Biochemical and Biophysical Research Communications 415 (2011) 163–167

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Protein kinase CK2 modulates IL-6 expression in inflammatory breast cancer

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ARTICLE INFO

Article history:
Received 30 September 2011
Available online 18 October 2011

Keywords:
CK2
IL-6
CX-4945
Inflammatory breast cancer

ABSTRACT

Inflammatory breast cancer is driven by pro-angiogenic and pro-inflammatory cytokines. One of them Interleukin-6 (IL-6) is implicated in cancer cell proliferation and survival, and promotes angiogenesis, inflammation and metastasis. While IL-6 has been shown to be upregulated by several oncogenes, the mechanism behind this phenomenon is not well characterized. Here we demonstrate that the pleotropic Serine/Threonine kinase CK2 is implicated in the regulation of IL-6 expression in a model of inflammatory breast cancer. We used siRNAs targeted toward CK2 and a selective small molecule inhibitor of CK2, CX-4945, to inhibit the expression and thus suppress the secretion of IL-6 in *in vitro* as well as *in vivo* models. Moreover, we report that in a clinical trial, CX-4945 was able to dramatically reduce IL-6 levels in plasma of an inflammatory breast cancer patient. Our data shed a new light on the regulation of IL-6 expression and position CX-4945 and potentially other inhibitors of CK2, for the treatment of IL-6-driven cancers and possibly other diseases where IL-6 is instrumental, including rheumatoid arthritis.

1. Introduction

Inflammatory breast cancer (IBC) is a highly aggressive and proliferative form of breast cancer that disproportionally affects younger women. While it comprises only 1–6% of all breast cancer cases in the US, the 5-year survival post-diagnosis is less than five percent (reviewed in [1]). IBC is a high grade aneuploidy cancer characterized by frequent absence of estrogen and progesterone receptor expression, high levels of E-cadherin and activation of RhoC. IBC is also characterized by overexpression of pro-inflammatory cytokines such as Interleukin-6 (IL-6), angiogenic chemokines, and angiogenic growth factors such as Interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) [2].

The cytokine IL-6 possesses strong pro-carcinogenic activity due to its roles in tumor cell proliferation and survival, angiogenesis, inflammation and metastasis (reviewed in [3]). Not surprisingly, serum IL-6 levels were found to negatively correlate with the clinical outcome for patients with various types of cancer, including breast, lung and colon [4–6]. Multiple therapeutic approaches targeting IL-6 activity, including anti-IL-6 monoclonal antibodies (CNTO 136, Siltuximab/CNTO 328, Elsilimomab), anti-IL-6 avimer protein (C326), hemoperfusion device (CytoSorb), anti-IL-6 receptor monoclonal antibody (MRA) and angiotensin II receptor antagonists (Valsartan, Irbesartan, Olmesartan) are currently being investigated in pre-clinical settings and in clinical trials, highlighting the importance of IL-6 as a therapeutic target [7–11]. As such, Tocilizumab (anti-IL-6 receptor monoclonal antibody) has already been approved by the FDA for the treatment of rheumatoid arthritis [12].

Rather than directly targeting IL-6, like the therapeutic options described above, an alternate approach would be to disrupt the production of IL-6 by targeting upstream regulatory factors. One such potential upstream regulatory target is protein kinase CK2. Like IL-6, CK2 has been shown to positively regulate tumor cell proliferation and survival, angiogenesis, inflammation and metastasis [13,14]. CK2 is a Ser/Thr kinase that is often overexpressed in cancer, and is also known to control the expression of IL-8 and VEGF [15,16]. This rationale prompted an investigation of the potential link between CK2 and IL-6 in a model of IBC. For this purpose we employed CX-4945, a first-in-class small molecule selective inhibitor of CK2, currently in clinical development [17,18]. Treatment of the IBC cell line SUM-149PT with CX-4945 resulted in dose-dependent reductions in the expression and secretion of IL-6, demonstrating that CK2 modulates IL-6 expression. These data were supported by siRNA studies targeting the catalytic sub-

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doi:10.1016/j.bbrc.2011.10.046

units of CK2. To further investigate the pharmacologic effects of CK2 inhibition on IL-6 expression, we treated SUM-149PT xenograft bearing mice with CX-4945. Oral administration of CX-4945 resulted in reduced plasma levels of human IL-6. Likewise, in the clinical setting, an IBC patient responded to CX-4945 treatment by a major reduction in her IL-6 plasma levels. Collectively, these data demonstrate a link between CK2 activity and IL-6 expression and position CK2 as a potential therapeutic target for the treatment of IL-6-driven disorders.

