

Metabolomics
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1 ORIGINAL ARTICLE

2 **Cyclin-dependent kinases 4 and 6 control tumor progression**
3 **and direct glucose oxidation in the pentose cycle**

4 **Miriam Zanuy · Antonio Ramos-Montoya · Oscar Villacañas · Nuria Canela ·**
5 **Anibal Miranda · Esther Aguilar · Neus Agell · Oriol Bachs · Jaime Rubio-Martinez ·**
6 **Maria Dolors Pujol · Wai-Nang P. Lee · Silvia Marin · Marta Cascante**

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9 **Abstract** Cyclin-dependent kinases CDK4 and CDK6 are
10 essential for the control of the cell cycle through the G₁
11 phase. Aberrant expression of CDK4 and CDK6 is a hall-
12 mark of cancer, which would suggest that CDK4 and CDK6
13 are attractive targets for cancer therapy. Herein, we report
14 that calcein AM is a potent specific inhibitor of CDK4 and
15 CDK6 in HCT116 human colon adenocarcinoma cells,
16 inhibiting retinoblastoma protein (pRb) phosphorylation and
17 inducing cell cycle arrest in the G₁ phase. The metabolic
18 effects of calcein AM (the calcein acetoxymethyl-ester) on

HCT116 cells were also evaluated and the flux between the 19
oxidative and non-oxidative branches of the pentose phos- 20
phate pathway was significantly altered. To elucidate whe- 21
ther these metabolic changes were due to the inhibition of 22
CDK4 and CDK6, we also characterized the metabolic 23
profile of a CDK4, CDK6 and CDK2 triple knockout of 24
mouse embryonic fibroblasts. The results show that the 25
metabolic profile associated with the depletion of CDK4, 26
CDK6 and CDK2 coincides with the metabolic changes 27
induced by calcein AM on HCT116 cells, thus confirming 28
that the inhibition of CDK4 and CDK6 disrupts the balance 29
between the oxidative and non-oxidative branches of the 30
pentose phosphate pathway. Taken together, these results 31
indicate that low doses of calcein can halt cell division and 32
kill tumor cells. Thus, selective inhibition of CDK4 and 33
CDK6 may be of greater pharmacological interest, since 34

Miriam Zanuy and Antonio Ramos-Montoya contributed equally to this work.

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M. Zanuy · A. Ramos-Montoya · A. Miranda · E. Aguilar ·
S. Marin · M. Cascante (✉)
Department of Biochemistry and Molecular Biology,
Faculty of Biology (Edifici Nou), University of Barcelona,
Av. Diagonal 645, 08028 Barcelona, Spain
e-mail: martacascante@ub.edu

M. Zanuy · A. Ramos-Montoya · A. Miranda · E. Aguilar ·
S. Marin · M. Cascante
Institute of Biomedicine of the Universitat de Barcelona (IBUB)
and CSIC Associated Unit, Barcelona, Spain

O. Villacañas · J. Rubio-Martinez
Department of Physical Chemistry, Institut de Recerca en
Química Teòrica i Computacional (IQTCUB), Universitat de
Barcelona, Martí i Franqués 1, 08028 Barcelona, Spain

N. Canela · N. Agell · O. Bachs
Department of Cell Biology, Immunology and Neurosciences,
Institut d'Investigacions Biomèdiques August Pi i Sunyer
(IDIBAPS), Faculty of Medicine, Universitat de Barcelona,
Casanova 143, 08036 Barcelona, Spain

M. D. Pujol
Department of Pharmacology and Therapeutic Chemistry,
CSIC Associated Unit, Faculty of Pharmacy, Universitat de
Barcelona, Joan XXIII, s/n, 08028 Barcelona, Spain

W.-N. P. Lee
Department of Pediatrics, Los Angeles Biomedical Research
Institute at the Harbor-UCLA Medical Center, RB1,
1124 West Carson Street, Torrance, CA 90502, USA

Present Address:
A. Ramos-Montoya
Uro-Oncology Research Group, Cancer Research UK
Cambridge Research Institute, Li Ka Shing Centre,
Robinson Way, Cambridge CB2 0RE, UK

Present Address:
O. Villacañas
Intelligent Pharma S.L, C/Baldiri Reixac 4,
08028 Barcelona, Spain

35 inhibitors of these kinases affect both cell cycle progression
36 and the robust metabolic profile of tumors.

37
38 **Keywords** Cyclin dependent kinases · CDK-inhibitor ·
39 Tracer-based metabolomics · Pentose phosphate pathway ·
40 Phase plane analysis

41 Abbreviations

42 Calcein AM	Calcein acetoxymethyl-ester
43 CDK	Cyclin dependent kinase
44 DMEM	Dulbecco's modified eagle medium
45 FCS	Fetal calf serum
46 Ct MEF	Mouse embryonic fibroblast
47 PBS	Phosphate buffer saline
48 PPP	Pentose phosphate pathway
49 pRb	Retinoblastoma protein
50 TKO MEF	Mouse embryonic fibroblast knockout for CDK4, CDK6 and CDK2

51
52

53 1 Introduction

54 Typical proliferation of eukaryotic cells involves an orderly
55 progression through four distinct phases of the cell cycle: G₁,
56 S, G₂, and M (Malumbres and Barbacid 2001; Sherr 1996).
57 The first step of the G₁/S transition of the cell cycle is regu-
58 lated by cyclin-dependent kinases (CDKs: EC 2.7.11.22),
59 CDK4 and CDK6 and their inhibitors, p16^{INK4a} and
60 p15^{INK4b}. According to the long-prevailing model of cell
61 cycle control in mammalian cells, cyclin D-CDK4, cyclin
62 D-CDK6 and cyclin E-CDK2 complexes are sequentially
63 required to promote cell cycle entrance from quiescence,
64 progression through the G₁ phase and transition from the G₁
65 to the S-phase in response to mitogenic stimulation. Cell
66 culture and biochemical studies have indicated that cyclin
67 D-CDK4, cyclin D-CDK6 and cyclin E-CDK2 complexes
68 are essential and rate-limiting for the phosphorylation and
69 inactivation of the tumor suppressor retinoblastoma protein
70 (pRb) and the subsequent induction of the E2F-dependent
71 transcriptional program required to enter the S-phase
72 (Lundberg and Weinberg 1998; Malumbres et al. 2004; Sherr
73 and Roberts 2004). This step of the cell cycle is critical. If the
74 cell passes through the restriction point (R), it becomes
75 insensitive to extracellular stimuli and is committed to
76 entering the S-phase. Since almost all the regulators of this
77 cell cycle phase are mutated in cancer (Graf et al. 2009), this
78 phase has been considered as a valid therapeutic target. Since
79 most mutations in human cancers affect CDK4 and CDK6 or
80 their regulators (Hall and Peters 1996), and preclinical data
81 indicate that the inhibition of cyclin D-dependent kinase
82 activity may have therapeutic benefits (Graf et al. 2009;

Malumbres and Barbacid 2006; Shapiro 2006; Yu et al. 2006), interest in CDK4 and CDK6 as promising targets for inhibiting cell cycle progression has been generated.

