BAY 1000394, a Novel Cyclin-Dependent Kinase Inhibitor, with Potent Antitumor Activity in Mono- and in Combination Treatment upon Oral Application

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Running title: BAY 1000394 CDK Inhibitor

Key words: CDK, pharmacological inhibitor, in vitro profile, xenograft, combination

List of abbreviations: CDK, cyclin-dependent kinase; Mcl-1, myeloid cell leukemia sequence 1; pRb, retinoblastoma protein

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Disclosure of Potential Conflicts of Interest: Authors are employees of Bayer Pharma AG.

Notes about the manuscript: 195 words abstract; 4,961 words of text; 4 figures; 30 references
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ABSTRACT

Deregulated activity of cyclin-dependent kinases (CDK) results in loss of cell cycle checkpoint function and increased expression of anti-apoptotic proteins, which has been directly linked to the molecular pathology of cancer. BAY 1000394 inhibits the activity of cell cycle CDKs CDK1, CDK2, CDK3, CDK4, and of transcriptional CDKs CDK7 and CDK9 with IC\textsubscript{50} values in the range between 5 and 25 nmol/L. Cell proliferation was inhibited at low nanomolar concentration in a broad spectrum of human cancer cell lines. In cell based assays the inhibition of phosphorylation of the CDK substrates retinoblastoma protein, nucleophosmin, and RNA polymerase II was shown. Cell cycle profiles were consistent with inhibition of CDK 1, 2 and 4 as demonstrated in cell cycle block and release experiments. The physicochemical and pharmacokinetic properties of BAY 1000394 facilitate rapid absorption and moderate oral bioavailability. The compound potently inhibits growth of various human tumor xenografts on athymic mice including models of chemotherapy resistance upon oral dosing. Furthermore, BAY 1000394 shows more than additive efficacy when combined with cisplatin and etoposide. These results suggest that BAY 1000394 is a potent pan-CDK inhibitor and a novel oral cytotoxic agent currently in phase I clinical trials.
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INTRODUCTION

Loss of cell cycle control and increased resistance to apoptosis represent major hallmarks of cancer (1). Cyclin-dependent kinases (CDKs) belong to a family of serine/threonine protein kinases which associate with an activating cyclin regulatory subunit. CDKs are involved in the regulation of fundamental cellular processes such as cell division cycle and gene transcription. Cell cycle CDKs 1, 2, 4 & 6 are required for the correct timing and order of the events of the cell division cycle. CDK7 is a component of the CDK-activating complex that contributes to the assembly of CDK1/cyclin B. In addition, CDK7 functions as a transcriptional CDK, as well as CDKs 8 & 9 which have been shown to be involved in gene transcription via regulation of RNA polymerase II activity (2). Deregulated CDK activity results in loss of cell cycle checkpoint function and increased expression of anti-apoptotic proteins, which has been directly linked to the molecular pathology of cancer (3). Inhibition of CDK activity by low molecular weight chemical compounds appears as an attractive option for cancer treatment. Results from CDK gene targeted mice provoked a discussion which cell cycle kinase should be preferentially targeted for cancer therapy. Whereas interphase CDKs CDK2, CDK4 and CDK6 are only required for proliferation of specific cell types, CDK1 is essential for cell division in the embryo and can substitute for the genetic loss of interphase CDKs (4, 5). However, recent results with genetically engineered human cells harbouring CDK2 variants which are selectively sensitive to inhibition by bulky adenine analogs revealed a nonredundant role of CDK2 in restriction point passage and S phase entry in tumor cells as well as in non-tumorigenic cells (6). Specific inhibition of CDK4 and CDK6 may be of advantage in certain tumor settings such as Her-2 positive breast cancers (7) but is critically reliant on the presence of an intact retinoblastoma tumor suppressor gene excluding indications with a high frequency of retinoblastoma loss of
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function mutations such as small cell lung cancer (8). The heterogeneity of the cell population within the tumor tissue with respect to their position in the cell cycle may argue for a broad spectrum CDK inhibition profile.

Several low molecular weight inhibitors of CDKs with various inhibitor profiles on the panel of CDKs (9-11), some with additional non-CDK targets (12, 13), have entered clinical trials (for recent review see: (3)). However, the therapeutic efficacy was modest and none has reached the market yet. This may be at least in part due to insufficient potency, pharmacokinetic and physicochemical properties, off-target toxicities, and suboptimal dosing schedules (14).

