# Resorufin: a lead for a new protein kinase CK2 inhibitor

Iben Skjøth Sandholt, Birgitte Brinkmann Olsen, Barbara Guerra and Olaf-Georg Issinger

**Screening a natural compound library led to the identification of resorufin as a highly selective and potent inhibitor of protein kinase CK2. Out of 52 kinases tested, only CK2 was inhibited, in contrast to emodin, a structurally related, known CK2 inhibitor that, in addition to CK2, inhibited ten other kinases by 90%. The $IC_{50}$ values determined for the CK2 holoenzymes were 1.5 μmol/l and for the free catalytic subunits *ca.* 4 μmol/l. Altogether four cell lines were subjected to resorufin and emodin treatment. In the case of the three prostate carcinoma cell lines (PC-3, DU-145, LNCaP), 24 h treatment with 40 μmol/l resorufin led to 15–20% dead cells; however, no caspase-mediated apoptosis was observed. In the case of the colorectal carcinoma HCT116 cell line, a similar picture was obtained, yet, when resorufin was administered to cells treated with doxorubicin, apoptosis was strongly induced within 24 h. Endogenous protein kinase CK2 was inhibited by resorufin by *ca.* 80% in the three prostate cell lines. In the case of the HCT116 cells, the inhibition was only 40% supporting the notion of cell line-specific selectivity. Moreover, we analysed the effect of resorufin and emodin on selected signalling molecules in the cell lines under investigation. *Anti-Cancer Drugs* 20:238–248 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

CK2 is a ubiquitous and essential protein kinase implicated in a wide variety of cellular processes such as proliferation, apoptosis, differentiation and transformation (reviewed in Refs [1–5]). Although more than 300 substrates have been identified [6], attempts to link CK2 to individual steps of signal transduction pathways so far have failed.

Protein kinase CK2 is composed of two catalytic (α/α′) subunits attached to a dimer of noncatalytic chains (β). In contrast to most other protein kinases, CK2 has never been found in an inactive conformation, because of intramolecular restraints [7], thus yielding a constitutively active enzyme.

CK2 is detected in all eukaryotic organisms and its subunits CK2α and CKβ are essential for viability [8–10], whereas CK2α′ is required for spermatogenesis [11]. Although CK2α′ has been found predominantly in brain and testis [12] its precise role in these tissues is still unknown. However, knowledge concerning a specific interaction of potential small molecule inhibitors could be of value in treating diseases associated with these organs.

Owing to increasing reports of the involvement of CK2 in various diseases, especially cancer [1], the enzyme has been established as a 'druggable' kinase [13–16]. Six major classes of ATP-site-directed CK2 inhibitors (type I inhibitors) have been described to date (for review [16,17]): (i) flavonoids (apigenin and quercetin), (ii) hydroxyanthraquinones/xanthenones/hydroxycoumarines (emodin, 1,8-dihydroxy-4-nitroxanthen-9-one, 3,8-dibromo-7-hydroxy-4-methylchromen-2-one, 8-hydroxy-4-methyl-9-nitrobenzo[g]chromen-2-one), (iii) halogenated (aza) imidazoles [5,6-dichloro-1(β-D-ribofuranosyl)-benzimidazole, 4,5,6,7-tetrabromobenzotriazole (TBB), 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT), tetrabromocinnamic acid (TBCA)], (iv) indolo quinazolines [(5-oxo-5,6-dihydroindolo-(1,2-a)quinazolin-7-yl)acetic acid], (v) pyraxolo[1,5-a]triazines [18,19] and (vi) carboxamides/carboxylic acids [20].

Currently, TBB and DMAT are among the most popular CK2 inhibitors. Although both compounds exhibit a remarkable potency with respect to CK2 inhibition, [21–23] their selectivity for CK2 is not optimal [24].

Recent screening efforts using various compound libraries [25] led to the identification of several promising compounds, foremost the identification of polyoxometalates (POMs) as nanomolar inhibitors of CK2 [26].

In our laboratory screening of the 'NCI Mechanistic Set' led to the identification of several potential CK2 inhibitors, including phosphomolybdic acid as described

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by Prudent *et al*. [26]. However, this compound does not inhibit endogenous CK2 in all the cell lines tested so far (Krysiak *et al*., unpublished results, [26]). The reason for this lack of inhibition may be due to a lack of cell penetration, for reasons that, so far, have not been clarified. Hence, the search for small molecule inhibitors is pursued in preference to stable molecules with strong potency and selectivity and the ability to enter the cell.

Here, we report the results of a screening effort using the Natural Products Set from the 'Developmental Therapeutics Program NCI/NIH'. One of the several compounds we have identified is resorufin. The name 'resorufin' was coined almost 120 years ago [27]. Resorufin is *in senso stricto* not a natural compound since it was synthesized from resorcinol (resorcin), a natural product obtained from many resins, e.g. galbanum, asafoetida, and Brazilwood extract. Resorufin belongs to the class of compounds denoted as 'phenoxazin-3-ones'. Whereas resorcinol is used both medically (e.g. as an antiseptic and disinfectant) and chemically (e.g. for production of diazo dyes and plasticizers), resorufin, because of its strong fluorescent characteristics, is preferably used as a methodological tool in cell biology, and for various other applications such as in casein–resorufin, where it serves as a substrate in protease-detecting assays [28–30]. So far no medically relevant application for resorufin, in particular, has been reported, although a recent report has shown that phenoxazines exhibit tumour-specific cytotoxic activity [31].

The major objective for screening the natural compound library was to identify possible CK2 specific inhibitors with high selectivity and potency *in vitro* with the ability to penetrate cells and are thus able to efficiently inhibit endogenous CK2 activity.

We have identified and characterized resorufin as a very selective inhibitor of protein kinase CK2. From a panel of 52 kinases only protein kinase CK2 was inhibited. In contrast, with emodin, a structurally related known CK2 inhibitor, ten other kinases out of 32 were inhibited too, partly to the same extent as CK2, making this compound non-selective with respect to CK2. Resorufin was shown to inhibit endogenous CK2 activity in three prostate carcinoma cell lines (80% inhibition) and one colorectal carcinoma cell line (40% inhibition) at a concentration of 40 µmol/l within 24 h. Hence, the high selectivity, reasonable potency, and efficient inhibition of endogenous CK2 in cell lines make this compound a promising candidate to target protein kinase CK2.

The identification of resorufin as a highly specific and potent inhibitor for the anti-apoptotic protein kinase CK2 is a good example for the rationale to screen known compounds, even when known for more than 100 years, for possible present day applications [32,33].

