ICEC0942, an Orally Bioavailable Selective Inhibitor of CDK7 for Cancer Treatment

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Abstract

Recent reports indicate that some cancer types are especially sensitive to transcription inhibition, suggesting that targeting the transcriptional machinery provides new approaches to cancer treatment. Cyclin-dependent kinase (CDK)7 is necessary for transcription, and acts by phosphorylating the C-terminal domain (CTD) of RNA polymerase II (PolII) to enable transcription initiation. CDK7 additionally regulates the activities of a number of transcription factors, including estrogen receptor (ER)-α. Here we describe a new, orally bioavailable CDK7 inhibitor, ICEC0942. It selectively inhibits CDK7, with an IC50 of 40 nmol/L; IC50 values for CDK1, CDK2, CDK5, and CDK9 were 45-, 15-, 230-, and 30-fold higher. In vitro studies show that a wide range of cancer types are sensitive to CDK7 inhibition with GI50 values ranging between 0.2 and 0.3 μmol/L. In xenografts of both breast and colorectal cancers, the drug has substantial antitumor effects. In addition, combination therapy with tamoxifen showed complete growth arrest of ER-positive tumor xenografts. Our findings reveal that CDK7 inhibition provides a new approach, especially for ER-positive breast cancer and identify ICEC0942 as a prototype drug with potential utility as a single agent or in combination with hormone therapies for breast cancer. ICEC0942 may also be effective in other cancers that display characteristics of transcription factor addiction, such as acute leukemia and small-cell lung cancer. Mol Cancer Ther; 1–11. ©2018 AACR.

Introduction

Despite considerable evidence for dysregulation of transcription in cancer, inhibition of transcription has traditionally been viewed as being likely to suffer from toxicity, due to its requirement in normal tissues and consequent presumed lack of selective action in cancer cells. However, studies highlighting the potential of inhibiting bromodomains and extra-terminal proteins (BET) proteins that interact with the positive transcription elongation factor (P-TEFb; comprised of the CDK9 proteins (BET) proteins that interact with the positive transcription elongation factor (P-TEFb); refs. 1–3), show promise for cancers dependent on transcriptional drivers, such as c-Myc, the androgen receptor (AR) and ER (4–6). Our group, through the development of BS-181, has demonstrated the marked sensitivity of many cancer types to selective CDK7 inhibition (7).

More recently, a covalent CDK7 inhibitor, THZ1, has also been found to selectively inhibit growth of cancer cells at doses at which normal cells are insensitive (8–11). The cancer selectivity in the latter studies appears to be due to the particular sensitivity of genes encoding key transcriptional drivers to inhibition of PolII activity, such as RUNX1 (T-cell acute lymphoblastic leukemia; ref. 8) and N-myc (neuroblastoma; ref. 9).

CDK7 is a component of TFIIH, the basal transcription factor that is recruited to transcription start sites alongside PolII, and which phosphorylates serine-5 in the C-terminal domain (CTD) repeat region of PolII, to facilitate transcription initiation. CDK7 also phosphorylates CDK9 (P-TEFb), which in turn is responsible for phosphorylation of the PolII CTD at serine-2. PolII and CDK9 phosphorylation by CDK7 are required for transcription by PolII (12). In addition, CDK7 directly regulates the activities of several transcription factors, including nuclear receptors RARα, RARγ, and ER (13–17), as well as p53 (18, 19). For nuclear receptors, phosphorylation of CDK7 target sites in the N-terminal transcription activation functions is frequently mediated by ligand-dependent recruitment of TFIIH to the C-terminal hormone binding domain. CDK7 targeted phosphorylation of these receptors is required for full activity and directed ubiquitination and proteasomal degradation critical for the cyclical recruitment of these transcription factors to gene promoters (15, 16, 20, 21). The role of TFIIH/CDK7 in regulating the activities of specific transcription regulators further highlights the potential for cancer selective action of CDK7 inhibitors, for example in breast and prostate cancer, for which ER and AR respectively, are critical drivers.
In addition to its function in transcription, CDK7 directs cell-cycle progression by phosphorylating CDK1, 2, 4, and 6 (22, 23). Deregulation of cell-cycle CDKs, affected by, for example, cyclin D1 overexpression or silencing of CDK inhibitor expression, is a common feature in cancer (24). This may mean that cancer cells are more sensitive than normal cells, to inhibition of cell-cycle CDK activities. Indeed, several inhibitors of cell-cycle CDKs have entered advanced clinical trial settings, the most prominent of which, the CDK4/6 inhibitor palbociclib, shows promise, although there is significant toxicity, especially neutropenia, gastro-intestinal disorders and alopecia, associated with its use in the combination setting with anti-estrogens or aromatase inhibitors in ER-positive breast cancer (25, 26).