BAY 1000394 is a new CDK inhibitor with low nanomolar activity against cell cycle CDKs and transcriptional CDKs. The physicochemical and pharmacokinetic properties of the compound facilitate rapid absorption and potent efficacy in human tumor xenograft models upon oral application. Here we present the in vitro and in vivo pharmacological profile of BAY 1000304.
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MATERIALS and METHODS

Chemicals

BAY 1000394, (R)-S-Cyclopropyl-S-(4-{{4-{{1R,2R}-2-hydroxy-1-methylpropyl}oxy}-5-(trifluoromethyl)pyrimidin-2-yl}amino}phenyl)sulfoximide, was synthesized at Bayer Pharma AG. Paclitaxel was purchased from Bristol-Myers Squibb, cisplatin from Sigma, and etoposide from Hexal.

Kinase assays and counter-screening

Recombinant CDK1 and CycB-GST fusion proteins, and CDK2 and CycE-GST fusion proteins, purified from baculovirus-infected Sf9 insect cells, were purchased from ProQinase. Histone IIIS (Sigma) was used as kinase substrate. Inhibition of kinase activity was determined by substrate phosphorylation assays using ^{33}P-gamma adenosine triphosphate (for details see supplementary methods). BAY 1000394 was counter screened against a panel of 220 kinases using the Millipore Kinase profiler screen at 500 nmol/L. Kinases, which were inhibited to less than 10% residual activity, were retested at 10 nmol/L and 100 nmol/L, and IC_{50} values were estimated.

Cell lines and cell-based assays

Authentication of all human cell lines used was performed at the German Collection of Microorganisms and Cell Cultures via PCR-based DNA profiling of polymorphic short tandem repeats (for cell line details see Supplementary Table S1). Cells were propagated under the suggested growth conditions in a humidified 37°C incubator.

Proliferation assays were performed in 96 well plates at densities between 1,000 and 5,000 cells per well in the appropriate medium containing 10% FCS. Cells were treated in quadruplicates
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with serial dilutions of BAY 1000394 for 96 hours followed by quantification of relative cell
numbers upon crystal violet staining (15). IC$_{50}$ values were calculated by means of a 4 parameter
fit using the company’s own software. Activated caspases 3 and 7, as well as DNA
fragmentation were determined using the ApoONE Caspase-3/7 Assay (Promega) and the Cell
Death Detection ELISA Plus (Roche Diagnostics). BAY 1000394 was profiled on panels of 40
human lung and 24 human breast cell lines using the Invitrogen Oncology Profiling Service.

Assessment of pRb in tumor samples

Frozen xenograft tumor pieces (approximately 5 mm$^3$) were homogenized in lysing buffer
including phosphatase inhibitor I+II and protease inhibitor (Meso Scale Discovery) using a tissue
lyser machine (Qiagen). 20µg/well protein lysate, determined using a bicinchoninic acid protein
assay (Pierce), was analysed using a Phospho (Ser780) / Total Rb Whole Cell Lysate Kit (Meso
Scale Discovery) and a Meso Scale Discovery MSD-SECTOR™ reader.

Pharmacokinetic investigations

PK studies in rats and mice were performed in 3 animals/timepoint and blood was taken by
exsanguination via the carotid artery. In dogs, three individual animals were used and blood was
drawn from a limb vein. BAY 1000394 was solubilized in 50% polyethylene glycol 400, 5%
ethanol, 45% water for studies with rats and dogs. For studies in mice the compound was
solubilized in 30% polyethylene glycol, 5% ethanol, 65% water for i.v. application, and in 40%
polyethylene glycol, 60% water for i.g. dosing. In pharmacokinetic studies, plasma samples were
collected at various time points between 2 min and 24 h after i.v. administration and between 15
min and 24 h after i.g. administration and precipitated with ice cold acetonitrile (1:5).
Supernatants were analysed for BAY 1000394 via liquid chromatography-tandem mass
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spectroscopy. Pharmacokinetic parameters were estimated from the plasma concentration data e.g. using the lin-log trapezoidal rule for AUC estimation. Maximal plasma concentrations (Cmax) and time thereof (Tmax) were taken directly form the concentration time profiles.

Plasma protein binding was determined by ultrafiltration using the 14C-labelled compound at 0.1µmol/L to 2.5 µmol/L.