Another important and critical feature of tumor cells is their metabolic adaptation, which provides them with metabolites and energy to progress through the cell cycle. This adaptation includes the phenomenon known as the "Warburg effect" (high glycolysis in the presence of oxygen) (Warburg 1956), a high glutamine uptake, the activation of biosynthetic pathways and the over-expression of some glycolytic isoenzymes (Vizán et al. 2008). In recent years, it has become accepted that the metabolic adaptation of tumor cells also involves an enhancement of pentose phosphate pathway (PPP) fluxes and a specific imbalance between its two branches in favor of the oxidative branch versus the non-oxidative branch to maintain the high proliferative rates (Kuo et al. 2000; Poulsen and Frederiksen 1981; Ramos-Montoya et al. 2006). In previous studies, we have demonstrated that this balance between the oxidative and non-oxidative branches of the PPP is necessary to maintain the metabolic efficiency of the cancer cell for growth and proliferation, and that it can be a weakness in the robust tumor metabolic adaptation (Ramos-Montoya et al. 2006). PPP is also specifically regulated during cell cycle progression in tumor cells (Vizan et al. 2009).

In the present study, we identified calcein (4'5'-bis(*N,N*-bis(carboxymethyl) aminomethyl) fluorescein) as a putative inhibitor of CDK4 and CDK6 that mimics the natural inhibitor p16^{INK4a} in HCT116 cells, through the use of new bioinformatic tools (Villacanas et al. 2002; Villacanas and Rubio-Martinez 2006), docking procedures (Rubio-Martinez et al. 2005) and molecular assays. Moreover, we provide experimental evidence that this CDK4 and CDK6 inhibitor counteracts metabolic adaptations which are characteristic of tumor cells, and that this metabolic fingerprint coincides with that obtained from a mouse embryonic fibroblast knockout for CDK4, CDK6 and CDK2 cell line. We demonstrate not only that calcein is a promising agent that could be a key factor in the development of a new family of selective cyclin D-dependent kinase inhibitors, but also that inhibition of CDK4 and CDK6 impairs metabolic adaptations that support tumor cell cycle progression.

2 Materials and methods

2.1 Materials

Dulbecco's modified Eagle Medium (DMEM), F-12 HAM Nutrient mixture with L-glutamine, MEM-EAGLE non-essential aminoacid solution ×100, antibiotic (100 U/ml penicillin, 100 mg/ml streptomycin), Dulbecco's Phosphate buffer saline (PBS), Trypsin EDTA solution C (0.05%

132 trypsin–0.02% EDTA), L-glutamine solution 200 mM and
 133 sodium pyruvate solution 100 mM were obtained from
 134 Biological Industries; Fetal calf serum (FCS) and Trizol
 135 were from Invitrogen; SDS was from Fluka; Coomassie blue
 136 was from Biorad; HEPES and MgCl₂ were from Applichem;
 137 A-Sepharose was from Pierce; the [γ -³²P]ATP, 3000
 138 Ci/mmol, 10 mCi/ml and ECL were from Amersham; his-
 139 tone H1 was from Boehringer Mannheim; Bradford reagent
 140 (500-0006), Acrylamide (161-0158) and peroxidase-cou-
 141 pled secondary antibody were from Bio-Rad Laboratories;
 142 anti-CDK6 (sc-177), anti-CDK4 (sc-260-R), anti-cyclin
 143 D3 (sc-182) and anti-p16INK4a (sc-468) were from Santa
 144 Cruz Biotechnology; anti-cyclin D1 (06-137), anti-CDK2
 145 (06-505) and anti-cyclin B1 (05-158) were from Upstate
 146 Biotechnology; anti-actin (691001) was from MP Biomed-
 147 icals; anti-phospho-Rb (Ser780) was from Cell Signaling
 148 Technology; pGST-Rb (379-928) (gift of Dr Wang, San
 149 Diego, CA, USA) fusion protein was expressed and purified
 150 following Smith and Johnson (1988) and Frangioni and Neel
 151 (1993). All other reagents were from Sigma Chemical CO.

152 2.2 Molecular modeling

153 Construction and molecular dynamics simulations of the
 154 CDK6-p16^{INK4a} complex and the determination of their
 155 interactions were carried out as described Villacañas et al.
 156 2002. All hot spots of the CDK6-p16^{INK4a} interaction
 157 surface were monitored throughout the production time to
 158 obtain its pharmacophores. Catalyst (Accelrys, Inc., San
 159 Diego, CA, USA) software was then used to obtain com-
 160 pounds that matched the different interaction pharmaco-
 161 phores. Selected compounds were docked into CDK6 with
 162 an in-house program (Rubio-Martinez 2005) and, finally, a
 163 visual structure analysis was carried out to reduce the
 164 number of final modeled complexes. More details can be
 165 found in supplementary material.

166 2.3 Cell culture

167 Human colon carcinoma HCT116 cells (donated by Dr.
 168 Capellà, the Institut Català d'Oncologia, Barcelona, Spain)
 169 were grown in DMEM:HAM's F12 (1:1), supplemented
 170 with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM
 171 sodium pyruvate, 1% non-essential amino acids, 50 mU/ml
 172 penicillin and 50 μ g/ml streptomycin. All cell cultures
 173 were carried out at 37°C in a humidified atmosphere with
 174 5% CO₂.

175 Mouse embryonic fibroblast (Ct MEF) and mouse
 176 embryonic fibroblast knockout for CDK4, CDK6 and
 177 CDK2 (TKO MEF) cell lines, obtained from Dr. Barbacid
 178 (Centro Nacional de Investigaciones Oncológicas, Madrid,
 179 Spain) (Santamaria and Ortega 2006), were grown as a
 180 monolayer culture in minimum essential medium (DMEM

with L-glutamine, without glucose or sodium pyruvate) in
 the presence of 10% heat-inactivated FCS, 10 mM D-glu-
 cose and 0.1% streptomycin/penicillin in standard culture
 conditions. They were incubated at 37°C, 80% humidity,
 5% CO₂, and 3% O₂. Two different clones of each were
 used in order to discard the effect of immortalization: Ct
 MEF: LD179.10.1 and LD207.3.1 and TKO MEF:
 LD1043.7.1 and LD1043.6.1.

