Cancer Chemother Pharmacol (2007) 59:855–860
DOI 10.1007/s00280-006-0334-2

SHORT COMMUNICATION

The effect of hydroxyurea on P-glycoprotein/BCRP-mediated transport and CYP3A metabolism of imatinib mesylate

Roos L. Oostendorp · Serena Marchetti · Jos H. Beijnen · R. Mazzanti · Jan H. M. Schellens

Received: 23 May 2006 / Accepted: 18 August 2006 / Published online: 16 December 2006
© Springer-Verlag 2006

Abstract
Purpose It has been reported that the combination therapy of imatinib mesylate, a tyrosine kinase inhibitor, plus hydroxyurea, a ribonucleotide reductase inhibitor, is associated with remarkable antitumor activity in patients with recurrent glioblastoma multiforme. However, the mechanism of the added activity of hydroxyurea to imatinib is not known. The purpose of this study was to investigate in vitro, whether hydroxyurea could enhance the central nervous system penetration of imatinib, by inhibition of the ATP-dependent transporter proteins P-glycoprotein (ABCB1; MDR1; Pgp) and Breast Cancer Resistance Protein (ABCG2; BCRP), or by inhibition of cytochrome P450 3A (CYP3A) metabolism of imatinib.
Methods The effect of hydroxyurea on the Pgp and BCRP mediated transport of imatinib was investigated by the sulforhodamine-B (SRB) drug cytotoxicity assay and transepithelial transport assay. In vitro biotransformation studies with supersomes expressing human CYP3A4 were performed to investigate whether hydroxyurea inhibited CYP3A4.
Results In both in vitro cytotoxicity and transport assays, hydroxyurea did not affect Pgp and BCRP mediated transport of imatinib. In a biotransformation assay, hydroxyurea had no influence on the metabolic degradation of imatinib either.
Conclusion The results indicate that hydroxyurea does not interact with imatinib by inhibition of Pgp and BCRP mediated transport or by CYP3A4 mediated metabolism of imatinib.

Keywords Imatinib mesylate · Hydroxyurea · BCRP · P-glycoprotein · CYP3A4

Roos L. Oostendorp, Serena Marchetti contributed equally to this article.

R. L. Oostendorp · S. Marchetti · J. H. M. Schellens
Division of Experimental Therapy,
The Netherlands Cancer Institute,
Plesmanlaan 121, 1066CX Amsterdam,
The Netherlands

J. H. M. Schellens (✉)
Division of Medical Oncology,
The Netherlands Cancer Institute,
Plesmanlaan 121, 1066CX Amsterdam,
The Netherlands
e-mail: jhm@nki.nl

J. H. Beijnen · J. H. M. Schellens
Faculty of Pharmaceutical Sciences,
Utrecht University, Sorbonnelaan 16,
3584 CA Utrecht, The Netherlands

S. Marchetti · R. Mazzanti
Postgraduate School of oncology,
University of Florence, Florence, Italy

Introduction

Imatinib mesylate (STI-571, Gleevec®, imatinib), a potent and selective receptor tyrosine kinase inhibitor was shown to be clinically effective and well tolerated in Bcr/Abl-expressing chronic myeloid leukemia [6] and c-Kit-expressing gastro-intestinal stromal tumors (GIST) [4]. In addition, imatinib effectively inhibits platelet-derived growth factor (PDGF)-induced glioblastoma cell growth preclinically [11]. However, trials with imatinib in patients with recurrent glioblastoma multiforme showed limited penetration of imatinib into the

central nervous system and modest antitumor activity [19, 24]. A plausible explanation for this low efficacy of imatinib is the efficient protection of the brain against drugs by the blood–brain barrier, containing various efflux transporters, including P-glycoprotein (ABCB1; MDR1; Pgp) and Breast Cancer Resistance Protein (ABCG2; BCRP). Pgp and BCRP are located in apical membranes of epithelia and vascular endothelial cells, which can actively extrude a variety of structurally diverse drugs and drug metabolites from the central nervous system and from tumor cells into the blood circulation [7, 22]. In vitro and in vivo studies have shown that Pgp and BCRP play an important role in the transport of imatinib and limit the distribution of imatinib to the brain [1, 3]. Furthermore, effective Pgp and/or BCRP inhibitors, such as elacridar (GF120918), zosuquidar (LY335979) and pantoprazole, significantly improved the brain accumulation of imatinib [1, 3]. This concept raises the possibility that co-administration of a transport inhibitor improves therapy with imatinib.