Animal efficacy studies

Housing and handling of animals was in strict compliance with European and German Guidelines for Laboratory Animal Welfare. For tumor xenograft studies female athymic nu/nu mice (Taconic), 50 days old, average body weight 20-22 g, were used after an acclimatization period of 14 days. Feeding and drinking was ad libitum 24 hours per day. Human tumor cells derived from exponentially growing cell cultures were resuspended in 50% Matrigel (BD Biosciences) / 50% growth medium (without FCS) to a final concentration of 1.5 x10^7 cells /mL. Subcutaneous implants of 1.5 x 10^6 cells were inoculated into the inguinal region of mice. Tumor area (product of the longest diameter and its perpendicular) measured with a caliper, and body weight were determined twice a week. When tumors reached a size of approximately 21 mm², the animals were randomized to treatment and control groups (8 mice / group) and treated p.o. with BAY 1000394, vehicle (40% polyethylene glycol, 60% water), or chemotherapeutic agents as indicated in tables and Fig. legends. Animals were euthanized according to the German Animal Welfare Guidelines. Data were expressed as means ± SD. Statistical analysis included one way analysis of variance, and differences to the control were compared versus control group by pair-wise comparison procedure, using the Sigmastat software.
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RESULTS and DISCUSSION

Kinase inhibition profile and cellular mechanism of action studies

In biochemical assays BAY 1000394 (Fig. 1A) inhibits the kinase activity of the cell cycle CDKs CDK1 / cyclin B, CDK2 / cyclin E, and CDK4 / cyclin D with IC\(_{50}\) values of 7, 9, and 11 nmol/L, respectively (Supplementary Table S2). The transcriptional CDK´s CDK9 / cyclin T1 and CDK7 / cyclin H / MAT1 were inhibited in a similar range (5 and 25 nmol/L), as well as the other CDK family members tested, classifying BAY 1000394 as a pan-CDK inhibitor. In particular, the compound inhibits cell cycle and transcriptional CDKs with similar IC\(_{50}\)´s indicating simultaneous inhibition of cell cycle progression and of RNA polymerase II-mediated gene transcription. On a panel of 214 non-CDK Ser/Thr and Tyr kinases 16 additional kinases were found to be inhibited by BAY 1000394 with IC\(_{50}\) values below 100 nmol/L (Supplementary Table S2).

The antiproliferative activity of BAY 1000394 was tested on a panel of 25 human cancer cell lines that represent various tumor types. It demonstrated broad and uniform inhibitory activity on cell proliferation with IC\(_{50}\) values between 8 and 33 nmol/L (mean 16 nmol/L, Supplementary Table S3) and IC\(_{90}\) values between 13 and 110 nmol/L. These results were further corroborated by data from a panel of 40 human lung tumor cell lines (IC\(_{50}\)´s between 9 and 79 nmol/L, mean 39 nmol/L) and a panel of 24 human breast tumor and immortalized cell lines (IC\(_{50}\)´s between 6 and 84 nmol/L, mean 37 nmol/L) (Supplementary Table S4). Within these panels, which represent a broad range of genetic backgrounds (p53, pRB, K-Ras, PGP, etc), no cell line could be identified that was poorly sensitive towards treatment with BAY 1000394 (i.e., with an IC\(_{50}\) value >100 nmol/L).
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Similar *in vitro* cell line panels have been previously used to identify genomic markers which may help to select the patient population that could benefit most from the treatment, an approach that is highly recommended for molecularly targeted agents (16). For the CDK 4/6 inhibitor PD 0332991 IC_{50} values in the range of 4 nmol/L up to >1,000 nmol/L were reported for a panel of 44 breast cancer cell lines. Cell lines with IC_{50} <150 nmol/L were classified as 'sensitive' and those with IC_{50} >1,000 nmol/L as 'insensitive'. A statistical analysis resulted in the identification of differentially expressed genes in 'sensitive' and 'insensitive' cell lines (17). In contrast to the CDK4/6 inhibitor PD 0332991, BAY 1000394 is characterized by a remarkably uniform inhibition pattern on the cell line panels which does not provide any basis for a clear cut classification into ‘sensitive cells’ and ‘insensitive cells’, a feature of BAY 1000394 that can most likely be attributed to the potent inhibition of multiple CDKs. A statistical analysis against genomic data failed to reach the level of statistical significance. Of note, a couple of PD 0332991 insensitive cell lines respond quite well to treatment with BAY 1000394. In summary, BAY 1000394 has demonstrated a broad and potent antiproliferative activity on a wide range of human tumor cells representing various oncogenomic backgrounds.