BS-181 is a selective small molecule inhibitor of CDK7, which inhibits cancer cell growth in vitro and in vivo (7). However, absorption, distribution, metabolism, and excretion (ADME) and pharmacokinetic (PK) assays highlighted poor cell permeability and oral bioavailability, prompting us to develop BS-181 analogues that maintain CDK7 selectivity, but have improved drug-like properties. We reasoned that oral bioavailability was of importance in view of the potential need for co-administration with oral anti-endocrine agents, given over considerable periods of time in the adjuvant therapy of breast cancer. Iterative compound design using in silico modeling of BS-181 and the structurally related orally available CDK1/2/5/7/9 inhibitor BS-194 (27) in the CDK7 X-ray crystallographic structure, testing potency and CDK7 selectivity using in vitro and cell-based assays and in vivo PK, allowed development of structure activity relationships (SAR) for a larger series of compounds. These approaches have yielded a new CDK7 noncovalent ATP competitive inhibitor, ICEC0942, with good ADME/PK properties and significant oral bioavailability. We show that ICEC0942 inhibits the growth of multiple cancer cell lines and is effective as a single agent or in combination with endocrine therapies. Our results therefore provide a rationale for the use of CDK7 inhibitors in the treatment of cancer, especially ER-positive breast cancer.

**Material and Methods**

**Chemicals**

Synthesis of ICEC0942 has been described (28). FT-NMR and high-resolution mass spectrometry were used to confirm structure and material at a purity of $>$99% (by HPLC) was used. ICEC0942 was solubilized in DMSO at a concentration of 10 mmol/L. Tamoxifen (T5648), 4-hydroxytamoxifen (H7904), and fulvestrant (I4409) were purchased from Sigma-Aldrich. Tamoxifen was solubilized in DMSO at a concentration of 10 mmol/L.

**In vitro kinase assays**

Activities of purified CDK1/cycA1, CDK2/cycA1, CDK4/cycD1, CDK5/p35NCK, and CDK6/cycD1 from Proqinase GmbH were assayed using the RB-CTF peptide (Proqinase GmbH; catalog number: 0040-0000-6), according to manufacturer's protocols. A peptide having the sequence N-YSPTSPSYSPTSPSYSPTSPS-C (Polli CTD) was used as the substrate for CDK7/cycH/MAT1 and CDK9/cycT1 (Proqinase GmbH). Kinase inhibition was evaluated by determining ATP remaining at the end of the kinase reaction using a luciferase assay (PKLight assay; Cambrex), as described (27). Screening of a 117 kinase panel with 10 μmol/L ICEC0942 was performed by the International Centre for Kinase Profiling (http://www.kinase-screen.mrc.ac.uk/).

**Cell proliferation**

Cell lines were purchased from the ATCC (www.lgcstandards-atcc.org) as frozen stocks and were cultured in DMEM supplemented with 10% FCS. Once established in the laboratory, the authenticity of the lines was further confirmed using short tandem repeat (STR) analysis as described in 2012 in ANSi Standard (ASN-0002) Authentication of Human Cell Lines: Standardization of STR Profiling by the ATCC Standards Development Organization (SDO; see ref. 29). Cell stocks were not used 20 passages beyond initial thawing. Cell cultures were routinely tested for Mycoplasma infection by assay of culture supernatants, and found to be negative, using the MycoAlert Mycoplasma Detection Kit (Lonza, UK). Cell numbers were assessed using the sulforhodamine B (SRB) assay (30).

**Immunoblotting**

Whole cell lysates were prepared in RIPA buffer (Sigma-Aldrich), supplemented with protease and phosphatase inhibitor cocktails (Roche), as previously described (7).

**Antibodies**

- β-Actin (ab2380), CDK7 P-Thr170 (ab59987), CDK7 (ab9516), cyclin H (ab54903), Polli P-Ser2 (ab5095), Polli P-Ser5 (ab5131), Polli (ab817), RB P-Thr821 (ab4787), CTS (ab6313), MYC (ab32), CCND1 (ab16663), and PGR (ab821) were purchased from Abcam plc. ER P-Ser118 (sc12915), MAT1 (sc135981), and RB (sc-50) were supplied by Santa Cruz Biotechnology Inc. Cell Signaling Technology Inc. provided CDK1 (9112), CDK1 P-Thr161 (9114), CDK2 (2546), PARP1 (9542), RB P-Ser780 (9307), P-Ser795 (9301), P-Ser807/811 (9308), CHK2 (2662), CHK2 P-Thr68 (2661), and CHK2 P-Ser516 (2669) antibodies. The ER (VPE613), Polli P-Ser7 (61087), CDK2 P-Thr160 (PAB0438), and Ki-67 (Mib-1) antibodies were obtained from Vector Laboratories, Active Motif, Abnova, and DAKO, respectively.