## Materials and methods

$CK2\alpha^{1-335}$, CK2α-His, CK2α′-His, MBP-CK2α′, $CK2\alpha'_2\beta_2$, $CK2\alpha_2\beta_2$, $CK2\alpha^{1-335}{}_2\beta_2$ were expressed and purified as described earlier [23,34–36]. DYRK2-His was expressed in *E. coli* and the kinase domain of RIPK1 (His-RIPK1) was expressed in Sf9 cells. Both proteins were purified by Ni-sepharose chromatography (GE Healthcare, Brondby, Denmark). Protein kinases AKT1, AKT2, AKT3, CHK2, ERK1, ERK2, JNK2α2, MEK1, MKK6, PI3K (p110α/p85α), PKA, PKCα, p38α were from KinaseDetect, Odense, Denmark; AMPK, CaMKII, CDK1/CyclinB1, CHK1, CK1, GSK3β, LKB1/STRADα/MO25α, MLK1, PIM1, SRC were from Upstate Biotechnology, Frederikssund, Denmark and CDK2/CyclinA was from Carna Biosciences Inc., Kobe, Japan.

MBP, CaMKII substrate cocktail, Histone H1, SAMS peptide, S6 kinase/RSK2 peptide, Woodtide and PKC lipid activator were from Upstate. $PIP_2$ was from Jena Biosciences, Jena, Germany. Other substrate peptides were from KinaseDetect.

The following primary antibodies were used: monoclonal anti-β-actin (Sigma, Brondby, Denmark); anti-AKT1 and anti-GSK3β (both from BD Biosciences, San Jose, California, USA); anti-CHK1 and anti-MDM2 (both from Santa Cruz Biotechnology, Santa Cruz, California, USA); anti-phospho-ERK1/2 (T202/Y204) (Cell Signaling Technology, Beverly, Massachusetts, USA); anti-CHK2 and anti-PTEN (Upstate); anti-PARP (BD Pharmingen, San Diego, California, USA); anti-p53, anti-CK2α/α′ and anti-CK2β (all from Calbiochem, Nottingham, UK).

Polyclonal anti-cyclin A, anti-cyclin B were both from Santa Cruz; anti-phospho-AKT (T308), anti-phospho-CHK2 (T68), anti-phospho-CDC2 (Y15), anti-ERK1/2, anti-AR, anti-phospho-GSK3β (S9), anti-phospho-p53 (S15) were all from Cell Signaling; anti-phospho-AKT1 (S473) was from Biosource, Invitrogen Life Technologies, Inc., Carlsbad, California, USA; anti-$p21^{WAF1/CIP1}$ was from Calbiochem. Polyclonal anti-CK2α′ was raised in rabbits immunized with the peptide SQPCAD NAVLSSGTAAR from human CK2α′.

### Screening of natural compounds

Screening of 235 compounds from a natural compound library (Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, the National Cancer Institute (NCI), USA) was performed using a luminometric approach. Compounds were tested at a final concentration of 10 µmol/l. The experimental setup corresponded to that for the PI3K.

### Luminometric kinase activity test

Determination of PI3K activity was performed as described [37]. In short, 200 ng PI3K were assayed against 54 μmol/l $PIP_2$ in 25 mmol/l Tris–HCl pH 7.5, 5 mmol/l NaCl, 5 mmol/l DTT, 0.0125% Brij35. The reaction was initiated upon addition of 1 μmol/l ATP/20 mmol/l $MgCl_2$. Kinase activity was allowed for 30 min at 20°C in 40 μl in 96-well plates, 40 μl Easylite-Kinase reagent (PerkinElmer, Hvidovre, Denmark) were added and luminescence was measured in a luminometer (VictorLight, PerkinElmer). Activity was calculated as the reduction in luminescence relative to samples without PI3K.

### Radioactive kinase activity test

Radioactive kinase activity tests were performed for 10 min at 30°C in 40 μl (50 μl for PKCα due to addition of 10 μl lipid activator) containing 125 μmol/l ATP, 20 mmol/l $MgCl_2$ and 0.6 μCi [γ-$^{32}$P]-ATP ((3000 Ci/mmol), Hartmann Analytic, Braunschweig, Germany) according to the manufacturer's instructions. Samples were spotted onto P81 phosphocellulose paper and washed extensively in 0.85 mmol/l phosphoric acid. Incorporation of radiolabelled phosphate was measured by counting samples in a liquid scintillation counter (Canberra-Packard, Downers Grove, Illinois, USA).

For specificity analysis, 10 μmol/l resorufin or emodin were used, whereas increasing concentrations from 0.08 to 80 μmol/l were used for $IC_{50}$ determinations. $IC_{50}$ values were extrapolated from plots of CK2 activity against inhibitor concentration. For the kinetic analysis increasing resorufin and emodin concentrations were applied. Kinetic parameters ($K_M$ and $V_{max}$) were calculated using the program ENZPACK (Biosoft, Cambridge, UK) as average based on Lineweaver–Burk, Eadie–Hofstee plots and the direct linear method. $K_i$ values were determined based on these averages.

### Out-of-house kinase profiling

Protein kinases indicated by an asterisk (Table 2) were screened against resorufin by Carna Biosciences using a regular mobility shift assay as described by the company. Emodin was not included in this screening service except for protein kinases DYRK1a, HIPK2, PIM2, PIM3 and PKD1, which were also tested against inhibitors DMAT and TBB. The ATP concentrations used by Carna Biosciences vary between 5 and 150 μmol/l.

### Cell cultures and treatments

The human prostate carcinoma cell lines PC-3, DU-145 and LNCaP (all from the German Collection of Microorganisms and Cell Cultures (DSMZ) Braunschweig, Germany) were grown in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum. The human colorectal carcinoma cell line HCT116 (American Type Culture Collection (ATCC) Manassas, Virginia, USA) was grown in Dulbecco's modified Eagle's medium and 10% fetal bovine serum. All cell lines were grown at 37°C in a 5% $CO_2$ atmosphere. One day after seeding, cells were treated for 24 h with 40 μmol/l resorufin, emodin, 0.5 μmol/l doxorubicin or a combination as indicated in the figure legends. The trypan blue dye exclusion assay was performed as described earlier [38].

### Preparation of protein extracts, western blot analysis and protein kinase assays

Before harvesting, cells were washed extensively in PBS, collected by centrifugation and resuspended in cold lysis buffer (50 mmol/l Tris–HCl pH 7.5, 150 mmol/l NaCl, 1% Triton-X-100, 10% glycerol, 1 mmol/l DTT, 30 mmol/l $Na_4PP_i$, 10 mmol/l NaF, 1 mmol/l $Na_3VO_4$, 100 nmol/l okadaic acid) containing a protease inhibitor cocktail (Roche, Penzberg, Germany). Lysates were cleared by centrifugation at 4°C for 20 min at 13000*g*. Whole-cell extracts were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis, western blot or kinase activity assays.