2. Materials and methods

2.1. Cell culture

Human IBC cell line SUM-149PT was obtained from (Asterand, Detroit, MI) and passaged in Ham's media containing 5% FBS, 5 mg/mL insulin, 1 mg/mL hydrocortisone and 10 mM Hepes at 37 °C.

2.2. siRNA transfection

Cells were grown to sub-confluency in 6-well plates and transfected with ON-TARGETPlus™ Smartpool siRNAs using DharmaFECT1 reagent by following the manufacturer's protocol (Dharmacon, Lafayette, CO). Equal molar amounts of non-targeting negative control siRNA or CK2α and CK2α′- specific siRNAs were used for each transfection reaction. After transfection, cells were harvested at indicated times for western blot analyses.

2.3. Elisa

Hundred microlitres of conditioned media or murine plasma were tested for human IL-6 using BD OptEIA™ Human IL-6 ELISA Kit II (BD Biosciences, San Jose, CA).

2.4. qRT-PCR

The RNA was isolated from treated cells using RNeasy kit (QIAGEN, Valencia, CA) and relative levels of IL-6 mRNA were measured using Applied Biosystems' (Foster City, CA) proprietary primers-probe set. Analysis was run on 7900HT Real Time PCR System (Applied Biosystems, Foster City, CA).

2.5. In vivo

Animal experiments were performed with five to six week old female athymic (NCr nu/nu fisol) mice of Balb/c origin (Taconic Farms, Germantown, NY) in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by Cylene Pharmaceuticals' Institutional Animal Care and Use Committee. All efforts were made to ameliorate animal suffering. Mice were inoculated with 5×10^6 cells in 100 μL suspension subcutaneously in the right flank. Tumor measurements were performed by caliper analysis, and tumor volume was calculated using the formula $(l \times w2)/2$, where w = width and l = length in mm of the tumor. Established tumors ($\sim$200 mm^3) were randomized into untreated or treated groups. At the end of dosing the animals were sacrificed, their tumors were excised and weighed, plasma was collected and human IL-6 levels were measured using the ELISA kit as described previously.

2.6. Clinical data

In a Phase I, dose escalating, clinical trial, patient plasma samples were collected prior to treatment and again following 21 days of twice daily oral doses of CX-4945. The level of IL-6 was quantitated using the Quantikine® Human IL-6 Immunoassay Kit (R&D Systems, Inc, Minneapolis, MN). The study was performed in accordance with Helsinki Declaration and approved by Food and Drug Administration. All patients gave an informed written consent. The trial was registered in the ClinicalTrials.Gov Registry (No. NCT00891280).

3. Results

3.1. Inhibition of CK2 blocked the in vitro secretion of IL-6 by IBC cells

First we wanted to test if the inhibition of CK2 would translate into downregulation of IL-6 expression in IBC cells grown in cell culture. To modulate the activity of CK2 we used CX-4945, a first-in-class small molecule inhibitor that selectively and potently inhibits enzymatic activity of both catalytic isoforms CK2α and CK2α′ [17,18]. As a model of IBC we selected SUM-149PT cells [19], as these are readily available from a commercial source, well characterized in the literature and known to secrete significant levels ($\sim$1.5 ng/mL) of IL-6 into the culture media. qRT-PCR analysis of the total RNA isolated from SUM-149PT cells treated with various concentrations of CX-4945 for six hours demonstrated that CX-4945 inhibited transcription of IL-6 mRNA with an IC_{50} = 0.658 μM (Fig. 1A). To test if this observation translated into reduced IL-6 production, we used ELISA to measure the resulting levels of IL-6 in the culture media. ELISA analysis demonstrated that a six-hour treatment of SUM-149PT cells by CX-4945 caused dose-dependent (IC_{50} = 4.7 μM) depletion of human IL-6 from the cell culture media (Fig. 1B). Thus, CX-4945 reduced the expression and production of IL-6 from IBC cells *in vitro*.