Two recent reports suggested that the combination of imatinib plus hydroxyurea, a ribonucleoside reductase inhibitor, is a safe and effective therapy for a subpopulation of glioblastoma multiforme patients who have experienced disease progression after prior radiotherapy and at least temozolomide-based chemotherapy [5, 20]. This is the first report that a signal transduction inhibitor combined with a chemotherapeutic agent has activity in glioblastoma multiforme. However, the mechanism of action underlying the activity of this regimen is unknown. Based on the preclinical results of Dai et al. [3] and Breedveld et al. [1], we hypothesized that hydroxyurea interferes with the penetration of imatinib through the blood–brain barrier by inhibition of the efflux transporters Pgp and/or BCRP or by inhibition of cytochrome P450 3A (CYP3A) metabolism of imatinib.

Materials and methods

Chemicals

Imatinib, [^{14}C]imatinib (both as the mesylate salt) and its main metabolite N-desmethyl-STI (CGP74588) were kindly provided by Novartis Pharma AG (Basel, Switzerland). Pantoprazole (Pantozol®, Altana Pharma, Hoofddorp, The Netherlands) was obtained from the pharmacy of the Netherlands Cancer Institute. Zosuquidar trihydrochloride (LY335979) was kindly provided by Eli Lilly (IN, USA). Ritonavir was provided by Abbott (Chicago, IL, USA). Hydroxyurea was purchased from Sigma (St Louis, MO, USA).

Cell lines and culture conditions

The Madin–Darby Canine Kidney II (MDCKII) epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 units penicillin/streptomycin per ml [13]. Cells were grown at 37°C with 5% CO_2 under humidifying conditions. Polarized MDCKII cells stably expressing human MDR1 (ABCB1) or murine Bcrp1 (ABCG2) cDNA have been described previously [8, 10].

Cytotoxicity assay

MDCKII-parental, -MDR1 and -Bcrp1 cells were cultured as described above and used in a sulforhodamine B (SRB) drug cytotoxicity assay for single and combination experiments as described by Ma et al. [15]. Briefly, 1,000 exponentially growing MDCKII cells/200 µl/ well in 96-well plates were allowed to attach for 1 day followed by imatinib administration in the presence or absence of hydroxyurea for three more days.

Transport across MDCKII monolayers

MDCKII-parental, -MDR1 and -Bcrp1 cells were seeded on microporous polycarbonate membrane filters at a density of 1×10^6 cells/well in complete medium. Transepithelial transport assays were performed as described previously [2]. To exclude any contribution of Pgp in the MDCKII-Bcrp1 and MDCKII-parental cells, LY335979 was added. As the expression of BCRP in the MDCKII-parental and -MDR1 cells is negligible, co-administration of a BCRP inhibitor is redundant.

Biotransformation assay

The main metabolite of imatinib, CGP74588, was formed in in vitro incubations with supersomes that contained cDNA expressing human CYP3A4. Supersomal incubations (final volume = 50 µl) were performed at 37°C according to the BD Gentest procedure/catalogue (BD Bioscience, Erembodegem, Belgium) and contained, per incubation: supersomes CYP3A4 (10 pmol), NADPH regenerating solutions A (2.5 µl) and B (0.5 µl) from BD Gentest/Bioscience, 0.1 M phosphate buffer, water and imatinib 20 µM in the presence or absence of hydroxyurea 300 µM or the known CYP3A4 inhibitor ritonavir 100 µM. Supersomal incubations were started by the addition of imatinib in water. Control incubations were performed on ice instead of 37°C. Incubations were performed for 1 h

and stopped by the addition of 50 µl acetonitrile. Protein precipitations were obtained by the centrifugation of the incubates (8,000 rpm for 10 min). Supernatants were transferred and injected into the analytical column (method described below).

HPLC analysis of imatinib and CGP74588

Imatinib, CGP74588 and the internal standard 4-hydroxybenzophenone were separated using a narrow bore (2.1 × 150 mm) stainless steel packed column packed with 3.5 µm Symmetry C-18 material and detection was accomplished with a UV detector set at excitation and emission wavelengths of 265 nm and 460 nm, respectively. The mobile phase consisted of 28% (v/v) acetonitrile in 50 mM ammonium acetate buffer pH 6.8 containing 0.005 M 1-octane sulfonic acid and was delivered at 0.2 ml/min.