In wash out experiments in HeLa-MaTu cells IC_{50} values declined from 1,000 nmol/L to 12 nmol/L with increasing time of exposure to BAY 1000394. A time of exposure of approximately 16 h achieved almost the maximal effect (IC_{50} of 14 nmol/L; Supplementary Fig. S1) indicating that CDKs have to be inhibited for a sufficiently long period of time to achieve anti-tumor activity. Similar results had been obtained for the CDK1 inhibitor RO-3306 which showed fully reversible effects on cell viability up to an exposure time of 20 h (18).

Next we investigated whether the antiproliferative activity of BAY 1000394 was associated with the induction of apoptotic cell death. A concentration-dependent increase in activity of caspases...
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7 and 9 as well as in DNA fragmentation was observed upon treatment of HeLa cells for 17 h suggesting the induction of apoptotic cell death (Fig. 1B). These results were further corroborated by the observation that 32 of 66 lung and breast cancer cell lines within the antiproliferation panels showed a reduced cell number relative to the starting cell number upon exposure to BAY 1000394 at 111 nmol/L for 72 h indicating cell death (Supplementary Fig. S2).

Ample evidence indicate that activity of individual CDK isoforms is required for transition through the phases of the cell cycle (for recent review see: (19)). Exposure of asynchronously growing HeLa cells to BAY 1000394 for 24 h reduced the fraction of cells with 2N content, indicative for G0/G1 cell cycle phase, from 69% to 52% compared to vehicle treated cells and strongly increased the fraction of cells with <2N DNA content from 1% to 16%, indicative of apoptotic cell death (Fig. 1C). Only minor shifts were observed for cells in S, G2 or M phase (2<N<4, or 4N DNA content). For a more detailed analysis of the effects of BAY 1000394 on cell cycle distribution HeLa cells released from a double-thymidine block were used. At release about 60% of the cells had a 2N DNA content. 24 h after release into vehicle 33% of the cells were found with 2N whereas 44% were found with 4N DNA content indicating cell cycle progression. In contrast, 24 h after release into BAY 1000394 containing medium, 50% of the cells were 2N and only 11% were 4N, indicating that the compound arrests the majority of cells at the G1/S boundary. Similarly, cells treated with vehicle 3 h, 5 h, or 8 h after release progressed or completed the cell cycle. BAY 1000394 treated cells were found with intermediate (S phase) or 4N DNA contents and with cell cycle distribution patterns that resembled more the pattern at the time of treatment than the pattern of vehicle treated cells. These data suggest that BAY 1000394 arrests the cells at various CDK-dependent steps within the cell cycle, which differentiates BAY 1000394 from compounds with a more pronounced selectivity towards
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Specific CDK isoforms such as the CDK1-selective compound RO-3306 that did not affect transition through G1 and S phases but arrested the cells at the G2/M border (18).

The mechanism of action of BAY 1000394 was further investigated by analysis of downstream targets of the cell cycle kinases CDK1, CDK2 and CDK4, and of the transcriptional kinase CDK9. BAY 1000394 at 30 nmol/L completely suppressed the phosphorylation of retinoblastoma protein (P-pRb) in MCF7 human breast cancer cells stimulated to enter the cell cycle from quiescence indicative for cellular inhibition of CDK4 and CDK2 (Supplementary Fig. S3A). The amount of T199-phosphorylated nucleophosmin, a mitotic target of CDK1/cyclin B (20), was reduced at a concentration of 30 nmol/L of BAY 1000394 and completely abolished at 100 nmol/L in nocodazole-arrested HeLa cells (Supplementary Fig. S3B). BAY 1000394 inhibited Ser 2 phosphorylation of RNA polymerase II, a key substrate of CDK9/cyclin T1 (21), in a concentration-dependent manner in A549 cells and reduced the level of myeloid cell leukemia sequence 1 (Mcl-1) protein, which is under transcriptional control of RNA polymerase II (22), at ≥ 80 nmol/L (Supplementary Fig. S3C).