2.4 Immunoprecipitation and kinase assays

For the kinase assays, immunoprecipitations were performed
 as described by Harlow and Lane (Harlow and Lane 1988).
 HCT116 cells were lysed for 30 min at 4°C in IP buffer
 (50 mM HEPES pH 7.5, 150 mM NaCl, 2.5 mM EGTA,
 1 mM EDTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT,
 1 mM phenyl methyl sulfonyl fluoride, 1 μ g/ml aprotinin,
 10 μ g/ml leupeptin, 10 mM β -glycerophosphate, 0.1 mM
 Na₃VO₄ and 1 mM NaF). Lysates were sonicated twice for
 10 s at 4°C and clarified by centrifugation at 10,000 \times g for
 10 min. The supernatant fraction protein content was mea-
 sured using the Bradford method (Bradford 1976), and
 400 μ g of protein from the lysates were incubated with 4 μ g
 of antibody (CDK6, CDK4, cyclin-D1, cyclin-D3, cyclin-B1
 or CDK2) or with 1 μ l of normal rabbit serum or normal
 mouse serum (controls) O/N shaking at 4°C. Protein
 immunocomplexes were then incubated with 20 μ l protein
 A-Sepharose for 1 h at 4°C, collected by centrifugation and
 washed four times in IP buffer and twice in kinase buffer
 (50 mM HEPES pH 7.4, 10 mM MgCl₂, 2.5 mM EGTA,
 0.1 mM Na₃VO₄, 10 mM β -glycerophosphate and 1 mM
 DTT). They were then incubated in kinase buffer containing
 2 Ci [γ -³²P]ATP and 1 μ g pGST-Rb (379-928) fusion pro-
 tein for CDK6 and CDK4 kinase assays, or 3 μ g histone H1
 for CDK1 and CDK2 kinase assays, for 30 min. at 30°C in a
 final volume of 30 μ l. The samples were pooled and redis-
 tributed to assure equal amounts of all the reagents and
 immunoprecipitated CDK. Finally, the samples were boiled
 for 5 min and electrophoresed on SDS-polyacrylamide gels,
 essentially as described by Laemmli (1970), and the gels
 were stained with coomassie brilliant blue, dried, and
 exposed to X-ray films at –80°C. The intensity of radioac-
 tivity was measured with Typhoon Trio and Trio 9200 (GE
 Healthcare). p21^{Kip/Cip} and purified p16^{INK4a} were used as a
 positive control of inhibition.

2.5 Gel electrophoresis and immunoblotting

Cells were lysed in a buffer containing 2% SDS, 67 mM
 Tris–HCl pH 6.8 and 10 mM EDTA and sonicated twice
 for 10 s (4°C). Protein content was measured according to
 the Lowry procedure, using bovine serum albumin (BSA)
 as standard. The extracts were electrophoresed in SDS-

230 polyacrylamide gels, essentially as described by Comin-
231 Anduix et al. 2002 and Laemmli 1970. After electropho-
232 resis, the proteins were transferred to Immobilon-P strips
233 for 1.5 h at 70 V. The sheets were preincubated in TBS
234 (20 mM Tris-HCl pH 7.5, 150 mM NaCl), 0.05% Tween
235 20 and 3% BSA for 1 h at room temperature and then
236 incubated for 1 h at room temperature in TBS, 0.05%
237 Tween 20, 3% BSA containing anti-phospho-Rb (Ser780),
238 anti-CDK4 (sc-260), anti-CDK2 (06-505) or anti-actin
239 (60100) antibodies. After washing in TBS, 0.05% Tween
240 20 (three times, 10 min each), the sheets were incubated
241 with a peroxidase-coupled secondary antibody (1:3000
242 dilution) for 1 h at room temperature. After incubation, the
243 sheets were washed twice in TBS, 0.05% Tween 20 and
244 once in TBS. The reaction was visualized using ECL. The
245 Image LAS-3000 Photo Version 2.0 (Fujifilm) was used to
246 analyze the chemiluminescence.

247 2.6 Viability assay

248 The assay was performed using a variation of the method
249 described by Mosmann (Matito et al. 2003; Mosmann
250 1983; Ramos-Montoya et al. 2006). Growing concentra-
251 tions of the product were plated in 96-well flat-bottomed
252 microtiter plates to a final volume of 200 μ l where
253 1700 cells/well had been seeded 24 h before. After incu-
254 bation for 72 h, MTT at a final concentration of 0.5 mg/ml
255 was added. After 1 h of incubation, the generated *formazan*
256 was dissolved with 100 μ l of DMSO per well. The absor-
257 bance was measured on an ELISA plate reader (Merck
258 ELISA System MIOS version 3.2., Tecan Sunrise, Tecan
259 Group Ltd.) at 550 nm. The concentrations that caused
260 50% inhibition of cell viability (IC_{50}) were calculated.

261 2.7 Cell culture synchronization and cell cycle analysis

262 HCT116 cells were brought to 95% cell confluence and
263 kept confluent for 24 h with medium containing 0.5% FCS.
264 Cells were then seeded to 50–60% cell confluence in a
265 medium with 10% heat-inactivated FCS. Calcein AM
266 2 μ M was added.

267 In order to determine the proportion of cells in each cell
268 cycle phase (G_1 , S or G_2), cell cycle analysis was assessed
269 with flow cytometry using a fluorescence-activated cell
270 sorter (FACS). Approximately 500,000 cells were resus-
271 pended in 0.5 ml PBS followed by the addition of 4.5 ml
272 70% (v/v) ethanol (Matito et al. 2003). Cells were briefly
273 stained in PBS containing 50 μ g/ml propidium iodide,
274 10 μ g/ml DNase free RNase and 0.1% Triton[®] X-100.
275 FACS analysis was carried out at 488 nm in an Epics XL
276 flow cytometer (Beckman Coulter). Data from 12,000 cells
277 were collected and analyzed using the MultiCycle program
278 (Phoenix Flow Systems).

2.8 Isotopologue distribution analysis

280 Tracer studies were carried out by incubating the cells in the
281 presence of the corresponding incubation medium con-
282 taining 10 mM glucose enriched by 50% in the tracer
283 [1,2-¹³C₂]-D-glucose. After incubation for 72 h, the cell
284 medium was removed, thereby separating the incubation
285 medium from the cells adhered to the dishes, and all frac-
286 tions were frozen in liquid nitrogen and stored at -80° C
287 until processing.

288 Mass spectral data were obtained on an HP5973 mass
289 selective detector connected to an HP6890 gas chromato-
290 graph (HCT116 with calcein AM assays) and on a GCMS-
291 QP2010 selective detector connected to a GC-2010 gas
292 chromatograph from Shimadzu (Ct MEF and TKO MEF
293 assays). The settings were as follows: GC inlet 230 $^{\circ}$ C (200 $^{\circ}$ C
294 for lactate measurement), transfer line 280 $^{\circ}$ C, MS source
295 230 $^{\circ}$ C and MS Quad 150 $^{\circ}$ C. An HP-5 or a DB-5MS capillary
296 column (both: length (m), 30; internal diameter (μ m), 250;
297 film thickness (μ m), 0.25) was used. Spectral data were
298 corrected using regression analysis to extract natural ¹³C
299 enrichment from results (Lee et al. 1991). Measurement of
300 ¹³C label distribution determined the different relative dis-
301 tribution percentages of the isotopologues, m0 (without any
302 ¹³C labels), m1 (with one ¹³C), m2 (with two ¹³C), etc., which
303 were reported as molar fractions. $\sum m$ is the sum of the
304 labeled species ($\sum m = m1 + m2 + m3...$) and is repre-
305 sentative of the synthesized molecules of each metabolite.
306 The total label enrichment $\sum mn$ is the weighted sum of the
307 labeled species ($\sum mn = m1 \times 1 + m2 \times 2 + m3 \times 3...$)
308 and is representative of the contribution of the tracer used in
309 the synthesis of each metabolite.

