8 or corresponding amounts of DMSO. Proteins were extracted, resolved by SDS-PAGE and analyzed by Western blotting using appropriate antibodies.

Figure 5. MYCN, an unstable protein, is massively down-regulated following exposure to CR8
(A) Proteasome inhibition (upper panel) strongly stabilizes MYCN, while protein synthesis inhibition (lower panel) down-regulates MYCN protein level. IMR32 cells were exposed to MG132 (10 μM) or cycloheximide (CHX) (12 $\mu\text{g/mL}$) at time 0. Cells were sampled at various times after drug addition and proteins were resolved by SDS-PAGE followed by Western blotting with anti-MYCN antibodies. **(B)** CR8 triggers rapid down-regulation of MYCN in IMR32 cells. Cells were exposed for various times to 5 μM CR8 (C, control: corresponding volume of DMSO). Proteins were extracted, resolved by SDS-PAGE and analyzed by Western blotting using anti-MYCN antibodies. **(C)** CR8 triggers dose-dependent down-regulation of MYCN in MYCN overexpressing neuroblastoma cell lines. Nine different neuroblastoma cell lines were exposed to increasing concentrations of CR8 for 24 h. Proteins were resolved by SDS-PAGE followed by Western blotting with anti-c-

MYC (3 upper panels) or anti-MYC (6 lower panels) antibodies. The level of MYC amplification in the 9 cell lines is indicated on the right. No MYC was detected in the 3 non-amplified MYC cell lines.

Figure 6. Effect of different CDK and non-CDK inhibitors on MYCN levels

MYCN in IMR32 exposed for 10 hrs to 3 concentrations of CR8, roscovitine, MR4, N-&-N1, purvalanol A, SCH727965, AT7519, N6-methyl-CR8 and N6-methyl-roscovitine (kinase inactive derivatives of CR8 and roscovitine, respectively), SNS-032, flavopiridol, staurosporine (general kinase inhibitor), Leucettine L41 and harmine (DYRK1A inhibitors), TG003 (CLK1), IC261, DRF053 and D4476 (CK1), UO126 and PD098059 (MEK1). All drugs were tested at 5, 15 and 50 μM , except staurosporine (0.2, 0.6 and 2 μM). Cells were harvested and proteins were resolved by SDS-PAGE followed by Western blotting with anti-MYCN antibodies. Actin was used as a loading control.

Figure 7. CR8 down-regulates MYCN and reduces tumor growth of xenografted IMR32 cells in NOD scid gamma mice

(A, B), Time course of tumor growth following subcutaneous injection of IMR32 cells in NOD scid gamma mice. In experiment A, cells were injected as a free suspension while in experiment B, cells were injected as a Matrigel/cells slurry. Ten mice were treated by daily intraperitoneal injection of CR8 (final concentration: 6.7 mg/kg) or a corresponding volume of the DMSO/PEG300/water vehicle. Tumor size was monitored throughout the experiments. In B, only the CR8-sensitive tumors were kept in the graph. (C) Control and CR8-treated tumors (sensitive tumors only) were all collected at day 21 and their proteins analyzed by SDS-PAGE followed by Western blotting against MYCN and actin.

Table 1

NCI 60 mean graphs data for roscovitine (NSC 701554) and CR8 (NSC 749390). Data obtained from NCI's in vitro-oriented tumor cells screen. GI₅₀ is the molar concentration causing 50% growth inhibition of tumor cells. TGI is the molar concentration giving total growth inhibition. LC₅₀ is the molar concentration leading to 50% net cell death. Values are provided as in μM .