**Flow cytometry**

Cell-cycle analysis was carried out 24 hours following addition of ICEC0942 to 4 × 10^6 cells, as described (27). Propidium iodide (PI) stained cells were processed using the RXP cytomitos software on a Beckman FACS Canto (Beckman Coulter) and analyzed using FlowJo (TreeStar Inc.). For apoptosis, Annexin V and PI labeling and flow cytometry was undertaken exactly as previously described (7). Enrichment for cells in G0–G1 was achieved by culturing 1 × 10^5 cells in serum-free DMEM for 48 hours. For enrichment of cells in the S-phase, a double thymidine block was used, in which 2 mmol/L thymidine was added to cells in DMEM/10% FCS, for 18 hours, followed by washing in Hank's Balanced Salt Solution (HBSS), and addition of 2 mmol/L thymidine in DMEM/10% FCS, for a further 18 hours. For arrest in G2–M, cells were cultured in DMEM/10% FCS containing 2 mmol/L thymidine for 24 hours, washing in HBSS and culturing in medium containing 100 ng/mL nocodazole for 12 hours. At the end of synchronisation steps, cells were washed with HBSS and culturing in medium supplemented with ICEC0942 for 0 to 48 hours.

**Caspase 3/7 assay**

Cells were plated in optical quality 96-well plates and treated for 24 hours with the indicated concentration of ICEC0942 or
DMSO control. The Caspase-Glo 3/7 Assay (Promega) was used according to manufacturer’s instructions.

**Tumor xenografts**

All experiments were done by licensed investigators in accordance with the UK Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986 (HMSO, London, UK, 1990) and within guidelines set out by the UK National Cancer Research Institute Committee on Welfare of Animals in Cancer Research (31). Tumor xenograft studies were carried out as described previously (7, 27), following injection of 5 × 10^6 cells subcutaneously in a volume ≤0.1 mL, into the flanks of 7-week-old female nu/nu-BALB/c athymic nude mice (Harlan Olac). For MCF7 xenografts, a 0.72 mg 17β-estradiol 60-day release pellet (Innovative Research of America) was implanted subcutaneously, 24 hours before inoculation with cells. Animals were randomized and treated daily by oral gavage with ICEC0942, prepared in 10% DMSO/PBS (vehicle), once tumors size reached 100 to 200 mm^3. Tamoxifen (100 μg/mouse) was administered orally. Animals were weighed daily and tumors measured twice weekly. Tumor volumes were calculated using the formula 1/2[length (mm)] × [width (mm)]^2.

At the end of the study, tumors were divided in half for paraffin embedding or storage at −70°C. Protein lysates were prepared by homogenization of frozen tumors in RIPA buffer. Animal blood was acquired by cardiac puncture. For blood biochemistry, blood was allowed to clot at room temperature, centrifuged and 200 μL of serum analysed. Blood processing for cell counts and PolII phosphorylation assessment of peripheral blood mononuclear cells (PBMC) has been described (27). Blood biochemistry and cell counts were undertaken by the Clinical Biochemistry and the Haematology services at St Mary’s Hospital.

**Immunohistochemistry**

IHC was performed as described previously (27), using antibodies listed above. Images were acquired using the Automated Cellular Imaging System (ACIS; Carl Zeiss Ltd.); >1,000 cells were scored per section for three tumors.

**Results**

ICEC0942 is a selective inhibitor of CDK7

ICEC0942 inhibited CDK7 activity in vitro with an IC_{50} of 40 nmol/L (Fig. 1A and B; Supplementary Fig. S1A). IC_{50} values for CDK1, CDK2, CDK5, and CDK9 were 45-, 15-, 230- and 30-fold higher, whereas CDK4 and CDK6 were not substantially inhibited by ICEC0942, demonstrating CDK7 selectivity. ICEC0942 has a very similar spectrum of activity to our previously described, selective CDK7 inhibitor, BS-181 (7). Moreover, screening of 117 kinases representing different kinase classes confirmed CDK7 selectivity (Supplementary Fig. S1B). ERK8, STK33, CHK2, CLK2, and PHK were inhibited at this high concentration (10 μmol/L) of ICEC0942, but to an extent similar to the level of inhibition observed for CDK2.

ICEC0942 inhibited the growth of breast cancer cell lines with GI_{50} values between 0.2 and 0.3 μmol/L (Fig. 1C). The nontumourigenic breast epithelial cell line MCF10A and primary human mammary epithelial cells (HMEC) were at least 2 and 3.8 times less sensitive, respectively, to ICEC0942 than breast cancer cell lines, suggestive of a greater sensitivity of breast cancer cell lines to ICEC0942 inhibition. To extend the analysis to a more extensive set of cancer types, ICEC0942 was submitted to the National Cancer Institute’s Division of Cancer Treatment and whisker plot (5 to 95 percentile) showing that ICEC0942 inhibits proliferation of the NCI panel of 60 cancer cell lines. The black dots show cancer cell lines for which GI_{50} values were outside the 5 to 95 percentile.
in vitro screen of 60 human cell lines representative of diverse cancer types. ICEC0942 inhibited all 60 cancer cell lines, with median GI₅₀ = 0.25 μmol/L (Fig. 1D). These results indicate that ICEC0942 inhibits cancer cell growth with GI₅₀ values consistent with specific inhibition of CDK7.