The concentrations of BAY 1000394 that were sufficient to inhibit the phosphorylation of the substrate proteins of CDK1, CDK2, and CDK9 in the cellular mechanistic assays were in the range of the IC_{50}/IC_{90} values determined in antiproliferation assays suggesting that the antiproliferative activity of BAY 1000394 is mediated by intracellular inhibition of kinase activity of the CDKs. Cell cycle analyses, which indicated cell cycle arrest at CDK-dependent transition events, further suggest that the predominant mode of action of BAY 1000394 is through CDK inhibition despite biochemical inhibition of some non-CDK kinases.
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**In vivo pharmacokinetic parameters and pharmacodynamic mechanism of action of BAY 1000394**

Pharmacokinetic parameters were determined in mouse, rat and dog. After intravenous administration BAY 1000394 showed a low clearance and an intermediate half-life across species (Supplementary Table S5). A high volume of distribution suggests extensive tissue distribution of the compound. After oral application BAY 1000394 was readily absorbed and showed an intermediate oral bioavailability of approximately 50%. The compound is characterized by a high solubility in water of 170 mg/L at pH 7.0 and by moderate plasma protein binding with a fraction unbound between 5 to 7% in mouse, rat, dog and human.

Pharmacokinetic/pharmacodynamic relationship and *in vivo* mechanism of action were investigated in athymic mice bearing established HeLa-MaTu cervical xenograft tumors. In vehicle treated tumors concentration of P-pRb remained constant over the time course of the study (Fig. 2). 1 h after treatment with BAY 1000394 P-pRb was barely detectable and remained at a low level for 7 h. 48 h after dosing, phosphorylation of pRb recovered to the level of the vehicle controls. Unbound concentrations of BAY 1000394 reached approximately 70 nmol/L 1 h post dosing and declined over time. 7 h after dosing, when Rb protein still remains dephosphorylated, the unbound concentration declined to approximately 20 nmol/L, and 24 h after dosing, when phosphorylation of pRb partially recovered, the unbound concentration was approximately 12 nmol/L. As tumor and serum concentrations ran in parallel over time, the unbound serum levels were put in relation to the observed P-pRb inhibition in tumor tissue. This result demonstrates that BAY 1000394 inhibits CDK2 and CDK4 activity within the tumor tissue. The time course of pRb phosphorylation is consistent with the time course of the unbound concentration of BAY 1000394. Furthermore, the unbound concentration of BAY 1000394 after
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a single oral application of a dose of 2 mg/kg exceeded the IC$_{50}$ of inhibiton of HeLa-MaTu cell proliferation in vitro of 11 nmol/L for 24 h.

The pharmacokinetic and pharmacodynamic data indicate that by once daily dosing of BAY 1000394 a coverage of the cellular antiproliferative IC$_{50}$ can be achieved for one day. By twice daily dosing an almost complete target inhibition should be feasible over a time frame that is sufficient to cause profound antiproliferative and cell death inducing effects.

**BAY 1000394 in vivo efficacy**

The dose-response relationship was investigated using the HeLa-MaTu cervical cancer xenograft model. Athymic mice bearing established HeLa-MaTu xenograft tumors of approx. 25 mm$^2$ in size were treated orally with BAY 1000394 at doses of 0.5, 1.0, 1.5, and 2.0 mg/kg once daily for 21 days (Fig. 3A). A clear dose/response relationship was observed with T/C values of 0.61 at the lowest dose and of 0.03 and signs of tumor regression at the 2 mg/kg dose (Fig. 3C). Treatment was well tolerated as no body weight loss below the initial body weight was observed (Fig. 3D). Additional groups of mice were treated on a cyclic intermittent dosing schedule at doses of 1.5, 2.0, and 2.5 mg/kg twice daily for two days followed by five days without treatment (2on/5off). In total three treatment cycles were completed (Fig. 3B). Tumor growth was strongly inhibited in a dose-dependent manner with T/C values of 0.19 at the lower dose and of 0.02 (tumor regression) at the higher dose. Furthermore, BAY 1000394 strongly inhibited growth of HeLa-MaTu tumors which had been grown to a size of approx. 50 mm$^2$ before start of treatment (day 8 after inoculation). T/C values of 0.06 and 0.13 were achieved upon once daily 2 mg/kg and twice daily intermittent 2.5 mg/kg (2on/5off) treatment schedule (data not shown).
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These data indicate that BAY 1000394 can produce similar \textit{in vivo} responses upon various dosing schedules ranging from continuous once daily dosing to cyclic intermittent dosing. The full coverage of the antiproliferative IC$_{50}$ for about 24 h observed upon a single dose of 2 mg/kg and the suppression of P-pRb are in line with the observed efficacy upon once daily dosing in the xenograft study. The higher exposure upon twice daily treatment leads to a more pronounced target inhibition over the treatment period and allows introduction of drug holidays.