To ensure the effect of CX-4945 on IL-6 was due to the inhibition of CK2, we transfected SUM-149PT cells with siRNAs targeting the catalytic subunits of CK2 or a scrambled control siRNA. Seventy-two hours after the transfection, the effect on IL-6 secretion was determined by ELISA, while the CK2 knockdown was confirmed by western hybridization. A seen in Fig. 1C, knockdown of both CK2α and CK2α′ by siRNAs dramatically reduced the expression of their targets and had a significant effect on the phosphorylation of the CK2-specific site on Akt, Ser129. Interestingly, there was a clear subunit-dependence in the inhibition of IL-6 production, with CK2α knockdown having a significant effect on secretion of IL-6, while cells transfected with siRNA targeted toward CK2α' produced IL-6 levels similar to cells transfected with a scrambled control siRNA (Fig. 1D). These findings demonstrate that CK2, and in particular the CK2α isoform, modulates the expression of IL-6 and that CX-4945 acts to prevent IL-6 production through its inhibitory activity on CK2.

3.2. Suppression of IL-6 secretion by inhibition of CK2 downregulated activation of STAT3

IL-6 has been demonstrated to exert its oncogenic potential, at least in part, through the kinase JAK2-dependent activation of the transcription factor STAT3 [20]. We therefore asked if inhibition of CK2 by siRNAs or CX-4945 would translate also into downregulation of STAT3 activation. Treatment of SUM-149PT cells with siRNA against CK2α (for 72 h) and CX-4945 (for 6 and 24 h) significantly decreased phosphorylation of the JAK2-specific Y705 site on STAT3 (Fig. 2A). The inhibition of STAT3 activation by CX-4945 was dependent upon both concentration and time, such that substantial inhibition of STAT3 phosphorylation was observed with 1 μM CX-4945 after 24 h (Fig. 2B). These data demonstrate that inhibition of CK2 activity results in reduction of IL-6 production and suppression of the downstream activation of STAT3.

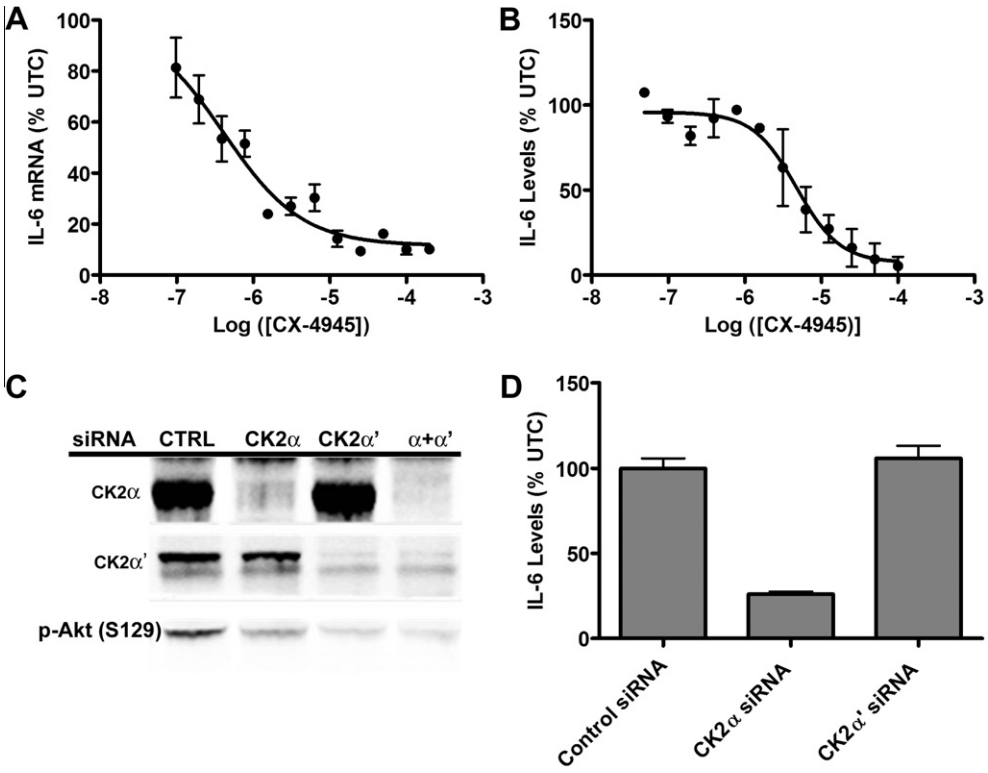

Fig. 1. CK2 controls expression of IL-6 in SUM-149PT IBC cell line. (A) CX-4945 inhibits the expression of IL-6. (B) CX-4945 inhibits the secretion of IL-6. (C) CK2 siRNAs affect CK2 protein levels and phosphorylation of AKT at S129. (D) CK2α siRNA inhibits the secretion of IL-6.