Statistical analysis

Statistical evaluation was performed using the two-sided unpaired Student's *t* test to assess the statistical significance of difference between two sets of data. Differences were considered to be statistically significant when $P < 0.05$.

Results

The effect of hydroxyurea on the cytotoxicity of imatinib

In the first part of this study we investigated in vitro the effect of hydroxyurea on the cytotoxicity of imatinib in parental MDCKII and MDCKII cells stably transfected with human Pgp or BCRP, for which we used the mouse homolog Bcrp1 (MDCKII-MDR1 or MDCKII-Bcrp1, respectively). Hydroxyurea and imatinib alone were not less cytotoxic to the MDCKII-MDR1 and MDCKII-Bcrp1 cells compared to parental MDCKII cells ($P > 0.05$; Table 1). Furthermore, the cytotoxicity of imatinib was not significantly affected by co-incubation with a non-toxic dose of 50 or 100 µM hydroxyurea ($P > 0.05$; Table 1).

Table 1 Cytotoxicity of imatinib in MDCKII-parental, -Bcrp1 and -MDR1 cell lines in the absence or presence of hydroxyurea

	MDCKII-parental	MDCKII-Bcrp1	MDCKII-MDR1
	$IC_{50\ (\mu M)}$	$IC_{50\ (\mu M)}$	$IC_{50\ (\mu M)}$
Hydroxyurea	740 ± 66	679 ± 69	698 ± 60
Imatinib	8.0 ± 0.2	9.1 ± 1.3	8.5 ± 0.9
Imatinib + Hydroxyurea 50 µM	8.4 ± 0.6	9.3 ± 2.0	8.4 ± 0.8
Imatinib + Hydroxyurea 100 µM	8.2 ± 0.4	9.2 ± 1.7	8.9 ± 1.6

Inhibiting concentrations of 50% (IC_{50}, in µM) of 3 day incubations are shown as mean ± SD from >3 independent experiments

The effect of hydroyurea on the active transport of imatinib by Pgp and Bcrp1

Secondly, we investigated in vitro, employing polarized MDCKII-parental, -MDR1 and -Bcrp1 monolayers, whether hydroxyurea is capable of inhibiting the active transport of imatinib by Pgp and Bcrp1. Imatinib alone resulted in an increased transport by Pgp and Bcrp1 from the basolateral to the apical side (BA) compared with the transport from the apical to the basolateral side (AB), i.e., active transport (BA/AB is 10.3 and 20.4, respectively) (Fig. 1a, b). These results are comparable to those shown previously by Dai et al. [3] and Breedveld et al. [1]. Furthermore, the effect of hydroxyurea and the Pgp and BCRP inhibitors, LY335979 and pantoprazole as positive controls, on the active transport of imatinib by Pgp and Bcrp1 were investigated. LY335979 and pantoprazole inhibited the MDR1 and Bcrp1-mediated transport of imatinib, respectively, as upon co-incubation the transport from BA was approximately equal to the transport from AB, i.e., no active transport. In contrast, hydroxyurea did not affect Pgp and Bcrp1-mediated transport of imatinib (BA/AB is 12.0 and 18.5, respectively) (Fig. 1a, b).

Imatinib biotransformation by human CYP3A supersomes in the absence and presence of hydroxyurea

We then tested whether hydroxyurea inhibited cytochrome P450 3A (CYP3A). Although hydroxyurea is not a known CYP substrate, recent studies of the 5-lipoxygenase inhibitor, zileuton, the structure of which includes a hydroxyurea moiety, indicate that it inhibits CYPs, including CYP3A, which isozyme is mainly responsible for the biotransformation of imatinib [14, 16]. We performed in vitro biotransformation studies with supersomes expressing human CYP3A4. The CYP3A4 supersomes metabolized 12.3 ± 1.2% of imatinib to its main metabolite CGP74588 over 1h of incubation. Subsequently, we incubated imatinib with hydroxyurea or ritonavir; the latter is a known CYP3A4 inhibitor. Ritonavir was able to inhibit imatinib biotransformation completely. In contrast, hydroxyurea had no inhibitory effect on the biotransformation of imatinib. The CYP3A4 supersomes metabolized 11.8 ± 0.9% of imatinib to CGP74588 in