310 Lactate from the cell culture medium was extracted with
311 ethyl acetate after acidification with HCl. Lactate was
312 transformed to its propylamide-heptafluorobutyric form
313 and the ion cluster around m/z 328 (carbons 1–3 of lactate,
314 chemical ionization) was monitored for the detection of m1
315 (recycled lactate through the pentose cycle) and m2 (lactate
316 produced by glycolysis). The relative amount of glucose
317 that is converted indirectly to lactate through the pentose
318 cycle, known here as pentose cycle activity, is calculated
319 by the $(m1/m2)/(3 + (m1/m2))$ ratio using lactate isoto-
320 pologues (Lee et al. 1998).

321 RNA ribose was isolated by acid hydrolysis of cellular
322 RNA after Trizol-purification of cell extracts. Ribose iso-
323 lated from RNA was transformed to its aldonitrile-acetate
324 form using hydroxylamine in pyridine and acetic anhy-
325 dride. We monitored the ion cluster around the m/z 256
326 (carbons 1–5 of ribose, chemical ionization) to find the
327 molar enrichment and positional distribution of ¹³C labels
328 in ribose (Boros et al. 1997; Lee et al. 1998). The m2 ribose
329 originated from [1,2-¹³C₂]-glucose that is converted to
330 ribose through transketolase enzyme reactions, whereas m1

331 ribose originated from glucose metabolized by direct oxida-
 332 tion via the oxidative steps of the PPP. The isotopo-
 333 logues m3 and m4 come from the recycling of the
 334 previously labeled riboses. The oxidative versus non-oxi-
 335 dative ratio was measured as $ox/non-ox = (m1 + m3)/$
 336 $(m2 + m3 + 2 \times m4)$.

337 2.9 Sugars-phosphate determination

338 Hexose, triose, pentose and fructose-1,6-bis-phosphates were
 339 determined in cell monolayers frozen in liquid nitrogen as
 340 described (Vizan et al. 2007). Frozen cells were briefly
 341 scraped off the plates and 100 mM acetic acid solution at 4°C
 342 was added. The obtained homogenates were centrifuged at
 343 $0.4 \times g$ for 10 min at 4°C, and the supernatants containing
 344 sugar phosphate molecules were separated and kept frozen at
 345 -80°C for the following liquid chromatography/mass spec-
 346 trometry (LC-MS) analysis. Chromatography was performed
 347 using an Agilent 1100 Quaternary Pump (Agilent Technolo-
 348 gies) equipped with a refrigerated autosampler. A Nucleodex
 349 β -OH high-performance liquid chromatography (HPLC)
 350 column, 200×4 mm i.d. (Panreac Química S.A.U.) with a
 351 binary gradient at a flow-rate of 0.75 ml/min was used. Sol-
 352 vent A consisted of 10 mM ammonium acetate pH 4.0. Sol-
 353 vent B consisted of acetonitrile. Before reaching the mass
 354 spectrometer, the flow-rate was split (1:3). To reduce the
 355 residual matrix effect reaching the mass spectrometer, a divert
 356 valve (VICI Valco Instruments) drained off the LC eluent
 357 during the time in which interference was detected in order to
 358 avoid contamination of the mass spectrometer. Identification
 359 of sugar phosphates was carried out in an API-3000 tandem
 360 mass spectrometer (Applied Biosystems). The multiple reac-
 361 tion monitoring (MRM) transitions were 259/97 for glucose-
 362 6-phosphate and fructose-6-phosphate (hexose phosphates),
 363 199/97 for glyceraldehyde-3-phosphate and dihydroxyace-
 364 tone phosphate (triose phosphates), 339/97 for fructose-1,6-
 365 bisphosphate and 229/97 for ribose-5-phosphate and xylulose-
 366 5-phosphate (pentose phosphates).

367 2.10 Data analysis and statistical methods

368 In vitro experiments were carried out using three cultures
 369 each time for each treatment regimen and then repeated
 370 twice. Mass spectral analyses were carried out by three
 371 independent automatic injections of 1 μl of each sample by
 372 means of the automatic sampler and accepted only if the
 373 sample standard deviation was less than 1% of the nor-
 374 malized peak intensity. Statistical analyses were performed
 375 using the parametric unpaired, two-tailed independent
 376 sample *t* test with 95, 99, and 99.9% confidence intervals,
 377 and $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered,
 378 respectively, to indicate significant differences in glucose
 379 carbon metabolism.

3 Results 380

381 3.1 Selection of a better CDK4 and CDK6 inhibitor 381

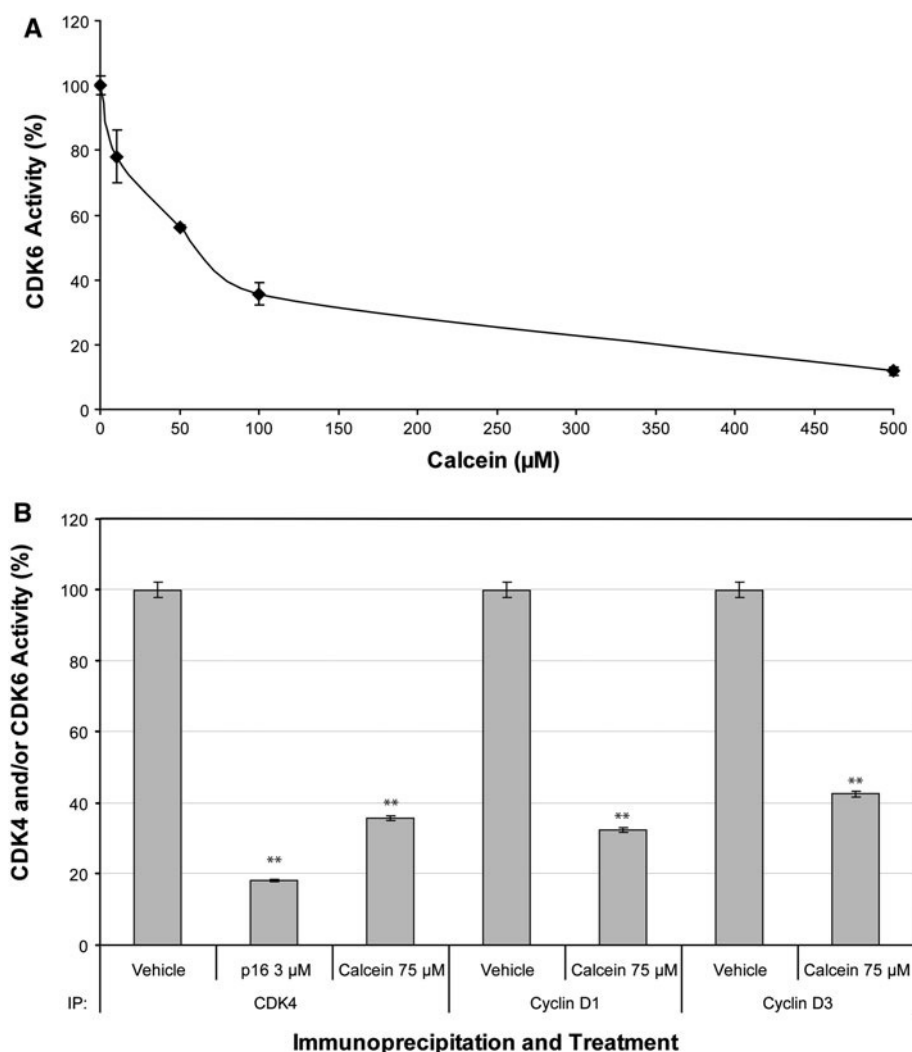
382 Results from CDK6-p16^{INK4a} complex dynamics were used 382
 383 to model the interaction pattern of the putative inhibitors. 383
 384 The ACD 3D database (available chemical database 3D) 384
 385 was screened for commercial compounds that matched our 385
 386 query. After docking procedures, eight compounds were 386
 387 selected for further experimental kinase assays, with cal- 387
 388 cein being the most active (Figure SM1, Supplementary 388
 389 Material). 389