Proteins were detected by using specific antibodies and protein–antibody complexes were visualized by a chemiluminescence western blotting detection system according to the manufacturer's instructions (CDP-Star, Applied Biosystems, Foster City, California, USA). Protein kinase CK2 activity was measured, similar to the radioactive kinase test on recombinant proteins, in a total volume of 50 μl containing 25 mmol/l Tris–HCl pH 8.5, 150 mmol/l NaCl, 5 mmol/l $MgCl_2$, 1 mmol/l DTT, 125 μmol/l [γ-$^{32}$P]-ATP, 200 μmol/l CK2 peptide and 5 μg protein extract.

## Results

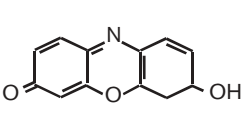
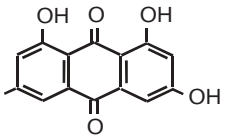
### Resorufin is a novel and selective inhibitor of protein kinase CK2

We have set up a luminometric-based assay, screening 235 natural substances from the Natural Products Set (DTP, NIH/NCI) in the search for novel compounds able to efficiently inhibit the activity of protein kinase CK2. We identified resorufin (7-hydroxy-3*H*-phenoxazin-3-one) as an effective inhibitor of both the catalytic CK2 subunits and the tetrameric holoenzymes.

To determine whether resorufin was an inhibitor of CK2, the $IC_{50}$ and $K_i$ values for four CK2 constructs were determined for resorufin and compared with emodin, a structurally related CK2 inhibitor [39].

The enzyme kinetic assays show that resorufin inhibits $CK2\alpha^{1-335}{}_2\beta_2$ (holoenzyme) and $CK2\alpha^{1-335}$ (catalytic subunit) activity as a competitive inhibitor against ATP with $K_i$ values of 0.8 and 1.3 μmol/l, respectively. The corresponding $K_i$ values of emodin were determined as 0.2 and 0.6 μmol/l, respectively. The enzyme kinetic assays were also performed with the CK2α′ subunit paralogue and

**Table 1** $IC_{50}$ and $K_i$ for resorufin and emodin towards protein kinase CK2

| | Resorufin | | Emodin | |
|---|---|---|---|---|
| |  | |  | |
| | $IC_{50}$ | $K_i$ | $IC_{50}$ | $K_i$ |
| $CK2\alpha^{1-335}{}_2\beta_2$ | 1.5 | 0.8 | 2.4 | 0.2 |
| $CK2\alpha'_2\beta_2$ | 1.4 | 0.7 | 2.5 | 0.2 |
| $CK2\alpha^{1-335}$ | 4.1 | 1.3 | 3.2 | 0.6 |
| CK2α′-His | 3.8 | 0.8 | 4.3 | 0.2 |

$IC_{50}$ values (in μmol/l) were calculated for the different CK2 constructs in the presence of 125 μmol/l ATP. The $K_i$ values were determined at different ATP concentrations. The values are averages of at least two independent experiments.

its corresponding holoenzyme. Both holoenzymes exhibit distinct lower $IC_{50}$ values towards resorufin and emodin than the free catalytic subunits (Table 1).

The $K_i$ values for the paralogue subunit (CK2α′) and its holoenzyme seem to differ slightly from the values measured for the CK2α-based constructs. In the case of resorufin, the $K_i$ values for the CK2α′-based holoenzyme and CK2α′ subunit were 0.2 and 0.3 μmol/l, respectively, and for emodin 0.3 μmol/l for both enzymes (Table 1).

We then analysed the selectivity of resorufin and, for comparison, emodin towards CK2 by using a panel of 52 kinases and the CK2 constructs whose activity was determined in the presence of 10 μmol/l resorufin and emodin, respectively. The residual kinase activity was expressed as the percentage of control activity determined in the absence of inhibitors (Table 2). The analysis of the obtained results revealed that resorufin did not exert a significant inhibitory effect on any of the tested protein kinases except for the CK2 constructs, where the highest residual kinase activity was 39% in the case of MBP-CK2α′ and the lowest 14% in the case of CK2α′-His. Resorufin affected the other protein kinases only marginally, but the degree of inhibition exceeded only 30% for the tyrosine kinase SYK and serine/threonine kinase HIPK2 and PIM3. In contrast, emodin also inhibited AMPK, CaMKII, DYRK1a, DYRK2, HIPK2, PI3K, PIM1, PIM2, PIM3 and PKD1 by more than 50% (Table 2). Part of the kinase screening was performed by Carna Biosciences (indicated by an asterisk). As emodin served as a mere control, it was not included in the out-of-house screening efforts, with the exception of DYRK1a, HIPK2, PIM2, PIM3 and PKD1. The latter kinases were also tested with established CK2 inhibitors DMAT and TBB. Strong inhibition was observed, confirming published data from Pagano *et al.* [24].

**Resorufin induces cell death in prostate carcinoma cells**

On the basis of the data obtained *in vitro*, we tested whether resorufin would be cell permeable, capable of inhibiting protein kinase CK2 *in vivo* and, thus, affect cell proliferation. A similar experiment was performed in the presence of emodin. We initially tested three human prostate cancer cell lines (i.e. PC-3, DU-145 and LNCaP) by incubating them in the presence of 40 μmol/l resorufin and emodin, respectively, for 24 h. As shown in Fig. 1a, both resorufin and emodin caused a reduction in adherent cell number in all three prostate carcinoma cell lines in comparison with untreated controls. Trypan blue staining (Fig. 1b) showed that both resorufin and emodin affected cell viability. PC-3 cells were the least affected by resorufin treatment. After 24 h, approximately 13% of the cells were dead. In the case of LNCaP cells, there was a 20% drop in viability after incubation with resorufin or emodin, whereas DU-145 cells appeared to be slightly more sensitive towards resorufin (i.e. 20% of cells were dead) than emodin where approximately 14% of cells were dead. To test whether the cytotoxic effect of resorufin was because apoptosis had been induced, we examined whole lysates from cells treated as indicated in Fig. 1c by western blot analyses. Immunoblots probed with anti-PARP antibody did not show caspase-mediated PARP cleavage in any of the cell lines incubated with resorufin, whereas in the case of emodin a weak signal was detected only in LNCaP cells. Consistent with these results are studies by Cha *et al.*, [12] showing that androgen-sensitive cell lines (i.e. LNCaP, PC-3-AR) exhibit higher sensitivity towards emodin than androgen-unresponsive cell lines (i.e. DU-145 and PC-3).

Next, we tested resorufin for its ability to inhibit endogenous protein kinase CK2. Lysates from the indicated cell lines grown for 24 h in the absence and presence of 40 μmol/l resorufin and emodin, respectively, were used as a source for measuring endogenous CK2 activity.