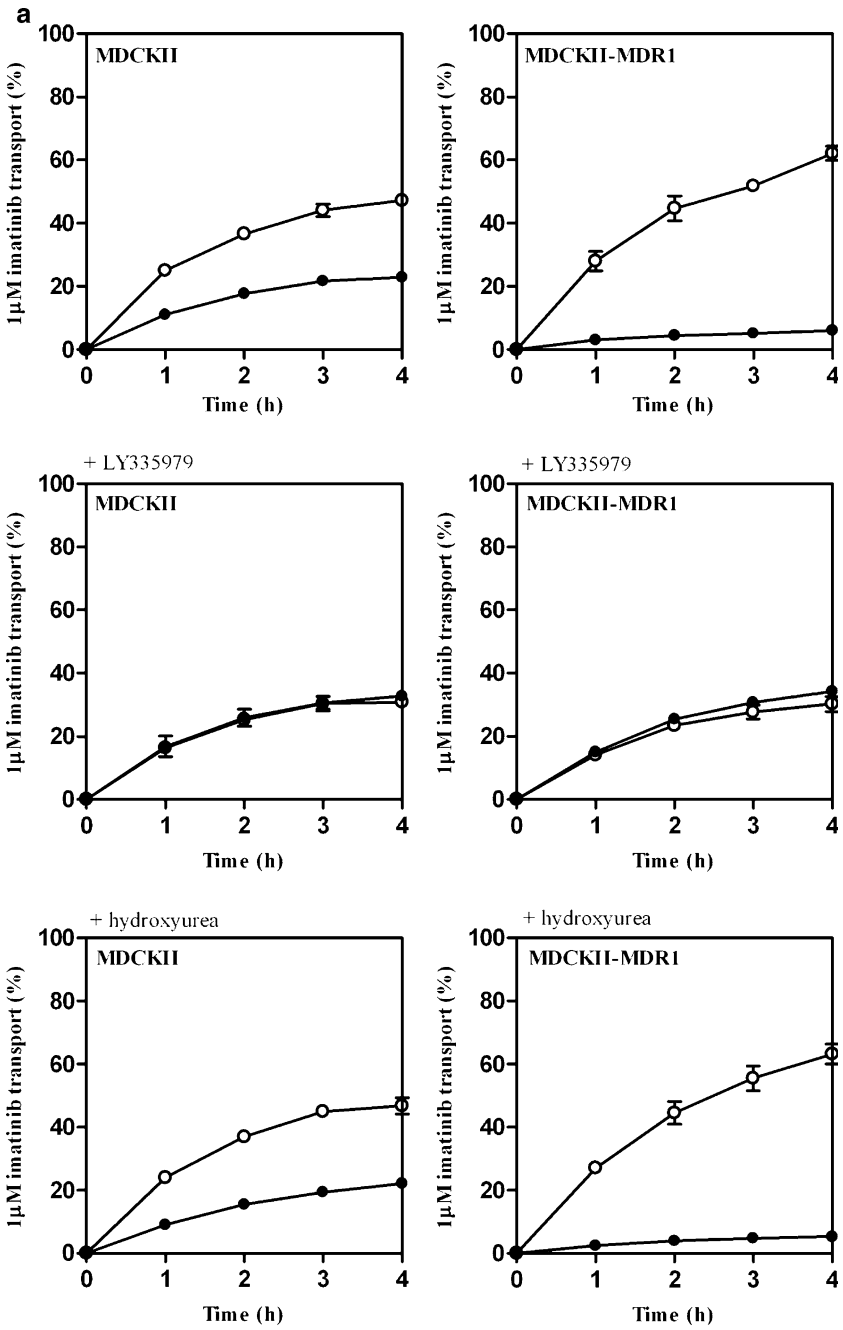


Fig. 1 Transport of imatinib by MDR1 (Fig. 1a) and Bcrp1 (Fig. 1b) in the absence or presence of hydroxyurea, LY335979 and pantoprazole. **a** MDCKII parental and MDCKII-MDR1 cells were pre-incubated for 2 h with and without (control) 5 μM LY335979 or 30 mM hydroxyurea. One μM of [^{14}C] imatinib and the indicated concentration of LY335979 or hydroxyurea were applied at $t = 0$ to the apical or basal side and the amount of [^{14}C] imatinib appearing in the opposite basal compartment (AB; *closed symbols*) or apical compartment (BA; *open symbols*) was determined. Samples were taken at $t = 1, 2, 3$ and 4 h. *Points*, means of each experiment in triplicate; *bars*, SD. **b** MDCKII parental and