Next we extended our \textit{in vivo} xenograft studies to models which are refractory to standard of care drugs. Treatment with BAY 1000394 of the triple negative MX-1 human breast cancer model, of the A2780-cis cisplatin-resistant human ovarian cancer model, and of the OVCAR-8-ADR (also known as NCI-ADR-Res) multidrug-resistant human ovarian cancer model was much more efficacious than treatment with the comparator drugs doxorubicin, cisplatin, and paclitaxel (Supplementary Table S6) indicating that BAY 1000394 was not recognized by the molecular mechanisms conveying resistance to the comparator drugs.

Small cell lung cancer (SCLC) represents a disease that is characterized on the molecular level by inactivation of the Rb tumor suppressor gene in more than 90% of the cases indicating a crucial role of the Rb – cell cycle pathway in tumorigenesis (8). BAY 1000394 was evaluated in cell line-derived and patient-tumor-derived SCLC xenograft models (Supplementary Table S6). The compound strongly reduced tumor growth with T/C values between 0.12 and 0.19 showing that BAY 1000394 was similar or even more efficacious as compared to cisplatin (T/C values between 0.06 and 0.55).

In summary, BAY 1000394 showed potent tumor growth inhibition in mono-treatment upon oral application in various dosing schedules in a dose-dependent manner, activity in models of
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treatment-refractory tumors, and efficacy in cell-line-derived as well as in patient-tumor-derived models.

**BAY 1000394 shows more than additive efficacy in combination with cisplatin and etoposide**

Based on the good anti-tumor activity of BAY 1000394 in SCLC models we asked whether cotreatment with BAY 1000394 could improve the treatment outcome of the standard of care first line medication for extensive-stage SCLC patients which consists of a combination treatment with platin derivatives (either cisplatin or carboplatin) and etoposide (23). Mono-treatment of NCI-H82 SCLC xenograft tumors with either cisplatin (6 mg/kg) or etoposide (12 mg/kg) on a 14 days treatment cycle was only weakly effective with T/C values of 0.58 and 0.88, respectively (Fig. 4A, B). The combination of cisplatin and etoposide produced a T/C value of 0.51. Note that cisplatin and etoposide were administered at the maximal tolerated doses. Treatment with BAY 1000394 at doses of 1.5 mg/kg and 1 mg/kg slowed tumor growth to T/C values of 0.15 and 0.62, respectively. Addition of BAY 1000394 to cisplatin resulted in a strong tumor growth inhibition with T/C values of 0.01 (1.0 mg/kg BAY 1000394) and -0.02 (1.5 mg/kg BAY 1000394) (Fig. 4A). The combination of etoposide (12 mg/kg) with a dose of 1.5 mg/kg of BAY 1000394 resulted in a tumor growth inhibition with a T/C of -0.05 (Fig. 4B). Calculating expected additive T/C values based on the mono-treatment results according to the Bliss algorithm (24) (0.31 and 0.08 for the cisplatin/BAY 1000394 combination), revealed that the observed T/C values were below the calculated values indicating more than additive efficacy of the combination. Similar results were found for the BAY 1000394/etoposide combination.
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Long-term tumor growth control (stasis) was observed in the cisplatin/BAY 1000394 combination groups over an observation period of 48 days. Treatments were well tolerated with acceptable body weight loss.

Next we asked whether a triple cisplatin/etoposide/BAY 1000394 combo would be feasible. The selected dose of 0.75 mg/kg of BAY 1000394 administered in mono produced a T/C value of 0.69. Cisplatin/etoposide combination treatment did also not achieve significant tumor growth inhibition (T/C 0.58). The triple combination of cisplatin (6 mg/kg), etoposide (12 mg/kg) and BAY 1000394 (0.75 mg/kg) treatment resulted in a strong tumor growth inhibition with a T/C value of 0.09 (Fig. 4C) which was clearly below expected additive T/C of 0.40 calculated according to the Bliss algorithm, indicating that BAY 1000394 adds efficacy to the cisplatin/etoposide combination in a more than additive manner. The strong tumor growth inhibition continued over the observation period of 32 days. Remarkably, BAY 1000394 did not increase the body weight loss in the triple combination over the body weight loss observed in the cisplatin/etoposide combination group (20%) indicating that the main drivers of toxicity are the standard of care drugs cisplatin and etoposide.