As shown in Fig. 2a, CK2 activity was significantly inhibited upon incubation with resorufin or emodin, because the calculated residual activity was 20% or below with respect to the control experiments, indicating that resorufin efficiently penetrated the cells. We also tested whether a reduction in the expression levels of the individual CK2 subunits was responsible for the lower kinase activity, which followed cell incubation with resorufin. As shown in Fig. 2b, we excluded this possibility, as we did not observe any change in the expression of the CK2 subunits in any of the three tested cell lines. Next, the aforementioned cell lines were tested with respect to the expression levels and/or the phosphorylation status of various signalling proteins, which play a role in cell proliferation and survival (Fig. 2c).

**Table 2 Specificity of resorufin and emodin**

| Kinase | | Resorufin | Emodin |
|---|---|---|---|
| ABL* | Tyr | 97 | – |
| AKT1 | Ser/Thr | 95 | 83 |
| AKT2 | Ser/Thr | 98 | 81 |
| AKT3 | Ser/Thr | 96 | 96 |
| AMPK | Ser/Thr | 95 | 41 |
| CaMKII | Ser/Thr | 94 | 46 |
| CDK1/Cyclin B1 | Ser/Thr | 98 | 70 |
| CDK2/Cyclin A | Ser/Thr | 93 | 55 |
| CHK1 | Ser/Thr | 143 | 83 |
| CHK2 | Ser/Thr | 106 | 78 |
| CK1 | Ser/Thr | 98 | 100 |
| $CK2\alpha_2\beta_2$ | Ser/Thr | 29 | 6 |
| $CK2\alpha^{1-335}{}_2\beta_2$ | Ser/Thr | 27 | 6 |
| $CK2\alpha'_2\beta_2$ | Ser/Thr | 31 | 8 |
| $CK2\alpha^{1-335}$ | Ser/Thr | 24 | 10 |
| CK2α′ (MBP) | Ser/Thr | 39 | 16 |
| CK2α (His) | Ser/Thr | 14 | 8 |
| CK2α′ (His) | Ser/Thr | 29 | 7 |
| CSK* | Tyr | 95 | – |
| DYRK1a* | Ser/Thr | 79 | 7 |
| DYRK2 | Ser/Thr | 84 | 27 |
| EGFR* | Tyr | 92 | – |
| EphA2* | Tyr | 99 | – |
| EphB4* | Tyr | 85 | – |
| ERK1 | Ser/Thr | 100 | 101 |
| ERK2 | Ser/Thr | 100 | 96 |
| FGFR1* | Tyr | 98 | – |
| FLT3* | Tyr | 75 | – |
| GSK3β | Ser/Thr | 96 | 64 |
| HIPK2* | Ser/Thr | 69 | 24 |
| IGF1R* | Tyr | 101 | |
| ITK* | Tyr | 94 | – |
| JNK2α2 | Ser/Thr | 90 | 52 |
| JAK3* | Tyr | 91 | – |
| KDR* | Tyr | 91 | – |
| LCK* | Tyr | 94 | – |
| LKB1/STRADα/MO25α | Ser/Thr | 89 | 92 |
| MEK1 | Ser/Thr | 101 | 101 |
| MET* | Tyr | 93 | – |
| MKK6 | Ser/Thr | 113 | 107 |
| MLK1 | Ser/Thr | 90 | 51 |
| PDGFRA* | Tyr | 98 | – |
| PI3K[a] | Lipid | 97 | 28 |
| PIM1 | Ser/Thr | 93 | 10 |
| PIM2* | Ser/Thr | 83 | 5 |
| PIM3* | Ser/Thr | 68 | 0 |
| PKAα | Ser/Thr | 95 | 93 |
| PKCα | Ser/Thr | 104 | 87 |
| PKD1* | Ser/Thr | 99 | 28 |
| PYK2* | Tyr | 103 | – |
| p38α | Ser/Thr | 95 | 93 |
| RIPK1 | Ser/Thr | 80 | 54 |
| SRC | Tyr | 100 | 83 |
| SYK* | Tyr | 52 | – |
| TIE2* | Tyr | 100 | – |
| TRKA* | Tyr | 98 | – |
| TYRO3* | Tyr | 97 | – |

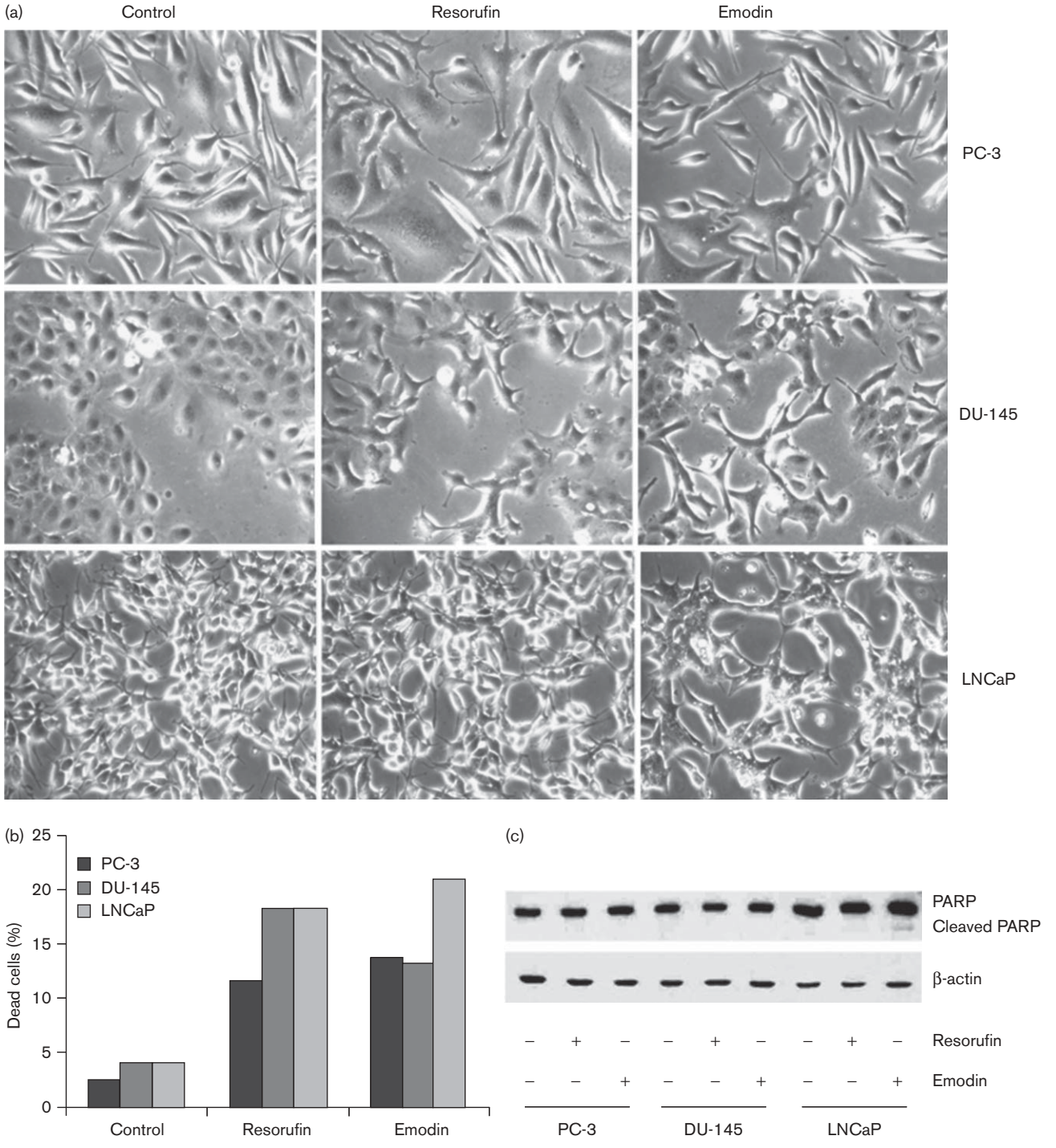
Residual kinase activities were determined in the presence of 10 μmol/l resorufin or emodin and 125 μmol/l ATP and are expressed as percentage of the control.
[a]PI3K was tested in the presence of 1 μmol/l ATP.
*Tested by Carna Biosciences at different ATP concentrations. Emodin showed inhibition in 10 out of 32 kinases (threshold was set to 50% inhibition).