MDCKII-Bcrp1 cells were pre-incubated for 2 h with 5 μM LY335979, and without (control) or with 500 μM pantoprazole or 30 mM hydroxyurea. One μM of [^{14}C] imatinib and the indicated concentration of LY335979 or hydroxyurea were applied at $t = 0$ to the apical or basal side and the amount of [^{14}C] imatinib appearing in the opposite basal compartment (AB; *closed symbols*) or apical compartment (BA; *open symbols*) was determined. Samples were taken at $t = 1, 2, 3$ and 4 h. *Points*, means of each experiment in triplicate; *bars*, SD

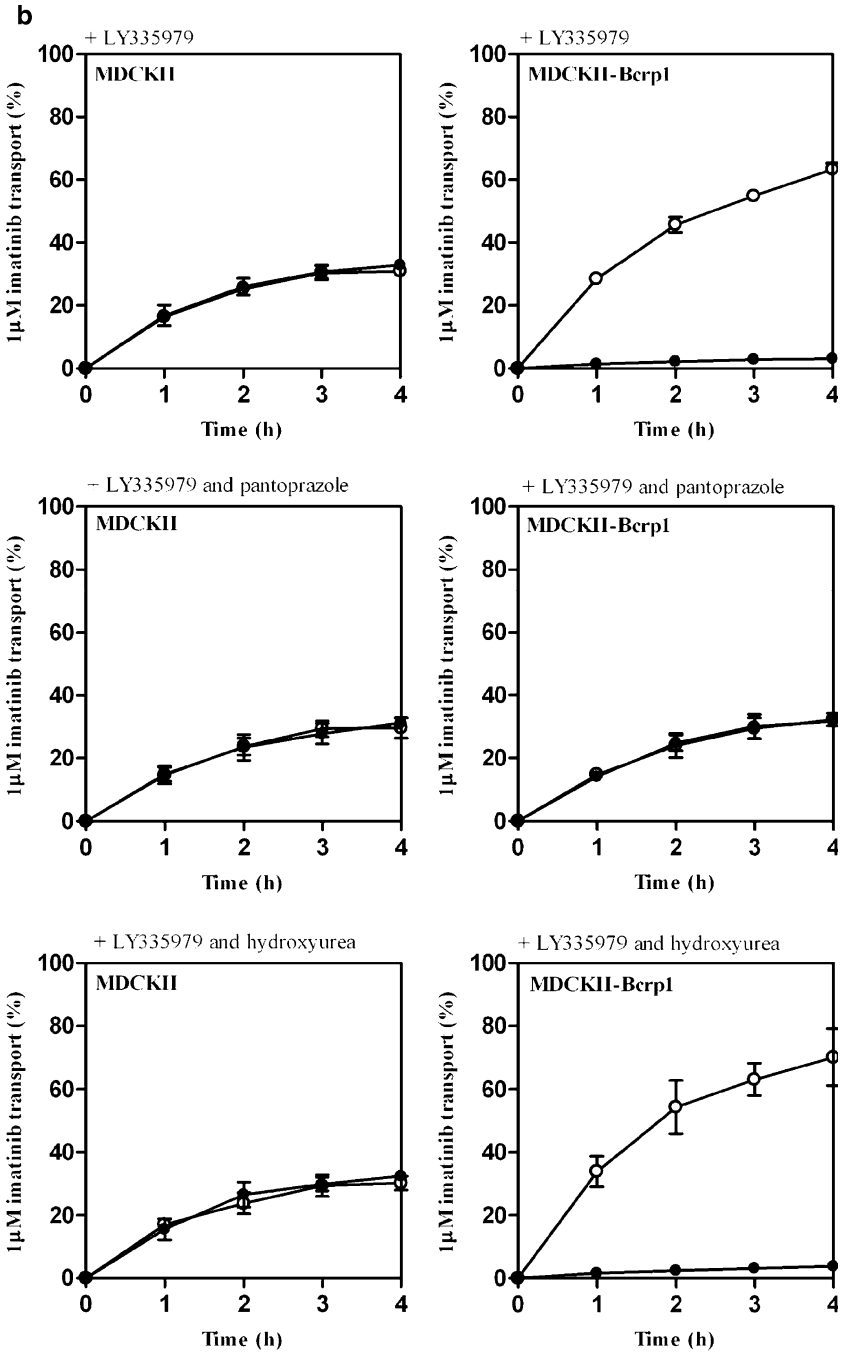
the presence of hydroxyurea, which is not significantly different from the rate of biotransformation of imatinib in the absence of hydroxyurea ($P > 0.05$).

