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MONOGRAPH

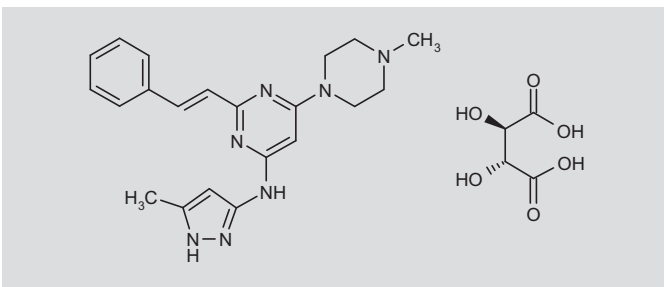
ENMD-2076

Aurora Kinase A Inhibitor
Protein Kinase Inhibitor
Oncolytic

ENMD-981693 (as free base)

6-(4-Methylpiperazin-1-yl)-*N*-(5-methyl-1*H*-pyrazol-3-yl)-2-[(*E*)-2-phenylvinyl]pyrimidin-4-amine L-tartrate

InChI: 1S/C21H25N7.C4H6O6/c1-16-14-20(26-25-16)23-19-15-21(28-12-10-27(2)11-13-28)24-18(22-19)9-8-17-6-4-3-5-7-17;5-1(3(7)8)2(6)4(9)10/h3-9,14-15H,10-13H2,1-2H3,(H2,22,23,24,25,26);1-2,5-6H,(H,7,8)(H,9,10)/b9-8+;/t;1-,2-/m.1/s1



$C_{25}H_{31}N_7O_6$

Mol wt: 525.5569

CAS: 934353-76-1 (as free base)

EN: 450149

SUMMARY

ENMD-2076 is a novel, orally active molecule that has been shown to have significant activity against Aurora kinases and multiple tyrosine-protein kinases, including tyrosine-protein kinase receptor FLT3, proto-oncogene c-Kit, vascular endothelial growth factor receptor VEGFR-2, basic fibroblast growth factor FGFR-1 and FGFR-3, and tyrosine-protein kinase JAK2. ENMD-2076 has potent activity against various cultured tumor cells through multiple effects, including induction of early caspase-dependent apoptosis, modulation of the expression of anti- and proapoptotic proteins to favor cell death, inhibition of the phosphatidylinositol 3-kinase (PI3K)/protein kinase Akt pathway and Aurora kinase A and B, signaling through FGFR-3 and VEGFR, and induction of G_2/M cell cycle arrest. Potent antitumor activity in mice bearing cancer cell lines or human tumor xenografts, including human colorectal cancer, multiple myeloma, leukemia and breast cancer, is also observed. Phase I trials of ENMD-2076 are currently ongoing in solid tumors and hematologic malignancies, with preliminary results showing that the drug has acceptable toxicity and shows promising activity in at least ovarian and colon cancer, multiple myeloma and acute myeloid leukemia. Studies directed at better defining the spectrum of antitumor activity of ENMD-2076 and the relative importance of its apparently different mechanisms of action will allow the design of rational combinations of ENMD-2076 with other anticancer drugs in future clinical trials.

S. Zhang and S.S. Farag. Division of Hematology and Oncology, Department of Medicine, Indiana University School of Medicine, Walther Hall-R3, C414, 840 West Walnut St., Indianapolis, IN 46202, USA. E-mail: ssfarag@iupui.edu.
*Synthesis prepared by J. Bolòs, R. Castañer. Thomson Reuters, Provença 388, 08025 Barcelona, Spain.