390 3.2 Calcein selectively inhibits CDK4 and CDK6 390 391 activities, disrupting cell growth, pRb and cell 391 392 cycle 392

393 To investigate whether calcein selectively inhibits CDK4 393
 394 and CDK6 activities, immunoprecipitations were per- 394
 395 formed, followed by kinase assays in the presence or absence 395
 396 of calcein. A dose-response curve with increasing doses of 396
 397 calcein from 10 μM to 500 μM was carried out with 397
 398 immunoprecipitated CDK6 (Fig. 1a), with an IC₅₀ of 75 μM . 398
 399 Calcein at this concentration produced similar effects when 399
 400 CDK4, cyclin D1 or cyclin D3 were immunoprecipitated 400
 401 (Fig. 1b), but did not inhibit CDK1 or CDK2 kinase activi- 401
 402 ties at any of the concentrations tested (Figure SM2.A sup- 402
 403 plementary material). As expected from *in silico* complex 403
 404 dynamics, the interaction of calcein with CDK6 seemed to be 404
 405 through the p16^{INK4a} binding site, as calcein was able to 405
 406 displace p16^{INK4a} from the immunoprecipitated enzyme 406
 407 (Figure SM2.B, supplementary material). These results 407
 408 demonstrate that calcein interacts selectively with CDK4 408
 409 and CDK6 at the p16^{INK4a} binding site, inhibiting their 409
 410 kinase activity without affecting CDK2 and CDK1 activities. 410

411 To examine whether calcein penetrates the cell mem- 411
 412 brane and inhibits intracellular CDK4 and CDK6 activities, 412
 413 we used human colon adenocarcinoma HCT116 cells, as 413
 414 they have a silenced wild-type p16^{INK4A} gene and only 414
 415 express a mutant allele (Myohanen et al. 1998). Increasing 415
 416 doses of calcein in the media induced a progressive inhi- 416
 417 bition of HCT116 cell viability, presenting a rather high 417
 418 IC₅₀ of 400 μM after 72 h of treatment. The calcein acet- 418
 419 oxymethyl-ester (calcein AM) and *tert*-butoxy methyl ester 419
 420 (calcein tBM), which are more lipophilic and diffusible 420
 421 through the cytoplasmic membrane than the non-esterified 421
 422 calcein, induced a stronger inhibition of HCT116 cell 422
 423 viability, with IC₅₀ values of 0.6 and 80 μM , respectively. 423
 424 Treatment of HCT116 cells with the non-esterified calcein 424
 425 or with calcein AM decreased the phosphorylation of the 425
 426 serine 780 of pRb, which is a specific target for CDK4 and 426
 427 CDK6 (Fig. 2a). In addition, calcein AM arrested the cell 427
 428 cycle in G₁ of synchronous HCT116 cells (Fig. 2b). 428

Fig. 1 Effect of calcein on kinase assays in immunoprecipitated CDK6, CDK4, cyclin D1, and cyclin D3. **a** Dose–effect curve of non-esterified calcein on CDK6 activity (10–500 μ M). **b** CDK4, cyclin D1 and cyclin D3 immunoprecipitations and kinase assays tested in the presence of 75 μ M of non-esterified calcein and p16^{INK4a} (3 μ M). pGST-Rb (379–928) fusion protein was used as a substrate. Mean \pm SD; $n = 3$. (*) indicates $P < 0.05$ and (**) indicates $P < 0.01$ compared to vehicle



429 All these data suggest a molecular mechanism of action
430 of calcein and its esters through the inhibition of CDK4 and
431 CDK6, which in turn affects cell cycle regulation.

432 3.3 Metabolic effects caused by the inhibition 433 of the CDKs responsible for G₁/S transition

434 HCT116 human colon adenocarcinoma cells exposed to
435 increasing concentrations of calcein AM were incubated
436 for 72 h with 10 mM glucose 50% enriched in [1,2-¹³C₂]-
437 D-glucose. The calcein AM concentrations were the IC₂₅
438 (0.36 μ M), IC₅₀ (0.61 μ M) and IC₇₅ (1.0 μ M) after 72 h of
439 treatment. In parallel, we also performed incubations with
440 immortalized mouse embryonic fibroblasts (Ct MEF)
441 control and knockout for CDK4, CDK6 and CDK2 (TKO
442 MEF) in the presence of the same tracer, to check whether
443 the metabolic changes induced by calcein AM on HCT116
444 cells were characteristic of the inhibition of the CDKs
445 responsible for the G₁/S transition. These MEF cell lines
446 (Ct and TKO) constitute an additional new tool that could

447 elucidate the effects of the permanent absence of these
448 CDKs in vivo and their contributions to cell cycle pro-
449 gression and the robust tumor metabolic adaptation.

450 Lactate and ribose from RNA synthesized from the
451 tracer [1,2-¹³C₂]-D-glucose were measured using gas
452 chromatography coupled to mass spectrometry (GC–MS).
453 Table 1 shows the pondered values of the ¹³C-enriched
454 isotopologues, m1/ Σ m and m2/ Σ m, of lactate and ribose
455 from RNA. The molar enrichment Σ mn of ribose from
456 RNA is also shown.