Western blot analyses of whole lysates from LNCaP cells, which are androgen responsive, PC-3 and DU-145, which are hormone unresponsive cell lines, revealed that the expression of the androgen receptor decreased upon resorufin or emodin treatment. Androgen receptor responsiveness has previously been shown to be reduced after treatment with the CK2 inhibitors, emodin and TBB, in human prostate carcinoma cell lines [12,40] making them valuable therapeutic compounds for the treatment of prostate cancer, as androgen signalling promotes the development and progression of prostate cancer. Next, we examined the phosphorylation and expression levels of p53 in DU-145 and LNCaP, which express mutant and wild-type p53, respectively, (PC-3 cells are p53 null). Both resorufin and emodin promote ATM mediated, ATR-mediated, DNA-PK-mediated phosphorylation of p53 at serine 15 in DU-145 cells, whereas in LNCaP cells only emodin treatment enhanced the expression and phosphorylation levels of p53 which is in agreement with the results from Yu *et al.* [41]. The analysis of the checkpoint kinases CHK1 and CHK2 revealed a significant decrease in CHK1 protein levels in PC-3 cells treated with resorufin or emodin as compared with untreated cells. In the case of DU-145 and LNCaP cell lines, only a modest decrease in CHK1 expression levels was observed in the case of emodin treatment. Although the treatment with the aforementioned compounds did not affect the expression level of CHK2 in any of the tested cells, the phosphorylation of threonine 68, which is known to be mediated by ATM upon DNA damage [42], slightly increased in all three cell lines after treatment with resorufin and emodin, respectively. Next we analysed components of the PI3K pathways, that is, PTEN, AKT/PKB and GSK3β after treatment with the indicated compounds. We did not observe a significant variation in the expression levels of PTEN (DU-145 is the only cell line expressing wild-type PTEN, whereas PC-3 and LNCaP are PTEN null), AKT1 and GSK3β before and after treatment with emodin or resorufin. The PDK1-mediated phosphorylation of AKT1 at threonine 308, although detectable only in LNCaP cells, remained unchanged with respect to control experiments whereas the Rictor-mTOR-mediated phosphorylation of serine 473 increased upon treatment with resorufin in PC-3 and DU-145 cells. In agreement with published data [43], it was noticed that loss of PTEN in PC-3 and LNCaP cells correlated with highly phosphorylated AKT1 at serine 473. The analysis of one of the major components of the MAP kinase signalling pathway, the extracellular-regulated kinase (ERK) also known as p44/42 MAP kinase, whose activation correlates with proliferation and cancer progression, did not reveal any change in the protein expression levels, whereas the MEK1-mediated phosphorylation at threonine 202 and tyrosine 204 amino acid residues detected only in DU-145 was inhibited by cell treatment with both resorufin and emodin.

### Resorufin sensitizes human colorectal carcinoma HCT116 cells to doxorubicin and induces apoptosis

In order to see whether the effects observed with the three prostate carcinoma cell lines upon resorufin treatment were general or only confined to this particular type of cancer, we extended our studies to a colorectal cancer cell line, that is, HCT116. Cells were treated with

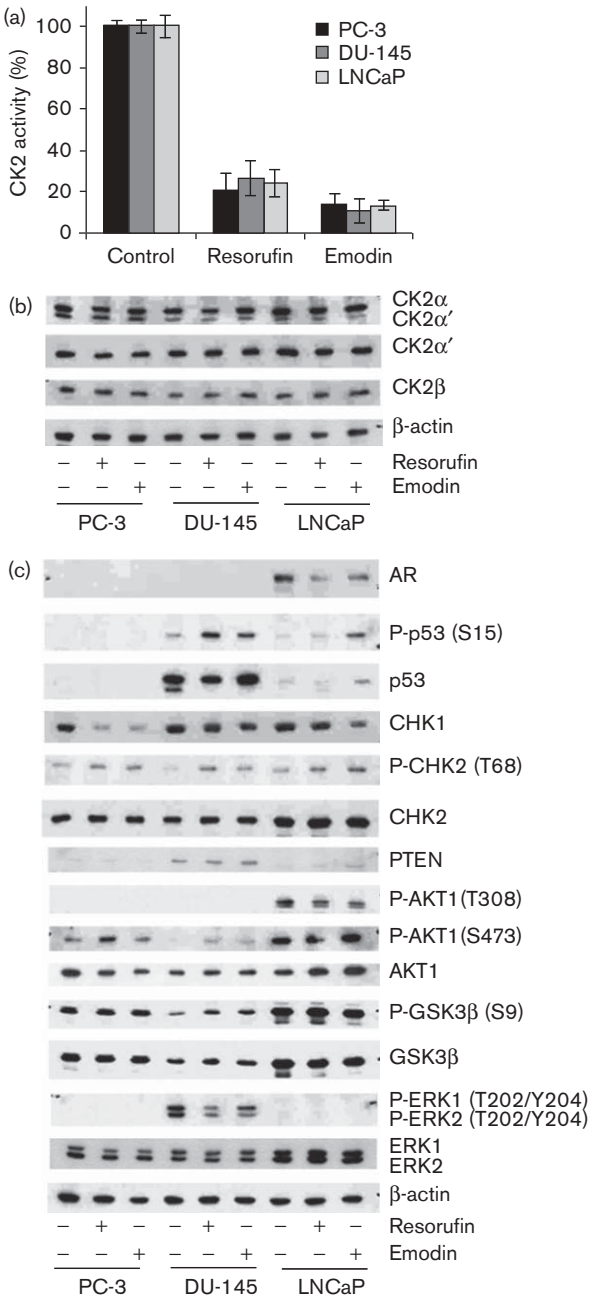
**Fig. 1**




Effect of resorufin and emodin on cell morphology and cell death. Prostate cancer cell lines (PC-3, DU-145, LNCaP) were treated for 24 h with 40 μmol/l resorufin or emodin. (a) Phase contrast microscopy of prostate cancer cell lines (PC-3, DU-145, LNCaP). Original magnification: × 200. (b) Cell death was measured as the percentage of trypan blue dye positive cells. (c) PARP cleavage analysis. β-actin was used as loading control.