ICEC0942 inhibits phosphorylation of CDK7 substrates and promotes cell-cycle arrest and apoptosis

Phosphorylation of the PolII CTD heptapeptide repeat at serines 2, 5, and 7 is required for transcription initiation and PolII procession (12). We investigated the effect of CDK7 inhibition in HCT116 cells, as they were particularly sensitive to ICEC0942 (Fig. 1D). PolII CTD phosphorylation was inhibited by ICEC0942 in a dose- and time-dependent manner in HCT116 colon cancer cells (Fig. 2A). ICEC0942 did not affect levels of CDK7, its cyclin partner (cyclin H), or the accessory protein MAT1. Nor was CDK7 phosphorylation in the T-loop (Thr170) altered by ICEC0942. CDK1 and CDK2 phosphorylation at Thr161 and Thr160, respectively, is mediated by CDK7- and ICEC0942-inhibited phosphorylation of these sites. Phosphorylation of retinoblastoma (Rb; ref. 32) was also reduced, further confirming that ICEC0942 inhibits the activities of CDK2/4/6. ICEC0942 similarly inhibited PolII, CDK1, CDK2, and RB phosphorylation in the MCF7 breast cancer cell line in a time- and dose-dependent manner.

Figure 2.
ICEC0942 inhibits phosphorylation of CDK7 substrates to promote cell-cycle arrest and apoptosis. A, HCT116 cells were treated with ICEC0942 at concentrations shown. Cell lysates were prepared at the indicated time points following ICEC0942 addition. B, Percentage of apoptotic HCT116 cells 24 hours following addition of ICEC0942, determined by Annexin V and propidium iodide staining (n = 3 independent experiments; *P < 0.05 relative to vehicle (0) control; t test. C and D, Cell lysates, prepared 24 hours following addition of ICEC0942 were immunoblotted for PARP cleavage or assayed for caspase 3/7 activity (n = 3). E, Flow cytometric analysis was carried out for HCT116 cells 24 hours following addition of ICEC0942 at concentrations shown. Mean percentage of cells in G1, S, and G2/M phases are shown for three independent experiments; error bars depict SEM; *, statistically significant (P < 0.05) difference between percentage of cells in G1 compared with the vehicle control; #, statistically significant (P < 0.05) difference between percentage of cells in G2/M, compared with the vehicle control. F, MCF7 cells were arrested in G0–G1, S-phase, or in G2/M. Cells were released from the block by washing and replenishment with fresh medium supplemented with ICEC0942. Shown are the FACS profiles 24 hours following addition of ICEC0942. The numbers below each graph are the percentage of cells in G1, S-phase, or G2/M for each enrichment condition for one experiment. These data are included in the time course in Supplementary Fig. S3A.
manner (Supplementary Fig. S2A). Interestingly, treatment with 10 μmol/L ICEC0942 reduced PolIII levels in both cell lines. Reduction in levels was not evident for CDK7, its associated proteins cyclin H, MAT1, or for other CDK7 substrates. Whether PolIII loss is due to inhibition of additional kinases at this high concentration of ICEC0942 is unclear, but it should be noted that at these high concentrations ICEC0942 can directly inhibit CDK9 (Fig. 1B), which phosphorylates PolIII and factors controlling transcription elongation. Phosphorylation of serine 118 (Ser118), the site in ER targeted by CDK9 (15), was also inhibited in MCF7 cells (Supplementary Fig. S2A).

Because ICEC0942 inhibited CDK7-mediated phosphorylation of CDK1 and CDK2, we investigated its effects on apoptosis and the cell cycle. Treatment with ICEC0942 for 24 hours induced caspase 3/7 and demonstrated PARP cleavage, both indicators of programmed cell death (Fig. 2B–D, Supplementary Fig. S2B). Further, cell-cycle analysis of ICEC0942-treated asynchronous HCT116 cells showed accumulation of cells in G2–M (Fig. 2E). Enrichment of cells in G2–M was also observed for asynchronous MCF7 cells (Supplementary Fig. S2C). To determine if ICEC0942 could block cells in other phases of the cell cycle, MCF7 cells were treated with thymidine-nocodazole to induce G2–M arrest (Supplementary Fig. S3A and S3B). Release from G2–M arrest by washing cells and replenishing with fresh medium, in the presence or absence of ICEC0942 showed that the cells remained blocked in G2–M, even at 48 hours following ICEC0942 addition. The reduced recovery from G2–M block was evident for 1.0 μmol/L ICEC0942, but was particularly strong at 10 and 25 μmol/L (Fig. 2E; Supplementary Fig. S3B). Interestingly, ICEC0942 also blocked release from G1 and slowed release from S-phase arrest, consistent with reports demonstrating that CDK7 is required for CDK2 (G1–S) and CDK4/6 (G1) activities in cancer cells (23, 33).