The pan-CDK inhibitor BAY 1000394 strongly improved the efficacy of the standard of care combination treatment with the DNA-damaging agents cisplatin and etoposide in the Rb negative NCI-H82 SCLC xenograft model without further worsening the tolerability of the therapy. CDKs play multiple roles in the DNA damage response pathway including activation of the DNA damage checkpoint, activation of DNA repair pathways, and G2 cell cycle arrest (recently reviewed in (25)). In line with the role of CDKs in DNA-damage response signaling combination treatment NCI-H460 NSCLC xenograft tumors with BAY 1000394 and carboplatin was more efficacious when carboplatin was administered a day before BAY 1000394 treatment compared
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to the schedules BAY 1000394 followed by carboplatin or concomitant administration of both
drugs (data not shown).

Overall, the novel pan-CDK inhibitor BAY 1000394 is characterized by a broad range of on
target activity and potent efficacy in various *in vitro* and *in vivo* models of human cancer,
however, a contribution of inhibition of non-CDK kinases can not be excluded. Although various
low molecular weight inhibitors of CDK’s have entered clinical trials in the past years the
therapeutic efficacy was modest and none has reached the market yet. This may at least in part be
due to insufficient potency, pharmacokinetic and physicochemical properties, off-target
toxicities, and suboptimal dosing schedules (14). R-roscovitine has low cellular activity, whereas
i.v. drugs administered on a 3 weeks treatment cycle, such as R547 or SCH 727965, have to
overcome the hurdle to cover the whole length of the cell-divison cycle at sufficient exposures
(3, 26). Another example is ZK 304709, an oral multitargeted CDK inhibitor, which showed a
promising activity profile in various human tumor xenografts at high daily doses of up to 100
mg/kg (13, 27). In phase I clinical trials blood concentrations of ZK 304709 increased in a sub-
dose proportional manner with high inter-patient variability. Concentrations expected to deliver
meaningful pharmacological activity were not reached and the trials were closed before the MTD
was determined (28, 29). The most likely reasons for the suboptimal human pharmacokinetics of
ZK 304709 were its low solubility and the high doses to be administered. BAY 1000394 clearly
overcomes these limitations and is characterized by a high solubility in water even at neutral pH
and low efficacious oral doses in the range of 1.5 to 2.5 mg/kg in mice. BAY 1000394 is
efficacious in human tumor xenograft models including patient-derived models which are
considered to provide a high probability of correct prediction of human tumor response (30). The
compound is active upon once daily dosing as well as upon intermittent dosing providing the
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room to optimize human dosing schedules with respect to efficacy and tolerability. Furthermore, BAY 1000394 has proven anti-tumor activity in xenograft models resistant to standard of care drugs such as doxorubicin, cisplatin or paclitaxel, and has demonstrated its potential for combination treatment with drugs on the market. BAY 1000394 is currently in phase I clinical trials (ClinicalTrials.gov identifier: NCT01188252).

References

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Fig. legends

Fig. 1. Cell cycle arrest and apoptosis induction by BAY 1000394. A. Chemical structure of BAY 1000394. B. BAY 1000394 induces apoptotic cell death in a concentration dependent manner. Proliferating HeLa cells were exposed to increasing concentrations of BAY 1000394 for 17h. Caspases 3 and 7 activity and DNA fragmentation were determined and normalized to vehicle controls and cell number. C. Cell cycle distribution upon BAY 1000394 treatment. HeLa cells were either grown asynchronously or were released from double-thymidine treatment-induced G1/S arrest. Cells were treated with vehicle (0.5% DMSO) or 100 nmol/L of BAY 1000394 at release and 3, 5, and 8 h after release. Cells were analyzed for DNA content at the time of treatment and 24 h after treatment by means of flow cytometry.

Fig. 2. Inhibition of pRb phosphorylation by BAY 1000394 in a mouse xenograft model. Athymic mice bearing established HeLa-MaTu human cervical tumors were treated orally (p.o.) once with vehicle or with BAY 1000394 at 2 mg/kg. Unbound concentrations of BAY 1000394 and P-pRb level in tumor tissue were determined 1, 7, 24, and 48 h after dosing.