resorufin or emodin as indicated in Fig. 3. Most cancer cells acquire resistance towards cell death-inducing drugs such as doxorubicin, making it necessary to design more effective therapeutic strategies, such as combination therapy, which would enhance the efficacy of chemotherapeutic agents. The ability of emodin and other polyphenol derivatives to potentiate the effect of numerous chemotherapeutic agents such as doxorubicin

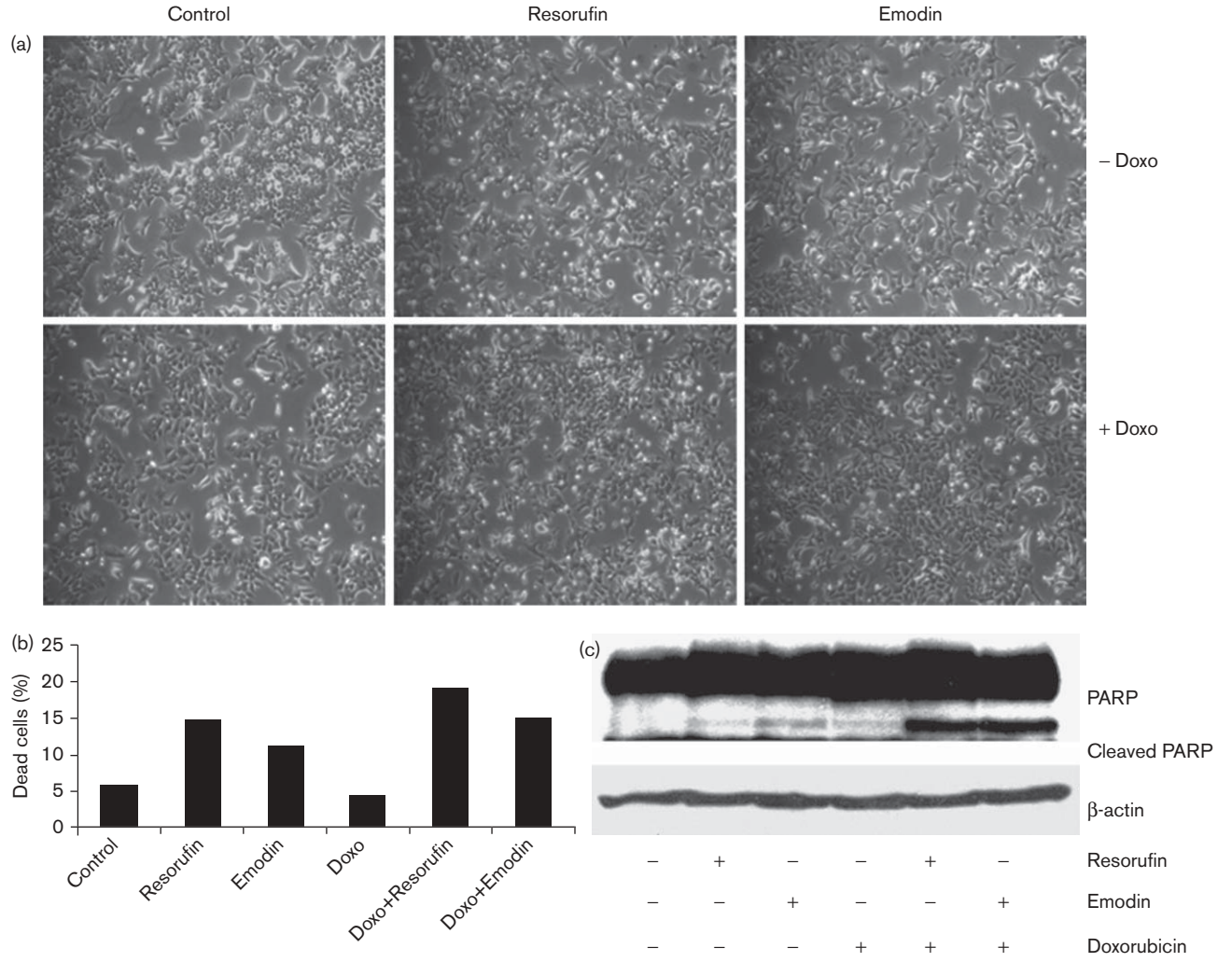
**Fig. 2**




Inhibition of endogenous CK2 by resorufin or emodin and their effect on signalling molecules. Prostate cancer cell lines (PC-3, DU-145, LNCaP) were treated for 24 h with 40 μmol/l resorufin or emodin. (a) CK2 kinase activity in cell lysates was determined against the synthetic CK2 peptide RRRADDSDDDDD in the presence of 125 μmol/l ATP. The activities represent the means ± standard deviation from three independent experiments. (b) Western blot analysis of CK2 subunits. (c) Protein levels and phosphorylation status were analysed by western blot in PC-3, DU-145 and LNCaP cell lysates. β-actin was used as loading control.