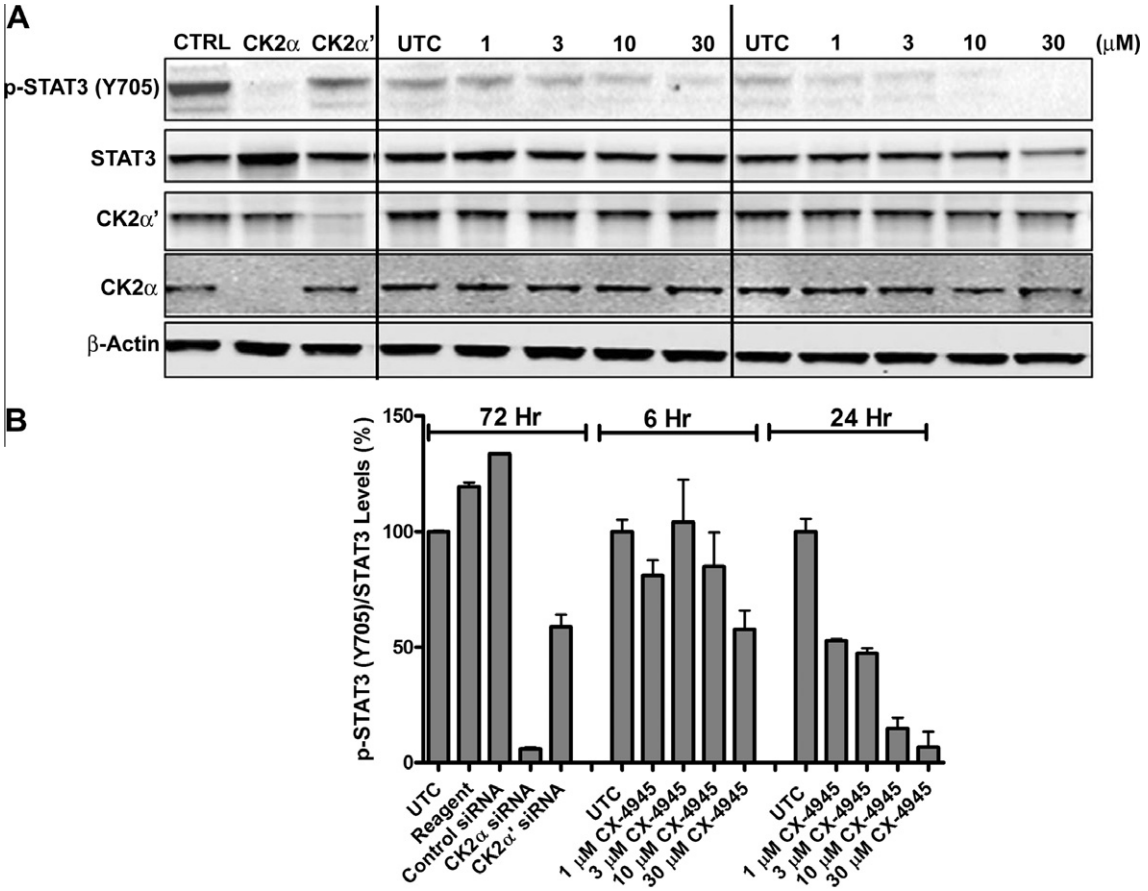

Fig. 2. Inhibition of CK2 by siRNA or CX-4945 suppresses phosphorylation of STAT3. (A) Western blot. (B) Graphic analysis.