457 Lactate m2 isotopologues (lactate molecules that con-
458 tain two ¹³C atoms) originated from [1,2-¹³C₂]-D-glucose
459 converted to lactate through glycolysis, whereas lactate m1
460 isotopologues originated from the metabolism of the
461 tracer through the oxidative step of the PPP and then
462 recycled to glycolysis via the non-oxidative PPP. Calcein
463 AM induced a dose–response decrease of m1/ Σ m and an
464 increase of m2/ Σ m in HCT116 cells. This drop of m1/ Σ m
465 suggests that calcein AM reduces the contribution of the
466 oxidative PPP flux in lactate synthesis. Similarly, when

Fig. 2 Phosphorylation of serine780 of pRb and cell cycle analysis. **a** HCT116 cells were treated with 400 μ M or with 0.6 and 2 μ M of calcein AM for 24 h and the extracts were blotted specifically against phosphoserine 780 of pRb. **b** Synchronous HCT116 cells in the G₁ phase at time 0 h (t₀ synchronous control) and after 24 h with or without treatment with calcein AM 2 μ M. (*) indicates $P < 0.05$ and (**) indicates $P < 0.01$. Both experiments were performed three times (Mean + SD; $n = 3$). One representative example is shown in each case

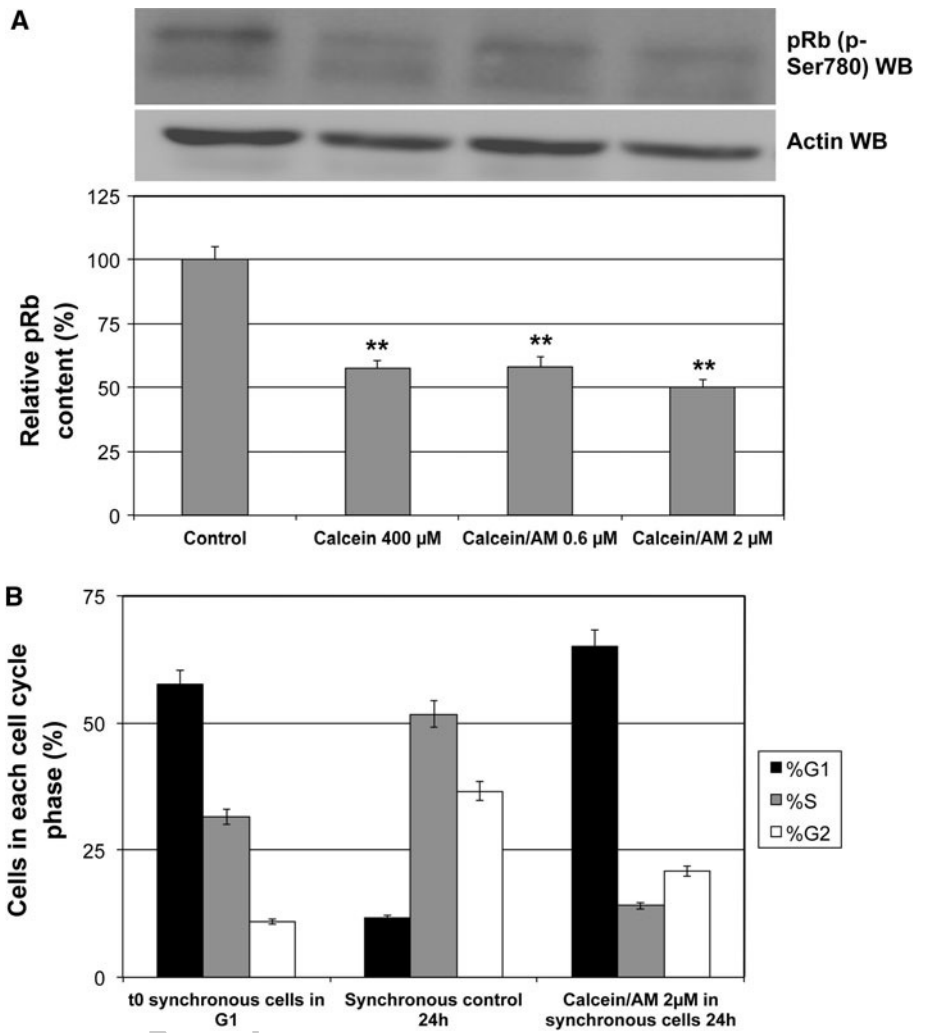


Table 1 Isotopologue distribution in lactate and ribose. M1/ Σ m and m2/ Σ m were determined in lactate isolated from incubation medium and in ribose isolated from RNA. Σ mn in ribose isolated from RNA was also measured

Isotopologue distribution analysis						
HCT116				MEF		
Lactate	Ct	IC ₂₅	IC ₅₀	IC ₇₅	Ct	TKO
m1/ Σ m	0.075 ± 0.003	0.075 ± 0.001	0.064 ± 0.003**	0.065 ± 0.002**	0.104 ± 0.000	0.070 ± 0.020*
m2/ Σ m	0.919 ± 0.003	0.918 ± 0.002	0.928 ± 0.002**	0.926 ± 0.002*	0.835 ± 0.049	0.843 ± 0.043
Ribose	Ct	IC ₂₅	IC ₅₀	IC ₇₅	Ct	TKO
m1/ Σ m	0.544 ± 0.003	0.522 ± 0.002***	0.496 ± 0.003***	0.474 ± 0.003***	0.450 ± 0.002	0.414 ± 0.002***
m2/ Σ m	0.303 ± 0.000	0.324 ± 0.001***	0.343 ± 0.004***	0.364 ± 0.002***	0.385 ± 0.002	0.420 ± 0.002***
Σ mn	0.839 ± 0.016	0.858 ± 0.030	0.811 ± 0.098**	0.668 ± 0.055*	0.754 ± 0.008	0.718 ± 0.008**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Experiments were performed twice. Results from one of them are shown (Mean + SD; $n = 3$)

467 MEF cell lines were incubated with [1,2-¹³C₂]-D-glucose, 468 the deletion of CDK4, CDK6 and CDK2 reduced m1/ Σ m 469 lactate, indicating that TKO MEF cells had a reduced 470 contribution of the oxidative pathway of PPP in lactate 471 synthesis. Moreover, the pentose cycle activity decreased 472 progressively in HCT116 cells treated with growing doses

of calcein AM, and was 13.75% lower in the condition 473 where the cells were treated with the calcein AM IC₇₅ 474 concentration (0.026 ± 0.001 in Ct vs. 0.023 ± 0.001 in 475 IC₇₅). Similarly, pentose cycle activity in TKO MEF cells 476 was 32.35% lower than in Ct MEF (0.040 ± 0.002 in Ct 477 vs. 0.027 ± 0.008 in TKO). This decreased pentose cycle 478

479 activity reinforced the hypothesis of a diminution of the
480 oxidative PPP flux and a decrease in its contribution to
481 glucose metabolism when the G₁/S-phase of the cell cycle
482 is perturbed.

483 Calcein AM treatment in HCT116 cells also resulted in
484 a slight decrease in the incorporation of ¹³C atoms from
485 glucose into nucleic acid ribose (Table 1). The average
486 number of ¹³C atoms per ribose molecule (Σmn) was
487 reduced by 20% at the dose of IC₇₅ of calcein AM in
488 HCT116 cells. As suggested by the above-described
489 decrease in lactate m1/ Σm data, the reduction of ribose
490 synthesis in HCT116 cells could be caused by reduced
491 substrate flux through the oxidative steps of the PPP.
492 Furthermore, calcein AM treatment in HCT116 cells
493 caused a dose-dependent m1/ Σm decrease as well as a
494 linear increase of m2/ Σm ribose (Table 1). This was in
495 accordance with the results obtained in lactate measure-
496 ments and denoted a clear attenuation of the flux through
497 the oxidative PPP.