[42,44], prompted us also to investigate whether resorufin sensitized colorectal cancer cells to doxorubicin treatment and to probe the molecular mechanism(s) of resorufin-mediated chemotherapeutic effects. Doxorubicin treatment was confined to HCT116 cells because of the ample reports in the literature using this cell line for combinatorial treatment experiments in the presence of doxorubicin. We treated cells as indicated in Fig. 3a. The treatment of HCT116 cells with 0.5 μmol/l doxorubicin for up to 24 h led to a progressive accumulation of cells in the G2/M phase because of doxorubicin-mediated DNA damage as indicated by the lower cell density observed with respect to control cells under the phase-contrast microscope (Fig. 3a) and by fluorescence activated cell sorting analysis of cells exposed for a longer period to doxorubicin as well (up to 48 h, results not shown). Cell treatment with resorufin, emodin or in combination with doxorubicin led to cell death instead (Fig. 3a). We performed a trypan blue assay for the quantification of cell viability, which was significantly lower in cells treated with resorufin and emodin, respectively, than in control cells, whereas co-treatment with doxorubicin did not significantly alter cell viability with respect to treatment with the individual inhibitors (Fig. 3b). Next, we examined whole lysates from cells treated as indicated in Fig. 3a by western blot analysis to verify whether the treatment with resorufin sensitized cells to doxorubicin through the induction of apoptosis (Fig. 3c). Immunoblots probed with anti-PARP antibody revealed that the caspase-mediated PARP cleavage was markedly visible in cells co-treated with doxorubicin and resorufin or emodin but not in control cells or in cells incubated solely with doxorubicin, emodin or resorufin. Native CK2 activity measured in whole lysates showed that emodin was more efficient than resorufin in inhibiting the CK2 kinase activity *in vivo* (Fig. 3). In cells treated with resorufin, the CK2 activity was inhibited by approximately 40%, whereas the remaining kinase activity was less than 20% in the case of emodin treatment (Fig. 4a) and the indicated treatments did not affect the expression levels of the CK2 individual subunits (Fig. 4b). It is worthwhile noting that the efficiency of resorufin inhibition of CK2 is cell line dependent, as endogenous CK2 was inhibited by 80% in the case of prostate cell lines. HCT116 cells that express wild-type p53, showed p53 accumulation upon doxorubicin treatment (Fig. 4c). In accordance with the expression level of p53 in these cells, upregulation of MDM2 and $p21^{WAF1/CIP1}$ proteins was also seen. When cells were co-treated with doxorubicin and resorufin or emodin we did not see a progressive accumulation of p53 and $p21^{WAF1/CIP1}$. Concomitantly, a decrease in MDM2 expression was also observed under the same treatment conditions. The analysis of cell cycle regulatory proteins from cells treated with resorufin or emodin in the absence or presence of doxorubicin, respectively, affected the expression levels of CHK1 (Fig. 4c). In contrast, we did not observe changes in the expression of CHK2. The cyclin A and cyclin B protein levels were also markedly reduced under the same conditions. Finally, the analysis

**Fig. 3**




Effect of resorufin and emodin on cell morphology and cell death. The human colorectal cell line, HCT116 was treated for 24 h with 40 μmol/l resorufin, 40 μmol/l emodin and/or 0.5 μmol/l doxorubicin (Doxo). (a) Phase contrast microscopy of HCT116 cells. Original magnification: × 100. (b) Cell death was measured as the percentage of trypan blue dye positive cells. (c) PARP cleavage analysis. β-actin was used as loading control.

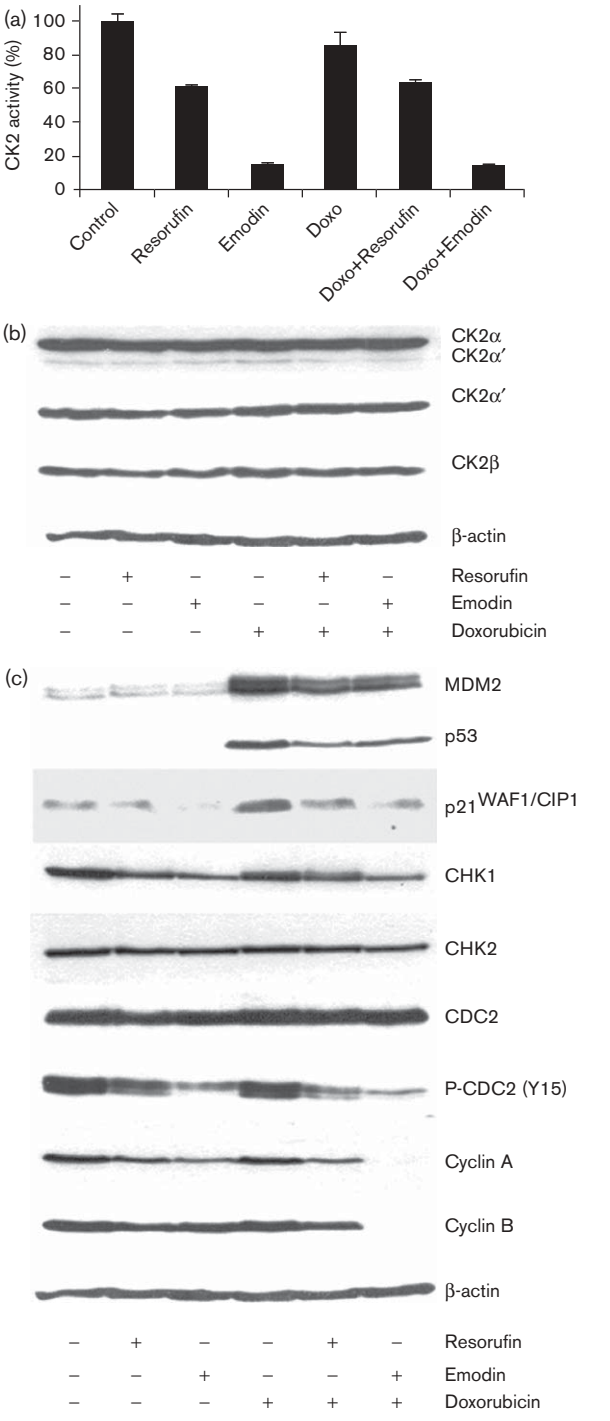
of the protein and phosphorylation levels of CDC2 kinase revealed no variations in the expression levels of CDC2, whereas the phosphorylation of the inhibitory tyrosine 15 markedly decreased after treatment with resorufin and emodin, respectively, and in the case of co-treatment with doxorubicin.

## Discussion

The $IC_{50}$ values obtained for resorufin and emodin for the two CK2 holoenzymes are distinctively lower than for the free CK2 catalytic subunits (Table 1). Yim *et al.* [39] measured an $IC_{50}$ value of 2 μmol/l for emodin by using the CK2α-based holoenzyme, which compares well with the $IC_{50}$ value we determined, that is, 2.4 μmol/l (Table 1). The $IC_{50}$ value for the CK2α′-based holoenzyme using emodin was determined to be 2.5 μmol/l. Hence, both CK2 holoenzymes show the same $IC_{50}$ values for emodin and the same is true for resorufin, albeit the values are lower. A similar picture is seen for the free catalytic subunits. Hence, the major conclusion drawn from these observations is that the holoenzymes are more specifically inhibited than the free subunits. Structural analyses of CK2 together with resorufin may possibly explain these observations.