3.3. CX-4945 inhibited IL-6 secretion in vivo

To test if modulation of IL-6 secretion by therapeutic inhibition of CK2 can be achieved in an *in vivo* setting, we examined the effect of CX-4945 on *nu/nu* mice bearing SUM-149PT xenografts tumors. When mice developed palpable tumors (approximately 200 mm^3 in size), they were randomized in two groups: treated, that received CX-4945 orally for eight days at 75 mg/kg BID, and untreated, that received vehicle on the same schedule. Eight hours after the last dose of CX-4945, animals from both groups were sacrificed, the tumors were weighed and whole plasma was collected from each animal. Treatment with CX-4945 significantly reduced plasma levels of human IL-6 in SUM 149PT xenograft-bearing mice: 61.03 ± 15.10 (pg of IL-6/mL of plasma/g of tumor) in treated group versus 143.6 ± 32.55 (pg of IL-6/mL of plasma/g of tumor) in untreated (Unpaired *t* test two-tailed $P = 0.025$, Fig. 3).

3.4. CX-4945 reduced IL-6 plasma levels in IBC patient

As an element of a Phase I clinical trial with CX-4945 delivered orally to patients with solid tumors, we investigated the potential of the drug to affect IL-6 plasma levels in an IBC patient that was treated orally with 300 mg/kg BID of CX-4945 and completed a full 21 days treatment cycle. Prior to treatment the patient had IL-6 plasma levels of 46.1 pg/mL. Following 21 days of treatment with CX-4945, the plasma IL-6 levels were substantially reduced to 6.4 pg/mL, a decrease of 86%. These data demonstrate that the effect of CX-4945 on CK2 can translate into *in vivo* reductions in IL-6 production in humans with tumors that express and rely on IL-6.

4. Discussion

Successful treatment of patients with IBC presents a major challenge for modern oncology. While positive results have been reported for the use of trastuzumab in a clinical setting in Her-2 positive IBC patients [21], there is still a severe unmet need for novel targeted approaches to treat this aggressively lethal form of breast cancer. IBC is highly angiogenic, invasive and metastatic due to the production of high levels of pro-angiogenic and pro-metastatic pro-inflammatory cytokines such as IL-6 [2]. High levels of IL-6 correlate with poor prognosis in breast, lung and colon cancer patients [4–6] and this cytokine was shown to play important roles not only in tumorigenesis, but also in inflammatory diseases such as rheumatoid arthritis, for the treatment of which an anti-IL-6 receptor antibody was approved by the FDA last year [12]. While the role of IL-6 in inflammation and carcinogenesis is clearly established, the exact mechanism that regulates IL-6 expression is not well defined. Several transcription factors, including NF-kB, have been shown to control the transcription of IL-6 and activation of certain oncogenes, such as mutant EGFR or RhoC, are known to increase IL-6 levels [2,22,23]. CK2 has been shown to be regulated by EGFR signaling and in turn can activate NF-kB [24,25], which may explain our findings that the small molecule CK2 inhibitor CX-4945, as well as siRNA targeted to CK2α, can downregulate the expression of IL-6 in a model of IBC. Interestingly, siRNA for CK2α′, while having no effect on IL-6 expression, inhibited the phosphorylation of STAT3 that is downstream from IL-6 signaling. The possible explanation for this observation is that both isoforms of CK2 may affect activation of STAT3 at different stages, with CK2α being involved in the regulation of IL-6 expression and CK2α′ (and possibly CK2α) playing a different role in the signaling cascade that leads to the phosphorylation of STAT3. This hypothesis is supported by data published by Piazza and coworkers, which demonstrated that treatment of primary CD138 + multiple myeloma cells with the two moderately selective CK2 inhibitors TBB and K27 suppressed activation of STAT3 when stimulated with exogenous IL-6 [26].

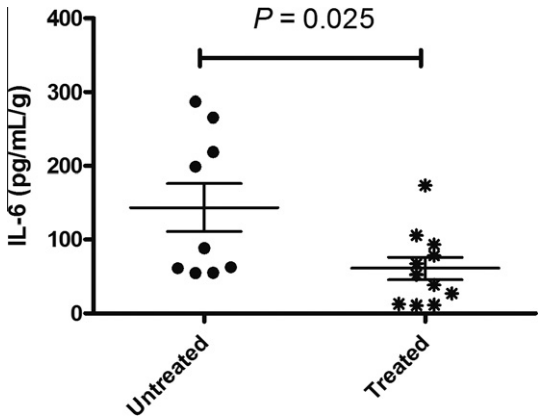

Fig. 3. CX-4945 inhibits the secretion of IL-6 by SUM-149PT xenografts.