498 Furthermore, TKO MEFs had a lower proliferation rate
499 than Ct MEFs (Ct MEF: 0.26 h⁻¹ vs. TKO MEF:
500 0.12 h⁻¹), the total label incorporation in ribose (Σmn)
501 being lower than that of Ct MEFs (Table 1). Moreover,
502 deletion of CDK4, CDK6 and CDK2 resulted in a decrease
503 in the percentage of ribose m1/ Σm and an increase in
504 ribose m2/ Σm , which suggests a decrease in the use of the
505 oxidative branch of the PPP. This was in accordance with
506 the results obtained in lactate measurements and denoted a
507 clear attenuation of the flux through the oxidative PPP.
508 Similarly, the oxidative/non-oxidative ratio of PPP was
509 14% lower for TKO MEFs than for Ct MEFs (0.78 ± 0.00
510 and 0.91 ± 0.01, respectively). Equally, all calcein AM
511 treatments showed a lower oxidative/non-oxidative ratio of
512 PPP compared to the control treatments (8.42, 18, and
513 31.90% lower for the IC₂₅, IC₅₀, and IC₇₅ treated HCT116,
514 respectively, 1.27 ± 0.00 being for control HCT116 cells).
515 It has been reported that this ratio is higher in tumor cells
516 compared to normal cells (Ramos-Montoya et al. 2006).

517 To provide information on the relative importance of the
518 two pathways of pentose phosphate production for the
519 viability of the cell, we used phenotype phase-plane analysis.
520 Phenotype phase-plane analysis is the analysis of
521 substrate production and utilization of cells and is an
522 important aspect of reaction network analysis (Edwards
523 et al. 2002; Lee 2006). Figure 3 contains the phase-plane
524 analysis of the normalized ribose isotopologues m1 and
525 m2, where values for oxidative ribose synthesis are plotted
526 against non-oxidative ribose synthesis. The line of opti-
527 mality is arbitrarily defined as the line drawn through the
528 point for the basal state (Ct Control treatment or Ct MEF)
529 corresponding to conditions satisfying the optimal condi-
530 tions for growth (objective function). The slope of the line
531 represents the optimal ratio of ribose formed through the

oxidative pentose phosphate pathway to a given level of
non-oxidative ribose synthesis for the tumor cells. When a
line is drawn from a phenotype (a point on the phase-
plane), parallel to the major axis, the intersection between
the line of optimality and the parallel line indicates the
degree of optimality relative to the basal state. Using
metabolic phenotype phase-plane analysis, we saw that
increasing doses of calcein AM or the deletion of the main
CDKs of the G₁/S-phase transition resulted in a more
dramatic imbalance between oxidative/non-oxidative PPP.

According to these data, the representation of m1/ Σm vs
m2/ Σm in a phenotype phase-plane analysis confirmed the
same tendency as in calcein AM-treated cells: the deletion
of the CDKs, which phosphorylate pRb, caused an imbal-
ance of the PPP towards the non-oxidative branch (Fig. 3).

3.4 Sugar phosphate pool decreases when cell cycle does not progress

Changes in the absolute concentrations of the intermediary
sugar phosphates reflect variations in the metabolic flux
profile distribution. Pentose phosphate, triose phosphate
and hexose phosphate pools were quantified in HCT116
cells treated with IC₅₀ of calcein AM (0.6 μM) and control

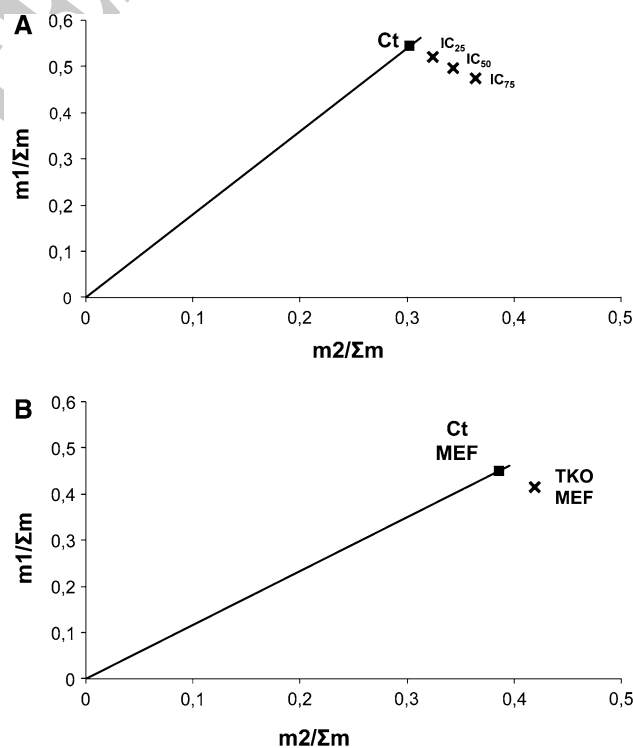


Fig. 3 ¹³C ribose label distribution. Phase-plane analysis of the normalized ribose isotopologues m1 and m2. **a** HCT116 cells treated without (Ct) or with IC₂₅, IC₅₀, and IC₇₅ doses of calcein AM; and **b** the control mouse embryonic fibroblasts (Ct MEF) and the MEF knockout for CDK4, CDK6, and CDK2 (TKO MEF)

554 and TKO MEFs (data not shown). Inhibition of CDK4 and
555 CDK6 function using a calcein AM inhibitor or the
556 knockout cell model resulted in decrease in the concen-
557 tration of fructose-1,6-bisphosphate, pentose and triose
558 phosphate intermediaries. Although, these changes were
559 not significant, they showed a tendency in which the arrest
560 in the G₁ phase of the cell cycle alters the profile of sugar
561 phosphate concentrations.

562 4 Discussion

563 Evidence indicates that CDK4 and CDK6 are excellent
564 targets for the design of new anti-tumor drugs (Landis et al.
565 2006; Yu et al. 2006; Malumbres and Barbacid 2006;
566 Marzec et al. 2006). However, the design of good specific
567 inhibitors against the activity of these kinases has not been
568 successful until now. Different strategies have been
569 employed in the search for good inhibitors but almost none
570 of them have been successful due to their unspecificity and
571 the subsequent side effects (Fry et al. 2004; McInnes 2008;
572 Menu et al. 2008). Thus, there is emerging interest in
573 developing new strategies to search for selective inhibitors
574 of CDK4 and CDK6 for cancer chemotherapy (Mahale
575 et al. 2006). To this end, in this study we used a new set of
576 bioinformatic tools to design CDK4 and CDK6 inhibitors
577 that mimic their natural inhibitor p16^{INK4a}. One of these
578 inhibitors was calcein.