	Roscovitine	CR8	CR8 versus roscovitine (fold decrease)
GI ₅₀	19.3	0.19	101.6
TGI	78.3	2.57	30.5
LC ₅₀	99.4	6.84	14.5

Table 2

Protein kinase selectivity of roscovitine and CR8 in a kinase interaction assay; (DiscoverX KinomeScan®). Roscovitine and CR8 were tested at a 10 μ M concentration on a 402 kinases interaction panel. A semi-quantitative scoring of this primary screen was obtained. This score relates to a probability of a hit rather than strict affinity. Scores >10, between 1 to 10 and < indicate the probability of a being a false positive is <20%, <10%, <5%, respectively. The 15 best scores are presented. Full results are available in the Supplementary Table S1.

roscovitine		
Kinases	Abbreviation	Score
Casein kinase 1 ϵ	CSNK1E	0.65
Cyclin-dependent kinase 7	CDK7	0.75
Cdc2-like kinase 2	CLK2	2.0
Casein kinase 1 δ	CSNK1D	2.6
Homeodomain interacting protein kinase 4	HIPK4	4.5
Casein kinase 1 α 1	CSNK1A1L	5.0
Homeodomain interacting protein kinase 1	HIPK1	5.9
Dual specificity tyrosine-phosphorylation-regulated kinase 1A	DYRK1A	6.4
Dual specificity tyrosine-phosphorylation-regulated kinase 1B	DYRK1B	7.1
Dual specificity protein kinase TTK	TTK/MPS1	8.8
Casein kinase 1 γ ²	CSNK1G2	9.2
Cdc2-like kinase 1	CLK1	10.0
Casein kinase 1 γ ³	CSNK1G3	15.0
Cdc2-like kinase 4	CLK4	19.0
Cyclin-dependent kinase 5	CDK5	21.0
CR8		
Kinases	Abbreviation	Score
Casein kinase 1 ϵ	CSNK1E	0.35
Dual specificity tyrosine-phosphorylation-regulated kinase 1A	DYRK1A	0.5
Cyclin-dependent kinase 7	CDK7	1.6
Casein kinase 1 α 1	CSNK1A1L	2.7
Casein kinase 1 δ	CSNK1D	3.2
Cyclin-dependent kinase 9	CDK9	3.2
Casein kinase 1 γ ³	CSNK1G3	4.6
Dual specificity tyrosine-phosphorylation-regulated kinase 1B	DYRK1B	4.8
Cdc2-like kinase 2	CLK2	4.8
Cdc2-like kinase 1	CLK1	5.1
Casein kinase 1 γ ²	CSNK1G2	5.8
Cdc2-like kinase 4	CLK4	6.0
Cyclin-dependent kinase 5	CDK5	11.0

Table 3

Roscovitine and CR8 -binding proteins from human neuroblastoma SH-SY5Y cells as identified by competition affinity chromatography (KinAffinity®, Evotec). Extracts prepared from SH-SY5Y cells were loaded on KinAffinity®; matrix and bound proteins were identified before and after competition with increasing concentrations of roscovitine or CR8. Non kinase proteins (i.e. potential associated proteins and substrates) are indicated in italics on the right side of the left column. $K_{d,free}$ values are reported in μM . Full results are available in Supplementary Table S3.

Roscovitine			
Kinases	Associated proteins	Abbreviation	$K_{d, free}(\mu\text{M})$
Ser/Thr protein kinase PAK 4	<i>CDK5 and ABL1 enzyme substrate 2</i>	<i>CABLES2</i>	0.081
		PAK4	0.129
	<i>Cyclin T2</i>	<i>CCNT2</i>	0.347
	<i>Cyclin H</i>	<i>CCNH</i>	0.372
	<i>Cyclin T1</i>	<i>CCNT1</i>	0.414
Cyclin-dependent kinase 7		CDK7	0.430
	<i>CDK-activating kinase assembly factor MAT1</i>	<i>MNAT1</i>	0.521
Cell division cycle 2-related protein kinase 7		CDK12, CRKRS	0.537
Cyclin-dependent kinase 9		CDK9	0.597
Casein kinase 1 δ		CSNK1D	0.726
Calcium/calmodulin-dependent proteinkinase kinase 2		CaMKK2	0.798
Casein kinase 1 ϵ		CSNK1E	0.920
Cdc2-like kinase 2		CLK2	1.134
	<i>TFIIH basal transcription factor complex helicase subunit</i>	<i>ERCC2</i>	1.307
	<i>CDK5 and ABL1 enzyme substrate 1</i>	<i>CABLES1</i>	1.989
Dual specificity tyrosine-phosphorylation-regulated kinase 1A	<i>Cyclin K</i>	<i>CCNK</i>	4.400
		DYRK1A	4.898
		CDK5	6.019
Cyclin-dependent kinase 5	<i>WD repeat-containing protein 68</i>	<i>WDR68</i>	6.987
		CDK2	11.386
Casein kinase 1 γ^2		CSNK1G2	17.997
Cyclin-dependent kinase 10		CDK10	18.966
ALK tyrosine kinase receptor		ALK	20.392
Serine/threonine-protein kinase 33		STK33	21.256
Casein kinase 1 γ^1		CSNK1G1	21.388
Ribosomal protein S6 kinase a 4		RPS6KA4	21.840
Casein kinase 1 γ^3		CSNK1G1	24.223
CR8			
Kinases	Associated proteins	Abbreviation	$K_{d, free}(\mu\text{M})$
Cell division cycle 2-related proteinkinase 7	<i>Cyclin T2</i>	<i>CCNT2</i>	0.294
		CDK12, CRKRS	0.302