While the regulation of IL-8 and VEGF by CK2 has been previously described [15,16], our work represents the first report that CK2 also regulates the expression of IL-6. We determined that the regulation occurs at the transcription level; however a more detailed mechanism remains to be defined. In addition to our preclinical *in vitro* and *in vivo* data, our findings were supported by the observations in a Phase I clinical trial, that an IBC patient who received a full cycle of treatment with CX-4945 exhibited a significant decrease in IL-6 plasma levels.

Here we present the first evidence that protein kinase CK2 regulates the expression of IL-6. We have shown previously that CX-4945, through its action on CK2, has anti-angiogenic, anti-proliferative and pro-apoptotic properties in various models of cancer [17]. Demonstration herein that CX-4945 also inhibits IL-6 production offers further validation for targeting CK2 for the treatment of cancer. Furthermore, given the role of both CK2 and IL-6 in pathologic processes in diseases beyond cancer, this work positions CX-4945, and other CK2 inhibitors, as potential therapies for multiple diseases that are driven by inflammation.

Funding/competing interests disclosures

This study was funded by Cylene Pharmaceuticals. All authors are affiliated with Cylene Pharmaceuticals in being current or past employees of the company. However, this did not influence the conduct of the research described in this manuscript and had no bearing on decision to submit paper for publication.

References

[1] F.M. Robertson, M. Bondy, W. Yang, H. Yamauchi, S. Wiggins, S. Kamrudin, S. Krishnamurthy, H. Le-Petross, L. Bidaut, A.N. Player, S.H. Barsky, W.A. Woodward, T. Buchholz, A. Lucci, N.T. Ueno, M. Cristofanilli, Inflammatory breast cancer: the disease, the biology, the treatment, CA Cancer J. Clin. 60 (2010) 351–375.

[2] K.L. van Golen, Z.F. Wu, X.T. Qiao, L. Bao, S.D. Merajver, RhoC GTPase overexpression modulates induction of angiogenic factors in breast cells, Neoplasia 2 (2000) 418–425.

[3] T. Ara, Y.A. Declerck, Interleukin-6 in bone metastasis and cancer progression, Eur. J. Cancer 46 (2010) 1223–1231.

[4] F. De Vita, M. Orditura, A. Auriemma, S. Infusino, A. Roscigno, G. Catalano, Serum levels of interleukin-6 as a prognostic factor in advanced non-small cell lung cancer, Oncol. Rep. 5 (1998) 649–652.

[5] G.J. Zhang, I. Adachi, Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma, Anticancer Res. 19 (1999) 1427–1432.

[6] H. Knupfer, R. Preiss, Serum interleukin-6 levels in colorectal cancer patients – a summary of published results, Int. J. Colorectal Dis. 25 (2010) 135–140.