Discussion

The combination therapy of imatinib plus hydroxyurea is associated with remarkable antitumor activity in patients with recurrent glioblastoma. Thus far the mechanism of the added activity of hydroxyurea to imatinib is unknown. We hypothesized that the effect could be due to increased exposure of the tumor to imatinib. As imatinib is a high affinity substrate drug for Pgp and BCRP and is extensively metabolized by CYP3A, we investigated the effect of hydroxyurea on Pgp/BCRP mediated transport and CYP3A metabolism of imatinib. This study shows for the first time that hydroxyurea does not interact with imatinib by inhibition of Pgp and BCRP mediated transport or by CYP3A mediated metabolism of imatinib.

There are several other possible mechanisms of action that have to be investigated and could underlie the positive activity of this regimen. First, preclinical

Fig. 1 continued



studies support that imatinib may enhance hydroxyurea mediated cytotoxicity by improving its delivery to the tumor microenvironment [9, 18]. Imatinib can diminish the tumor interstitial pressure. This could lead to increased capillary-to-interstitium transport and enhanced chemotherapy delivery, e.g., of hydroxyurea [17]. A clinical trial of imatinib with temozolomide, a cytotoxic agent with more established single-agent activity against glioblastoma multiforme than hydroxyurea, may be of interest in this respect.

Imatinib can also diminish tumor cell DNA repair after radiotherapy or chemotherapy by reducing Rad51 expression. Rad51 is an essential component of the DNA double-strand break pathway and has been implicated as a determinant of cellular radiosensitivity [21]. Imatinib-related decreased DNA repair may potentiate the cytotoxicity of hydroxyurea.

A final potential mechanism of action may be that PDGFR inhibitors exhibit significant antiangiogenic activity primarily by targeting perivascular cells, as shown in preclinical models [12, 23]. Furthermore, several chemotherapeutic agents suppress tumor angiogenesis and enhance the antitumor activity of vascular endothelial growth factor inhibitors. Therefore,

PDGFR inhibition by imatinib combined with chemotherapy, e.g., hydroxyurea may provide complementary antiangiogenic activity, thereby limiting tumor growth, e.g., in glioblastoma multiforme.

In conclusion, hydroxyurea and imatinib do not interact at the level of Pgp, BCRP and CYP3A4 and further research is needed to clarify the beneficial activity against glioblastoma multiforme of the combination of hydroxyurea and imatinib.