Although G2–M arrest has been reported for THZ1 (8), screening of 117 kinase showed that ICEC0942 can inhibit CHK2 at high concentrations (Supplementary Fig. S1B). As CHK2 activation by DNA damage arrests cells in G1–S and G2–M (34), we determined if ICEC0942 inhibits CHK2 activation. In the absence of DNA damage conditions, there was only low-level CHK2 phosphorylation in MCF7 cells, although phosphorylation of the ATM-directed Thr68 was stimulated by 10 μmol/L ICEC0942 (Supplementary Fig. S3C). Phosphorylation of Ser516, a CHK2 autophosphorylation site that is indicative of CHK2 activity (e.g., see ref. 35), was also not greatly affected with ICEC0942 treatment. To investigate the effect of ICEC0942 on CHK2 activity, MCF7 cells were treated with etoposide, which promotes ATM-directed CHK2 phosphorylation at Thr68. Etoposide stimulated Thr68 phosphorylation, which was not affected by ICEC0942 (Supplementary Fig. S3D). P-Ser516 levels were unaffected at concentrations of ≥ 1.0 μmol/L ICEC0942, with a small reduction in P-Ser516 with 2 μmol/L ICEC0942 and strong inhibition at higher ICEC0942 concentrations, suggesting that CHK2 inhibition could be involved in cell-cycle arrest at high concentrations of ICEC0942.

ICEC0942 is an orally bioavailable small molecule inhibitor of CDK7

ADME assays were consistent with acceptable or excellent properties for aqueous solubility, plasma protein binding (PPB), and absence of hERG liability (Supplementary Fig. S4A–S4C). To investigate pharmacokinetics, CD1 male mice were treated intravenously, subcutaneously, or by oral gavage with 10 mg/kg ICEC0942 (Supplementary Fig. S4D). In plasma, ICEC0942 levels declined in a bi-phasic manner (Supplementary Fig. S4E), indicating rapid distribution into tissues. In vitro human PPB was moderate to high at 90.8% [fraction unbound (fu) = 9.2%; Supplementary Fig. S4A]. However, volume of distribution (Vd), at 13.0 L/kg, was large (total body water in mice 0.6 L/kg; Supplementary Fig. S4F), indicating that PPB would not be ‘restrictive’ in distribution for this compound. In keeping with the high Vd, drug levels in the tumors and in liver were found to be maintained at high levels compared to blood. A logDv, of 1.68 (Supplementary Fig. S4A) indicated an approximate 100-fold preference for the compound for the organic over the aqueous phase, predicting tissue distribution of ICEC0942.

Following intravenous administration of ICEC0942 at 10 mg/kg in male CD1 mice Clplasma was calculated at 78 mL/min/kg. A blood/plasma ratio (Bl/Pi) of 1.81 was observed, which indicates a GROWTH of 43 mL/min/kg, about 48% of hepatic blood flow in the mouse. ICEC0942 has a half-life of 1.9 hours, a moderate half-life in this species. Pharmacokinetic analysis of plasma recovered 2 and 4 hours following a single oral administration (100 mg/kg) showed that only a small proportion (13.5%) of ICEC0942 was metabolized to give oxidation or dehydrogenation products (Supplementary Fig. S4G). Thus, the greater proportion of the parental CDK7 inhibitor remains unmetabolized even at 4 hours.

Comparing exposure (AUC) after single oral and intravenous administration at 10 mg/kg, oral bioavailability (%) was calculated at 30% (Supplementary Fig. S4D and S4F). Median Tmax for oral administration was 2 hours and was unaffected by increasing dose (Supplementary Fig. S4H–S4K). Over this dose range, Cmax was linearly associated with dose, as was the total exposure over time (AUC). Taken together, these findings indicate that ICEC0942 can be developed as an oral drug.

Oral administration of ICEC0942 inhibits tumor growth in vivo

We previously showed that immunostaining and flow cytometry of PBMCs allows determination of CDK7 inhibitor activity in vivo (27). Single-dose oral administration of ICEC0942 resulted in a concentration-dependent reduction in PolII phosphorylation within 6 hours (Supplementary Fig. S5), with no change in total PolII levels. To assess tumor growth inhibition, we treated established MCF7 tumor xenografts with 100 mg/kg/day ICEC0942 orally. At day 14, tumor growth was inhibited by 60% (P = 0.0001, n = 12), accompanied by highly significant reductions in PolII Ser2 and Ser5 phosphorylation in PBMCs and in tumors (Fig. 3A–C), consistent with distribution of the drug to tumors. ER Ser118 phosphorylation was also reduced in ICEC0942 treated tumours, as was phosphorylation of CDK1 and CDK2 (Fig. 3D).

We also treated nude mice with established HCT116 tumor xenografts daily with 100 mg/kg of ICEC0942. Tumor growth was inhibited by 60% (P < 0.0001, n = 15) by day 13, without significant loss in animal weight (Fig. 4A and B). Resected tumors showed substantial reduction in P-Ser2 and P-Ser5 (Fig. 4C), accompanied by only a slight reduction in total PolII. P-Ser2 and P-Ser5 levels were reduced by 40% and 60%, respectively, in PBMCs collected at the end of the experiment (Fig. 4D). ICEC0942 appeared to be well-tolerated, as treated mice showed...
no significant weight loss and blood biochemistry was normal (Fig. 4E). The only observable adverse event was reduced lymphocyte counts in ICEC0942-treated animals (Fig. 4F).