[7] Z. Xu, E. Bouman-Thio, C. Comisar, B. Frederick, B. Van Hartingsveldt, J.C. Marini, H.M. Davis, H. Zhou, Pharmacokinetics, pharmacodynamics, and safety of a human anti-IL-6 monoclonal antibody (Sirukumab) in healthy subjects in a first-in-human study, Br. J. Clin. Pharmacol. (2011).
[8] M. Trikha, R. Corringham, B. Klein, J.F. Rossi, Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence, Clin. Cancer Res. 9 (2003) 4653–4665.
[9] J. Silverman, Q. Liu, A. Bakker, W. To, A. Duguay, B.M. Alba, R. Smith, A. Rivas, P. Li, H. Le, E. Whitehorn, K.W. Moore, C. Swimmer, V. Perlroth, M. Vogt, J. Kolkman, W.P. Stemmer, Multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains, Nat. Biotechnol. 23 (2005) 1556–1561.
[10] J.A. Kellum, M. Song, R. Venkataraman, Hemoadsorption removes tumor necrosis factor, interleukin-6, interleukin-10, reduces nuclear factor-kappaB DNA binding, and improves short-term survival in lethal endotoxemia, Crit. Care Med. 32 (2004) 801–805.
[11] R. Asmar, Targeting effective blood pressure control with angiotensin receptor blockers, Int. J. Clin. Pract. 60 (2006) 315–320.
[12] M. Mircic, A. Kavanaugh, The clinical efficacy of tocilizumab in rheumatoid arthritis, Drugs Today (Barc) 45 (2009) 189–197.
[13] B. Guerra, O.G. Issinger, Protein kinase CK2 in human diseases, Curr. Med. Chem. 15 (2008) 1870–1886.
[14] M. Ruzzene, L.A. Pinna, Addiction to protein kinase CK2: a common denominator of diverse cancer cells?, Biochim Biophys. Acta 1804 (2010) 499–504.
[15] K. Parhar, J. Morse, B. Salh, The role of protein kinase CK2 in intestinal epithelial cell inflammatory signaling, Int. J. Colorectal Dis. 22 (2007) 601–609.
[16] D. Mottet, S.P. Ruys, C. Demazy, M. Raes, C. Michiels, Role for casein kinase 2 in the regulation of HIF-1 activity, Int. J. Cancer 117 (2005) 764–774.
[17] A. Siddiqui-Jain, D. Drygin, N. Streiner, P. Chua, F. Pierre, S.E. O'Brien, J. Bliesath, M. Omori, N. Huser, C. Ho, C. Proffitt, M.K. Schwaebe, D.M. Ryckman, W.G. Rice, K. Anderes, CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits prosurvival and angiogenic signaling and exhibits antitumor efficacy, Cancer Res. 70 (2010) 10288–10298.
[18] F. Pierre, P.C. Chua, S.E. O'Brien, A. Siddiqui-Jain, P. Bourbon, M. Haddach, J. Michaux, J. Nagasawa, M.K. Schwaebe, E. Stefan, A. Vialettes, J.P. Whitten, T.K. Chen, L. Darjania, R. Stansfield, K. Anderes, J. Bliesath, D. Drygin, C. Ho, M. Omori, C. Proffitt, N. Streiner, K. Trent, W.G. Rice, D.M. Ryckman, Discovery and SAR of 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid (CX-4945), the first clinical stage inhibitor of protein kinase CK2 for the treatment of cancer, J. Med. Chem. 54 (2010) 635–654.
[19] M.R. Hoffmeyer, K.M. Wall, S.F. Dharmawardhane, In vitro analysis of the invasive phenotype of SUM 149, an inflammatory breast cancer cell line, Cancer Cell. Int. 5 (2005) 11.
[20] S. Akira, IL-6-regulated transcription factors, Int. J. Biochem. Cell Biol. 29 (1997) 1401–1418.
[21] M. Cristofanilli, Novel targeted therapies in inflammatory breast cancer, Cancer 116 (2011) 2837–2839.
[22] C. Munoz, D. Pascual-Salcedo, M.C. Castellanos, A. Alfranca, J. Aragones, A. Vara, J.M. Redondo, M.O. de Landazuri, Pyrrolidine dithiocarbamate inhibits the production of interleukin-6 interleukin-8 and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediators: modulation of NF-kappa B and AP-1 transcription factors activity, Blood 88 (1996) 3482–3490.
[23] S.P. Gao, K.G. Mark, K. Leslie, W. Pao, N. Motoi, W.L. Gerald, W.D. Travis, W. Bornmann, D. Veach, B. Clarkson, J.F. Bromberg, Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas, J. Clin. Invest. 117 (2007) 3846–3856.
[24] H. Ji, J. Wang, H. Nika, D. Hawke, S. Keezer, Q. Ge, B. Fang, X. Fang, D. Fang, D.W. Litchfield, K. Aldape, Z. Lu, EGF-induced ERK activation promotes CK2-mediated disassociation of alpha-Catenin from beta-Catenin and transactivation of beta-Catenin, Mol. Cell. 36 (2009) 547–559.
[25] R. Romieu-Mourez, E. Landesman-Bollag, D.C. Seldin, G.E. Sonenshein, Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells, Cancer Res. 62 (2002) 6770–6778.
[26] F.A. Piazza, M. Ruzzene, C. Gurrieri, B. Montini, L. Bonanni, G. Chioetto, G. Di Maira, F. Barbon, A. Cabrelle, R. Zambello, F. Adami, L. Trentin, L.A. Pinna, G. Semenzato, Multiple myeloma cell survival relies on high activity of protein kinase CK2, Blood 108 (2006) 1698–1707.