CR8			
Kinases	Associated proteins	Abbreviation	K_{d, free} (μM)
Cyclin-dependent kinase 9	<i>Cyclin T1</i>	<i>CCNT1</i>	0.378
		CDK9	0.449
Ribosomal protein S6 kinase α 4	<i>Cyclin K</i>	<i>CCNK</i>	0.475
		RPS6KA4	2.289
Casein kinase 1δ		CSNK1D	2.652
	<i>WD repeat-containing protein 68</i>	<i>WDR68</i>	2.999
Cyclin-dependent kinase 10		CDK10	3.049
Dual specificity tyrosine-phosphorylation-regulated kinase 1A		DYRK1A	3.299
	<i>CDK5 and ABL1 enzyme substrate 1</i>	<i>CABLES1</i>	3.451
Cyclin-dependent kinase 2		CDK2	4.751
Dual specificity tyrosine-phosphorylation-regulated kinase 1B		DYRK1B	4.879
Casein kinase 1 ε		CSNK1E	5.726
Cdc2-like kinase 2		CLK2	6.243
Cyclin-dependent kinase 5		CDK5	7.424
Phosphatidylinositol-5 -phosphate 4-kinase type-2 α		PIP4K2A	8.675
Ser/Thr-protein kinase PAK 4		PAK4	10.620
Casein kinase 1 γ ¹		CSNK1G1	12.481
Phosphatidylinositol-5 -phosphate 4-kinase type-2 β		PIP4K2B	12.550
Casein kinase 1 γ ²		CSNK1G2	14.130
Phosphatidylinositol-5 -phosphate 4-kinase type-2 γ		PIP4K2C	14.537
Cyclin-dependent kinase 7		CDK7	20.049
	<i>Cyclin H</i>	<i>CCNH</i>	24.189
Ribosomal protein S6 kinase α 3		RPS6KA3	27.078
Maternal embryonic leucine zipperkinase		MELK	27.747
<i>CDK-act i</i>	<i>ivating kinase assembly factor MAT1</i>	<i>MNAT1</i>	27.914
	<i>Cyclin B2</i>	<i>CCNB2</i>	28.228

Table 4

Transcriptomics study: genes showing a >10 fold down-regulation, following exposure of SH-SY5Y cells to roscovitine or CR8. The absolute fold change is indicated for each gene. Two values indicate that the gene was represented by two different probes.

Gene	Roscovitine	CR8
Cell cycle regulators		
CCDC16	55.38	27.6
CDKN1B (p27 ^{kip1})	11.57	6.39
DUSP1	21.7	84.67
GADD45A	10.02	7.25
GSG2 (haspin)	27.86	19.55, 15.45
ING1	-	10.77
NEDD9	15.38	-
PPM1D	10.62	17.06
RGS2	87.67	54.58
SIAH1	13.51	10.37
TXNIP	3.82	11.79
Apoptosis regulators		
DDIT4	5.08	41.16
FADD	37.59, 28.81	19.44
GADD45A	10.02	7.25
GADD45G	14.37	12.64
c-MYC	52.94	41.75
MYCN	36.00	20.63
PHLDA1	-	10.51
PLEKHF1	14.63, 11.73	12.6
SIAH1	13.51	10.37
NFκB pathway		
FADD	37.59, 28.81	19.44
TRAF6	53.23	39.59