ICEC0942 in vivo pharmacokinetics in tumor-bearing mice

In both MCF7 and HCT116 tumor xenografts, ICEC0942 plasma concentrations followed similar patterns; thus, in HCT116-bearing mice, at 6 hours post-initial administration, ICEC0942 concentrations in plasma were almost twice as high for the 100 mg/kg, compared with a 50 mg/kg dose (Supplementary Fig. S6A). There was appreciable accumulation of ICEC0942 in tumors 6 hours post administration, although the tumor levels of ICEC0942 were only 1.36-fold higher for 100 mg/kg versus 50 mg/kg.

We also noted that, plasma concentrations of ICEC0942 after 13 days of daily ICEC0942 administration (100 mg/kg) were approximately twice as high as levels at first administration, possibly due to inhibition of CYP2D6, CYP3A3, and CYP2B6 (Supplementary Fig. S4C). This was not predicted from the plasma kinetics of ICEC0942 in CD1 mice, which indicated a half-life of 1.9 hours (Supplementary Fig. S4D). However, it is notable that the plasma concentrations of ICEC0942 were similar in female nu/nu BALB/c mice bearing MCF7 xenograft tumors and in nontumor bearing male CD1 animals, following a single oral administration of ICEC0942 (Supplementary Fig. S6B) and so are unlikely to reflect mouse strain differences. Following oral administration of 100 mg/kg ICEC0942 in nude mice, mean plasma C\text{max} [1.6 μg/mL (3.7 μmol/L)] was reached at 1 hour (Supplementary Fig. S6B). Thereafter, ICEC0942 levels decreased, being below the limit of detection by 48 hours. A similar plasma profile was obtained for male CD1 animals. ICEC0942 levels in tumors lagged behind plasma levels; thus, the mean maximum concentration [0.93 μg/mL (2.1 μmol/L)] was observed at 6 hours. Levels subsequently decreased 3.7-fold between 6 and 12 hours [0.25 μg/mL (0.6 μmol/L)], but remained at this level throughout the remainder of the time course, indicating retention of the compound in the tumor tissue. This observation is the likely explanation for the greater than expected levels of ICEC0942 in tumor tissue upon repeat dosing and may explain the elevated levels of drug in plasma achieved at the end of the repeat dosing.

Co-administration of CDK7 inhibitors with endocrine therapy

ER is the key transcriptional driver in breast cancer; its activity requires estrogen binding and is regulated by phosphorylation (36). In agreement with our previous studies which showed that estrogen binding promotes TFIIH recruitment to the ER ligand binding domain, to facilitate phosphorylation of Ser118 by CDK7 (15), ICEC0942 inhibited Ser118 phosphorylation in MCF7 cells in culture and in tumor xenografts (Fig. 3D; Supplementary Fig. S2A), indicating that ICEC0942 inhibits ER activity. These results prompted us to investigate whether ICEC0942 might be effective in combination treatment with anti-estrogens. Over the course of a 9-day growth assay, 1.0 μmol/L ICEC0942 completely inhibited MCF7 growth. For 0.1 μmol/L ICEC0942, MCF7 growth was inhibited by about 50%, consistent with its GI\text{50} (Fig. 5A; Supplementary
Fig. S7A and S7B). At this dose of ICEC0942, cotreatment with tamoxifen resulted in considerably greater growth inhibition than was achieved for the single agents. A similar combinatorial growth inhibition was achieved for ICEC0942 with fulvestrant. At these concentrations, tamoxifen did not affect PolII phosphorylation, nor was the ICEC0942-dependent inhibition of PolII P-Ser2 P-Ser5 abolished.
Figure 5.
Co-operativity between the CDK7 inhibitor ICEC0942 and hormone therapies in vitro and in vivo. A, MCF7 cells were treated with ICEC0942 in the presence or absence of the anti-estrogens Tamoxifen or Faslodex over a 12-day period. Growth is shown relative to the vehicle control (n = 3). Asterisks represent significant difference (P < 0.05) in growth relative to cells grown in the absence of ICEC0942. The hash symbol (#) shows significant (P < 0.05) difference in growth between cells cultured in the presence of anti-estrogen and 0.1 μmol/L ICEC0942, compared with cells cultured only in the presence of 0.1 μmol/L ICEC0942. B, Immunoblotting was performed for MCF7 cells treated for 24 hours, with ICEC0942 and/or tamoxifen at the concentrations shown. C, Flow cytometric analysis was carried out for MCF7 cells 24 hours following addition of ICEC0942 and/or tamoxifen, at concentrations shown. Mean percentage of cells in G1, S, and G2-M phases are shown for three independent experiments; error bars depict SEM; * statistically significant (P < 0.05) difference between percentage of cells in G2-M compared with the vehicle control; #, statistically significant (P < 0.05) difference between percentage of cells in G1 compared with the vehicle control. D, Percentage of apoptotic MCF7 cells 24 hours following addition of ICEC0942 and/or tamoxifen, determined by Annexin V and propidium iodide staining (n = 3 independent experiments; *, P < 0.05 relative to vehicle (0) control; t test. E, Animals with MCF7 tumor xenografts treated once daily with vehicle, 100 mg tamoxifen and/or 50 mg/kg ICEC0942 (n = 8 for each arm of the study). Multiple comparison test using one-way ANOVA analysis of slopes of linear regression lines was statistically significant (P = 0.0002) for the different treatment groups. Shown are the adjusted P values for treatment pairs from the multiple comparison testing. F, IHC was performed for tumors from three animals and scoring done as for Fig. 3. Asterisks show significant differences (P < 0.05) from the vehicle treated tumors. All statistical analyses were undertaken using the t test.
PolII phosphorylation greater in the presence of tamoxifen (Fig. 5B). ICEC0942 did inhibit the tamoxifen stimulation of ER phosphorylation at Ser118. Inhibition of ER target genes PGR and CTSD was greater for ICEC0942 than for tamoxifen. Interestingly, at low concentrations (0.1 μmol/L) ICEC0942, reduction in levels of the ER regulated MYC and CCND1 was greater for the ICEC0942 and tamoxifen combination than for either agent alone, suggestive of a combinatorial effect of the two drugs for a subset of ER-regulated genes. At this concentration (0.1 μmol/L) ICEC0942, there was an increase in the proportion of cells in G1, but no apparent effect of the combination on apoptosis (Fig. 5C and D).