References

1. Breedveld P, Pluim D, Cipriani G, Wielinga P, van Tellingen O, Schinkel AH, Schellens JH (2005) The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. Cancer Res 65:2577
2. Breedveld P, Zelcer N, Pluim D, Sonmezer O, Tibben MM, Beijnen JH, Schinkel AH, van Tellingen O, Borst P, Schellens JH (2004) Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles: potential role for breast cancer resistance protein in clinical drug-drug interactions. Cancer Res 64:5804
3. Dai H, Marbach P, Lemaire M, Hayes M, Elmquist WF (2003) Distribution of STI-571 to the brain is limited by P-glycoprotein-mediated efflux. J Pharmacol Exp Ther 304:1085
4. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H (2002) Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 347:472
5. Dresemann G (2005) Imatinib and hydroxyurea in pretreated progressive glioblastoma multiforme: a patient series. Ann Oncol 16:1702
6. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 344:1031
7. Eisenblatter T, Huwel S, Galla HJ (2003) Characterisation of the brain multidrug resistance protein (BMDP/ABCG2/BCRP) expressed at the blood-brain barrier. Brain Res 971:221
8. Evers R, Kool M, Smith AJ, van Deemter L, de Haas M, Borst P (2000) Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. Br J Cancer 83:366
9. Hwang RF, Yokoi K, Bucana CD, Tsan R, Killion JJ, Evans DB, Fidler IJ (2003) Inhibition of platelet-derived growth factor receptor phosphorylation by STI571 (Gleevec) reduces growth and metastasis of human pancreatic carcinoma in an orthotopic nude mouse model. Clin Cancer Res 9:6534
10. Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JH, Schinkel AH (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J Natl Cancer Inst 92:1651
11. Kilic T, Alberta JA, Zdunek PR, Acar M, Iannarelli P, O'Reilly T, Buchdunger E, Black PM, Stiles CD (2000) Intracranial inhibition of platelet-derived growth factor-mediated glioblastoma cell growth by an orally active kinase inhibitor of the 2-phenylaminopyrimidine class. Cancer Res 60:5143
12. Laird AD, Vajkoczy P, Shawver LK, Thurnher A, Liang C, Mohammadi M, Schlessinger J, Ullrich A, Hubbard SR, Blake RA, Fong TA, Strawn LM, Sun L, Tang C, Hawtin R, Tang F, Shenoy N, Hirth KP, McMahon G, Cherrington (2000) SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. Cancer Res 60:4152
13. Louvard D (1980) Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. Proc Natl Acad Sci USA 77:4132
14. Lu P, Schrag ML, Slaughter DE, Raab CE, Shou M, Rodrigues AD (2003) Mechanism-based inhibition of human liver microsomal cytochrome P450 1A2 by zileuton, a 5-lipoxygenase inhibitor. Drug Metab Dispos 31:1352
15. Ma J, Maliepaard M, Nooter K, Boersma AW, Verweij J, Stoter G, Schellens JH (1998) Synergistic cytotoxicity of cisplatin and topotecan or SN-38 in a panel of eight solid-tumor cell lines in vitro. Cancer Chemother Pharmacol 41:307
16. Peng B, Lloyd P, Schran H (2005) Clinical pharmacokinetics of imatinib. Clin Pharmacokinet 44:879
17. Pietras K, Ostman A, Sjoquist M, Buchdunger E, Reed RK, Heldin CH, Rubin K (2001) Inhibition of platelet-derived growth factor receptors reduces interstitial hypertension and increases transcapillary transport in tumors. Cancer Res 61:2929
18. Pietras K, Rubin K, Sjoblom T, Buchdunger E, Sjoquist M, Heldin CH, Ostman A (2002) Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. Cancer Res 62:5476
19. Raymond E, Brandes A, Van Oosterom A, Dittrich C, Fumoleau P, Coudert B, Twelves C, De Balincourt C, Lacombe M, Van Den Bent M (2004) Multicentre phase II study of imatinib mesylate in patients with recurrent glioblastoma: An EORTC: NDDG/BTG Intergroup Study. Proc Am Soc Clin Oncol 23:107
20. Reardon DA, Egorin MJ, Quinn JA, Rich JN Sr, Gururangan I, Vredenburgh JJ, Desjardins A, Sathornsumetee S, Provenzale JM, Herndon JE, Dowell JM, Badruddoja MA, McLendon RE, Lagattuta TF, Kicielinski KP, Dresemann G, Sampson JH, Friedman AH, Salvado AJ, Friedman HS (2005) Phase II Study of Imatinib Mesylate Plus Hydroxyurea in Adults With Recurrent Glioblastoma Multiforme. J Clin Oncol 23:9359
21. Russell JS, Brady K, Burgan WE, Cerra MA, Oswald KA, Camphausen K, Tofilon PJ (2003) Gleevec-mediated inhibition of Rad51 expression and enhancement of tumor cell radiosensitivity. Cancer Res 63:7377
22. Schinkel AH, Smit JJ, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CA, van der Valk MA, Robanus-Maandag EC, te Riele HP, (1994) Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell 77:491
23. Shaheen RM, Tseng WW, Davis DW, Liu W, Reinmuth N, Vellagas R, Wieczorek AA, Ogura Y, McConkey DJ, Drazan KE, Bucana CD, McMahon G, Ellis LM (2001) Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms. Cancer Res 61:1464
24. Wen PY, Yung WK, Lamborn K (2004) PhaseI/II study of imatinib mesylate (STI571) for patients with recurrent malignant gliomas (NABTC 99-08). Neuro-Oncol 6:384 (abstr TA-57)