Emodin and resorufin are ATP competitive inhibitors. With resorufin, the $K_i$ values for $CK2\alpha^{1-335}{}_2\beta_2$ and $CK2\alpha'_2\beta_2$ are 0.8 and 0.7 μmol/l, respectively. The $K_i$ values for the free subunits – $CK2\alpha^{1-335}$ and CK2α′-His – are 1.3 and 0.8 μmol/l, respectively. The $K_i$ values for emodin in the case of the holoenzymes were both 0.2 μmol/l (Table 1). Yim *et al.* [39] found a $K_i$ value

**Fig. 4**




Inhibition of endogenous CK2 by resorufin or emodin and their effect on signalling molecules involved in cell cycle regulation. The human colorectal cell line HCT116 was treated for 24 h with 40 μmol/l resorufin, 40 μmol/l emodin and/or 0.5 μmol/l doxorubicin. (a) CK2 kinase activity in cell lysates was determined as described in the legend to Fig. 2. The activities represent the means ± standard deviation from triple determinations. (b) Western blot analysis of CK2 subunits. (c) Levels and phosphorylation status of proteins involved in cell cycle regulation were analysed by western blot of HCT116 cell lysates. β-actin was used as loading control.

of 7 μmol/l for emodin for the CK2α-based holoenzyme, which is significantly higher than our value of 0.2 μmol/l. The reason for this discrepancy is at the moment not clear.

We have tested 31 serine/threonine protein kinases including CK2 constructs, $CK2\alpha^{1-335}{}_2\beta_2$ and $CK2\alpha'_2\beta_2$, 20 tyrosine kinases and one lipid kinase together with five additional CK2 constructs in the presence of 10 μmol/l resorufin or emodin, respectively (Table 2). Resorufin inhibited only the CK2 constructs, the tyrosine kinase SYK (48% inhibition), HIPK2 (31%) and PIM3 (32%). Emodin inhibited, besides CK2 and its subunits, the lipid kinase PI3K and nine other protein kinases up to 90%. In particular, though, emodin caused drastic of DYRK1 (93%), DYRK2 (73%), PIM1 (90%), PIM2 (95%), PIM3 (100%), PKD1 (72%) and PI3K (72%). Although the structures of the two compounds are related they exhibit a distinct difference in their selectivity profile strongly supporting the notion that resorufin may bind very specifically to the ATP binding pocket of CK2.

A comparison of the remaining activities of protein kinase CK2 constructs between resorufin and emodin (Table 2) shows higher values for resorufin than emodin, albeit they exhibit similar $IC_{50}$ values (Table 1). In the case of the MBP-CK2α′, a higher remaining activity for both resorufin and emodin can be explained by the presence of the large tag that even exceeds the size of the kinase and thus may be responsible for the observed attenuation of inhibition.

The remaining activities seen for the CK2 holoenzymes in the presence of resorufin are 29 and 27% in comparison with emodin, where 6% remaining activity was measured. One reason for this discrepancy may be the different behaviour of CK2 towards inhibitors at concentrations of 10 μmol/l and above. Dose-dependent CK2 activity measurements by [23] using different known CK2 inhibitors, for example, DMAT, TBB, apigenin and emodin at concentrations from 0.1 to 10 μmol/l, showed interesting differences in the degree of inhibition. Most inhibitors were virtually ineffective until the threshold of greater than 1 μmol/l was reached, followed by significant differences in the degree of inhibition especially at a concentration of 10 μmol/l. This difference in dose-dependent inhibition may explain the observed differences between emodin and resorufin when used at concentrations of 10 μmol/l or above.

Moreover, it has been shown by Niefind *et al.* [45] and Raaf *et al.* [46] that CK2 is peculiar with respect to ATP binding. Although our data point to competitive binding of resorufin to the CK2 holoenzyme and the free catalytic CK2 subunit, specific interactions between enzyme and inhibitor, as revealed by structural investigations, exist

with respect to either one [46,47]. The CK2 holoenzyme has two ATP binding sites, albeit only one has been shown to be filled with ATP [45]. Longer incubation of CK2 with resorufin showed increased potency of the drug (results not shown), similar for what has been observed for BIRB 0796 inhibitor in the case of p38 kinase (for review see Ref. [48]).

Remarkable is the lack of inhibition from resorufin towards the other protein kinases tested, especially towards kinases such as PIM1 and DYRK1a, which have been targeted by all other CK2 inhibitors so far [24]. Hence, resorufin may indeed be a promising lead for developing highly specific and selective inhibitors targeting protein kinase CK2.

The data suggest that under our experimental conditions, the reduced cell viability that followed resorufin treatment could be accounted for by the efficient inhibition of protein kinase CK2 observed *in vitro*. The absence of PARP cleavage indicates that resorufin did not induce activation of caspase-mediated apoptosis or that a longer incubation time and/or a higher dosage was necessary for inducing apoptosis.

The effect of the two drugs with respect to cell death in the four investigated cell lines is interesting. Least affected were PC-3 and HCT116 cells by both drugs, whereas DU-145 and LNCaP cells showed a significant higher percentage of dead cells. With respect to LNCaP cells, the results could be explicable inasmuch as this cell line was derived from an early stage of prostate carcinoma, still harbouring wild-type p53 and being hormone responsive. DU-145 cells, however, resemble more PC-3 cells inasmuch that they harbour mutated p53, and are unresponsive to hormone treatment and are derived from an advanced prostate carcinoma.

Another example is CHK1 expression in the prostate cancer cell lines. Although emodin leads to a clear downregulation of CHK1 expression in LNCaP cells, this downregulation is also observed upon treatment with resorufin in the case of the colorectal HCT116 cell line. The latter observation is also seen in the p53 mutant containing PC-3 prostate carcinoma cell line. Hence, although p53 and CHK1 are protagonists of cell cycle regulation, and moreover, p53 is a substrate for CHK1, the response towards drugs, seems to be strongly dependent on the cell lines used, irrespective of whether they are derived from a tumour of the same site or not. This observation is also true for the degree of endogenous CK2 inhibition upon resorufin and emodin treatment, whereas in the prostate cell lines both drugs cause *ca*. 80% inhibition of CK2 activity. Resorufin treatment of HCT116 cells leads only to a mere 40%. Inhibition by emodin, however, affects endogenous CK2 activity in HCT116 cells by 85%. The molecular basis for these differences is unknown, yet it is suggestive that a major research focus in the future should be on elucidating the basis for these cell line-specific differences. The expectations that cell lines derived from tumours of the same site, as being well suited for comparative analyses, are perhaps set too high. One should take into consideration the possibility that irrespective of the site of tumour from which the cell line was derived, general pathway disturbances are more important. By this token it could well be that cell lines derived from tumours from different sites, but sharing the same molecular defects, are better suited for comparative analyses.

Taken everything into consideration, resorufin is a good example that screening of known compounds for discovery of new and so far unknown features may be useful, for example, for pharmaceutical purposes. This may prove to be rather a goldmine than a nuisance [32,33].

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