To test the combined action of hormone therapy and ICEC0942 in vivo, we administered 50 mg/kg/day ICEC0942 and 100 μg/day tamoxifen to nude mice bearing MCF7 xenograft tumors. This dose of tamoxifen was based on previous studies (37). Because treatment with 100 mg/kg/day ICEC0942 almost completely blocked MCF7 xenograft tumor growth (Fig. 3), we decided to use a lower dose of ICEC0942 was used to allow a combination effect to be readily observable. Note that at a dose of 50 mg/kg reductions in PolII phosphorylation were similar to those obtained with 100 mg/kg administration (Supplementary Fig. S5). Co-administration of ICEC0942 and tamoxifen resulted in substantially greater tumor growth inhibition in vivo than was observed for either agent alone (Fig. 5E). We quantified the Ki-67 proliferation marker for all treated tumors recovered at the end of the study. This showed significant reduction in Ki-67 (P < 0.05), but the reduction was not significant for animals treated with tamoxifen alone (Fig. 5F; Supplementary Fig. S7C). As expected, PolII and ER phosphorylation were significantly reduced only for the ICEC0942 treatments. Levels of PGR, a well-characterized ER target gene, were greatly reduced in tamoxifen treated tumors (P = 0.004), but were also strongly inhibited by ICEC0942 (P = 0.023), indicative of inhibition of transcription and/or ER activity by CDK7 inhibition in ER-positive breast cancer cells.

Consistent with earlier observations, animal weights were not different between the treatment arms and there were no evident blood biochemistry differences between the treatment groups, further confirming normal liver and kidney function in the treated animals (Supplementary Fig. S7D and S7E). Lymphopenia, observed in mice treated with ICEC0942 alone, was not seen in mice cotreated with tamoxifen at a dose of 50 mg/kg/day ICEC0942 (Supplementary Fig. S7F).

**Discussion**

We describe herein the first report of a specific, orally bioavailable noncovalent inhibitor of CDK7. The favorable characteristics of this compound comprise remarkable specificity at the doses used and tumor tissue penetration, combined with sufficient potency to reduce all relevant pharmacodynamic markers examined, including PolII and CDK1/2 phosphorylation, as well as ER phosphorylation. It has excellent characteristics, including aqueous solubility, plasma protein binding, and absence of hERG liability.

ICEC0942 has acceptable oral bioavailability and moderate clearance, with a large volume of distribution not restricted by PPB, with good tissue penetration, confirmed by analysis of ICEC0942 levels in tumours. Both Cmax and exposure (AUC0−t) increase in a linear manner over a range of 10 to 100 mg/kg. Over these doses tmax remains unchanged, indicating no saturation of absorption. ICEC0942 has a few undesirable features such as mild efflux, liver metabolism, and inhibition of some CYP genes. However, some drugs with high efflux ratios, such as gefitinib, have been used successfully in patients (38).

*In vivo* studies demonstrate a large volume of distribution (tissue penetration) for ICEC0942 and HCT116 xenograft data shows high levels of the compound in the tumor after both single and repeat dosing.

The compound was remarkably well tolerated, with no adverse histologic or functional effects on liver or kidney function. Although not directly tested for activity against mouse CDK7, the extremely strong conservation (>90% amino acid identity) between human and murine CDK7, together with their conserved functions in the two species, and ICEC0942-mediated inhibition of PolII phosphorylation in PBMCs, as well as in human cell lines, indicate that ICEC0942 inhibits murine, as well as human CDK7. On this basis, we would expect ICEC0942 to have effects on highly proliferating normal tissues, as indicated by the reduced lymphocyte counts in ICEC0942-treated mice in the xenograft studies. Detailed toxicologic studies will be required before progression to the clinic.