579 Calcein AM is a fluorescent dye that localizes intracel-
580 lularly after esterase-dependent cellular trapping and has
581 shown cytotoxic activity against various established human
582 tumor cell lines at relatively low concentrations (Jonsson
583 et al. 1996; Liminga et al. 2000). Furthermore, Liminga
584 and collaborators found that calcein AM caused a strong
585 apoptotic response within hours of exposure and tested it
586 on a panel of ten different cell lines, but they failed to find
587 its precise mechanism of action to inhibit cell proliferation
588 (Liminga et al. 1999; Liminga et al. 2000; Liminga et al.
589 1995). According to our results, calcein carboxylic esters
590 easily penetrate HCT116 cells, inhibiting cell viability at
591 relatively low doses compared with the non-esterified calcein.
592 We have also shown that calcein (the active form
593 inside the cell of the calcein AM ester) specifically inhib-
594 ited CDK4 and CDK6 (cyclin D-related activities),
595 inducing inhibition of pRb phosphorylation, which is
596 required for entering the S-phase of the cell cycle (Lund-
597 berg and Weinberg 1998; Malumbres et al. 2004). The
598 potential of calcein to avoid the entrance of treated cells
599 into the S-phase was further validated here, as calcein AM
600 treatment on HCT116 cells provoked a strong G₁-phase
601 cell cycle arrest.

602 Having elucidated the effects of calcein on the cell cycle,
603 we proceeded to characterize in depth the effects of

604 inhibiting CDK4 and CDK6 activities on the metabolic
605 profile of the HCT116 cells. We have previously demon-
606 strated that the balance between oxidative and non-oxidative
607 branches of the PPP is essential to maintain proliferation in
608 cancer cells and is a vulnerable target within the cancer
609 metabolic network for potential new therapies for over-
610 coming drug resistance (Ramos-Montoya et al. 2006). Our
611 results here show that increasingly high calcein AM con-
612 centrations result in a stronger imbalance of PPP in favor of
613 the non-oxidative branch (Fig. 4). Using metabolic pheno-
614 type phase-plane analysis, we deduced that the most efficient
615 doses of calcein AM in the inhibition of tumor cell growth
616 result in a more dramatic imbalance between oxidative and
617 non-oxidative branches of PPP. The perturbation of this
618 imbalance results in a state of metabolic inefficiency and
619 consequently could lead to a pause in cell proliferation or
620 even cell apoptosis. To ensure that the metabolic alterations
621 induced by calcein AM in HCT116 cells were due to the
622 specific inhibition of CDK4 and CDK6 activities induced by
623 this compound, we also characterized the metabolic profile
624 of control (Ct) and triple knockout (TKO) MEFs. These
625 results showed that the lack of functionality of CDK4, CDK6
626 and CDK2 induced changes in the metabolic profile of
627 fibroblasts that correlate with the alterations induced by
628 calcein AM in the metabolic profile of HCT116 tumor cells.
629 These results support our hypothesis that inhibition of CDK4
630 and CDK6 was responsible for the oxidative/non-oxidative
631 imbalance in PPP induced by calcein AM.

632 We recently reported a specific increase in the activities of
633 two key enzymes of PPP, glucose-6-phosphate dehydroge-
634 nase for the oxidative branch and transketolase for the non-
635 oxidative branch, during the S/G₂ phases of the cell cycle, in
636 particular during the S-phase, when the synthesis of nucle-
637 otides is required. Such an increase in the PPP enzyme

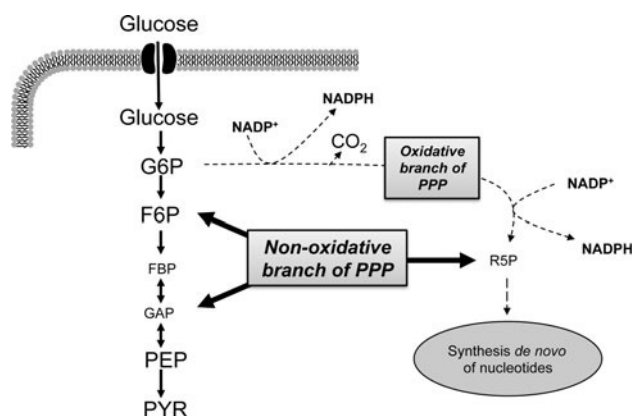


Fig. 4 Metabolic changes associated to CDK4/6 inhibition. CDK4 and CDK6 inhibition leads to an imbalance between the oxidative and non-oxidative branches of the pentose phosphate pathway towards the non-oxidative branch. *Thick lines* indicate enhanced metabolic routes. *Dotted lines* indicate less active metabolic routes and *smaller font sizes* indicate lower intermediate concentrations

638 activities correlates with a relative increase in the pentose
639 phosphate pool and a progressive increase in the balance
640 between oxidative and non-oxidative branches of PPP in the
641 S and G₂ phases (Vizan et al. 2009). This means that the
642 contribution of the oxidative branch to ribose-5-phosphate
643 synthesis is relatively increased when the cycle progresses
644 through the S-phase (Vizan et al. 2009). In this article, the
645 results support this assertion, showing a decrease in this
646 balance when HCT116 cells were treated with calcein AM or
647 when fibroblasts did not express functional CDK4, CDK6,
648 and CDK2 and their progress through the cell cycle was
649 compromised. Moreover, ¹³C incorporation from glucose
650 into RNA ribose was lower both in HCT116 treated with
651 calcein AM and in TKO MEFs, indicating that ribose-5-
652 phosphate synthesis decreases when the entrance of the cell
653 into the S-phase is inhibited. Additionally, in this work we
654 have shown that the imbalance in PPP induced by the inhi-
655 bition of CDK4 and CDK6 is able to slightly compromise the
656 balance in the overall central carbon metabolic network of
657 the cell, which is reflected in a non-significant change in the
658 levels of intermediary sugar phosphates (Fig. 4). The results
659 presented in this paper regarding the metabolic conse-
660 quences of the inhibition of CDK4 and CDK6 highlights the
661 metabolic requirements of the cell cycle and points to CDK4
662 and CDK6 as interesting drug targets to be explored in a
663 wider range of cancer types.

664 5 Concluding remarks

665 The forced imbalance of the PPP towards the oxidative
666 branch is a possible Achilles' heel in the robust tumor
667 metabolic adaptation. It has been shown that effective anti-
668 tumor strategies against this target can be designed not only
669 with drugs that force this imbalance even further (Ramos-
670 Montoya et al. 2006), but also using drugs that recover the
671 oxidative/non-oxidative balance in the non-tumor cells.
672 The data presented here demonstrate that the inhibition of
673 CDK4 and CDK6 using calcein AM not only inhibits the
674 progression of the cell cycle, but also disrupts this oxida-
675 tive/non-oxidative imbalance of PPP, which has been
676 described as essential for tumor proliferation, reinforcing
677 the interest of CDK4 and CDK6 as targets in cancer
678 therapy.

679 Furthermore, we suggest that calcein could be a key
680 factor in the development of a new family of selective
681 cyclin D-dependent kinases inhibitors based on its struc-
682 ture. The improved understanding of the specific effects of
683 the inhibition of CDK4 and CDK6 on tumor cell central
684 metabolic networks shown in this paper opens up new
685 avenues for the design of combination therapies with drugs
686 that directly inhibit those pathways and also to the use of

specific CDK4 and CDK6 inhibitors to impair metabolic
adaptations that support tumor cell cycle progression.

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