Fig. 3. Efficacy of BAY 1000394 in a mouse xenograft model. Athymic mice bearing established HeLa-MaTu human cervical tumors were treated p.o. with vehicle or BAY 1000394 at the doses and schedules as indicated. Treatment was started at day 3 after tumor inoculation. A. Tumor growth during the study course upon once daily oral dosing. B. Tumor growth during the study course upon intermittent oral dosing. C. Tumor weight at the end of the study (day 24). D. Animal body weight change during the study course.
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Fig. 4. Combination of BAY 1000394 with cisplatin, or etoposide, or cisplatin plus etoposide in human SCLC xenograft tumor model. Athymic mice bearing established NCI-H82 tumors were treated orally with vehicle or BAY 1000394 and/or intraperitoneally with cisplatin, or etoposide, or cisplatin plus etoposide at the indicated doses. A. Cisplatin/BAY 1000394 combination treatment. B. Etoposide/BAY 1000394 combination treatment. C. Cisplatin/etoposide/BAY 1000394 combination treatment. Treatment was started at day 4 after tumor inoculation. The following schedules were performed in 14-day cycles: BAY 1000394 monotherapy on days 1-3; cisplatin monotherapy on day 1; etoposide monotherapy on days 1-3; cisplatin on day 1 plus etoposide on days 3-5; cisplatin on day 1 plus BAY 1000394 on days 3-5; etoposide on days 4-6 plus BAY 1000394 on days 1-3; and cisplatin on day 1 plus etoposide on days 3-5 plus BAY 1000394 on days 8-10 of one treatment cycle (*Statistically significant vs. vehicle, P <0.05).
Figure 1

A

B

C

Synchronous HeLa: Time of treatment

Cells in cell cycle (%)

Asynchronous HeLa

Caspase 3/7 activity (x-fold over control)

DNA fragmentation (x-fold over control)

BAY 1000394 (micromol/L)

Caspase 3/7 activity

DNA fragmentation

BAY 1000394

DMSO

at treatment

at treatment

at treatment

at treatment

at treatment

at treatment

>4N

=4N

2<N<4

=2N

<2N

>4N

=4N

2<N<4

=2N

<2N

>4N

=4N

2<N<4

=2N

<2N

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2<N<4

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>4N

=4N

2<N<4

=2N

<2N

>4N

=4N

2<N<4

=2N

<2N
Figure 2

- BAY 1000394 (serum)
- in vitro IC50
- Vehicle - P-pRb
- BAY 1000394 - P-pRb

BAY 1000394 unbound concentration (nmol/L)

Phospho-pRb (Ser780) (RLU)

Time after dose [h]
Figure 4

A

- Vehicle
- BAY 1000394 1 mg/kg bid 3 on/11 off p.o.
- BAY 1000394 1.5 mg/kg bid 3 on/11 off p.o.
- Cisplatin 6 mg/kg 1 on/13 off i.p.
- Cisplatin 6 mg/kg + Etoposide 12 mg/kg
- Cisplatin 6 mg/kg + BAY 1000394 1 mg/kg
- Cisplatin 6 mg/kg + BAY 1000394 1.5 mg/kg

Tumor area [mm$^2$ ± SD]

Days after tumor inoculation

B

- Vehicle
- BAY 1000394 1.5 mg/kg bid 3 on/11 off p.o.
- Etoposide 12 mg/kg qd 3 on/11 off i.p.
- Cisplatin 6 mg/kg + Etoposide 12 mg/kg
- Etoposide 12 mg/kg + BAY 1000394 1 mg/kg
- Etoposide 12 mg/kg + BAY 1000394 1.5 mg/kg

Tumor area [mm$^2$ ± SD]

Days after tumor inoculation

C

- Vehicle
- BAY 1000394 0.75 mg/kg bid 3 on/11 off p.o.
- Cisplatin 6 mg/kg
- Cisplatin 6 mg/kg + Etoposide 12 mg/kg
- BAY 1000394 0.75 mg/kg

Tumor area [mm$^2$ ± SD]

Days after tumor inoculation

Body weight change [%]

Days after tumor inoculation
# Molecular Cancer Therapeutics

## BAY 1000394, a Novel Cyclin-Dependent Kinase Inhibitor, with Potent Antitumor Activity in Mono- and in Combination Treatment upon Oral Application

Gerhard Siemeister, Ulrich Lucking, Antje M Wengner, et al.

*Mol Cancer Ther* Published OnlineFirst July 19, 2012.

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