Although CDK7 is a ubiquitous kinase that regulates key events in cell-cycle progression and transcription, the excellent safety profile of ICEC0942 indicates that the compound exerts preferential inhibitory effects in tumor relative to normal tissue. Interestingly, knockout studies show that although CDK7 is required for embryonic development, it does not appear to be essential for tissues with low proliferation, such as the brain (39). However, CDK7 is important in highly proliferative tissues, such as skin and intestine. Hemizygous deletion of the gene encoding the largest PolII subunit (POLR2A) is frequent in human cancer, POLR2A most commonly being co-deleted with TP53 (40). Increased sensitivity of cancer cells lacking a POLR2A allele to transcription inhibitors implies that many cancers might be highly sensitive to CDK7 inhibition. The fact that transcriptional regulators are important drivers in many cancers types, provides a further explanation for the special sensitivity to inhibitors of transcription, such as CDK7 inhibitors (8–11).

For breast cancer, where ER is the key driver, our results show that ICEC0942 inhibits ER phosphorylation at Ser118, as predicted from prior studies by our group (15). Ser118 phosphorylation is an early event following estrogen binding and is important for cyclical association and dissociation of ER at regulatory regions of target genes that is required for ER-mediated gene expression in breast cancer cells (20, 21, 41, 42). In *vivo*, Ser118 phosphorylation is associated with patient response to endocrine therapies (43), is elevated in endocrine resistant cell lines and in tumors following relapse after tamoxifen treatment (44), highlighting the importance of Ser118 phosphorylation in breast cancer. The importance of Ser118 phosphorylation for ER activity, together with the direct role of CDK7 in transcription, provides a potential explanation for the effectiveness of combining inhibition of ER activity with endocrine agents, together with CDK7 inhibition. Finally, gene expression analysis and IHC have shown that expression of the CDK7 complex (CDK7, cyclin H, MAT1) is elevated in breast tumors compared with the normal breast and is highest in ER-positive, endocrine-resistant breast cancer (45), which is further suggestive of cancer selectivity of CDK7 inhibitors.
Several key questions remain before CDK7 inhibition can be considered as a strategy in the treatment of breast cancer. First, although ER is especially susceptible to CDK7 inhibition, presumably other transcription factors contribute to the effects observed. For example, transcriptomic studies for THZ1 have highlighted the special sensitivity of GATA3 expression in AML (8). GATA3 is a marker of luminal breast cancer that is coexpressed with ER and which acts as a pioneer factor promoting ER recruitment to chromatin (46). Indeed, GATA3 expression in MCF7 is as sensitive to THZ1 as is PolII phosphorylation (47). Second, the contribution of reduction in CDK1/2/4/6 activities in tumor responses to CDK7 inhibition, remains to be more fully investigated.

THZ1 has a phenylpyrimidine structure with a cysteine-reactive acrylamide moiety, which binds in the ATP binding pocket of CDK7 and covalently links to a cysteine residue, C312, lying outside the ATP pocket, to irreversibly inhibit CDK7 (8). It has potent activity against several cancer types, its reported mode of action being primarily to inhibit PolII activity. However, THZ1 also inhibits the activities of several other kinases, albeit at slightly higher concentrations, for example CDK12 and CDK13, both of which are predicted to be covalently bound by THZ1. Inhibition of these and other kinases, potentially in a covalent manner, may contribute to side effects, although results to date indicate strong cancer cell selectivity over normal cells. Notwithstanding, ICEC0942 is a valuable new selective CDK7 inhibitor with an cancer cell selectivity over normal cells. Notwithstanding, ICEC0942 is a valuable new selective CDK7 inhibitor with an alternative (noncovalent) mechanism of action and that is orally bioavailable and effective not simply as a single agent but also in combination with endocrine therapies. For ER-positive breast cancer, it will be necessary to establish the timing of CDK7 inhibitor therapy, for example to define whether combining ab initio is possible, or if they should be used following emergence of resistance to current therapies. Only well-controlled clinical trials will answer these questions.

Disclosure of Potential Conflicts of Interest
A. Bondke has ownership interest in a patent on these compounds, which is owned by Imperial College; and is a consultant/advisory board member at Consultant. B.W. Slafer has ownership interest in a patent. S.H.B. Kroll has ownership interest in a patent on disclosed compounds owned by Imperial College London. M. Barbazanges is a Maître de Conferences at Université Pierre et Marie Curie (France); and has ownership interest in a patent on compounds which is owned by Imperial College. M.J. Fuchter has ownership interest in a patent. A.G.M. Barrett has ownership interest (including patents) in inventor and beneficiary of the results. C. Coombes is a consultant/advisory board member at Carrick. S. Ali has ownership interest (including patents) in patent on CDK7 inhibitor. No potential conflicts of interest were disclosed by the other authors.

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Other (research and development for the chemical synthesis of ICEC0942): B.W. Slafer

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