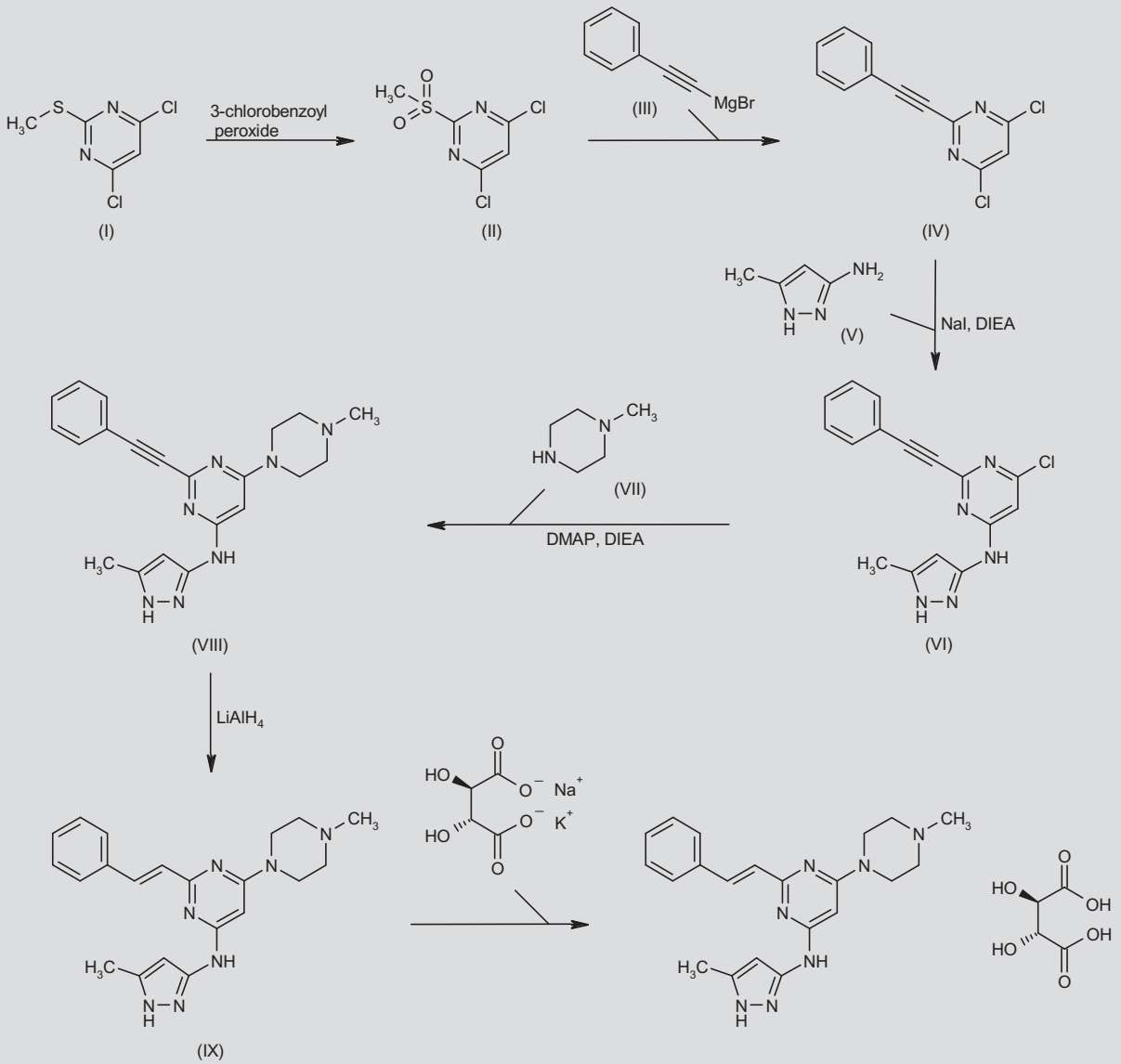
SYNTHESIS*

ENMD-2076 can be prepared by two different ways:

Oxidation of 4,6-dichloro-2-(methylsulfanyl)pyrimidine (I) with 3-chlorobenzoyl peroxide in CH_2Cl_2 gives the sulfonyl derivative (II), which is alkynylated with phenylacetylenyl magnesium bromide (III) in THF to provide 4,6-dichloro-2-(phenylethynyl)pyrimidine (IV). Condensation of the dichloropyrimidine derivative (IV) with 5-methyl-3-aminopyrazole (V) by means of NaI and DIEA in DMA at 90 °C affords amine (VI), which is coupled with *N*-methylpiperazine (VII) in the presence of DMAP and DIEA in 1,4-dioxane at 100 °C to furnish the piperazinyl derivative (VIII). Reduction of alkyne (VIII) by means of $LiAlH_4$ in THF gives alkene (IX), which is finally treated with sodium potassium tartrate (1). Scheme 1.

Treatment of cinnamonitrile (X) with HCl in toluene/EtOH gives the *O*-ethyl imidate.HCl (XI), which is aminated by means of methanolic ammonia in EtOH to provide amidine (XII). Cyclization of compound (XII) with dimethylmalonate (XIII) in the presence of NaOMe in MeOH at 90 °C affords the pyrimidindione (XIV), which is chlorinated with $POCl_3$ to generate the dichloro derivative (XV). Coupling of dichloro compound (XV) with 5-methyl-3-aminopyrazole (V) by means of NaI and DIEA in DMA at 90 °C affords the secondary amine (XVI), which is finally condensed with *N*-methylpiperazine (VII) to give the free base (IX) (1). Scheme 2.

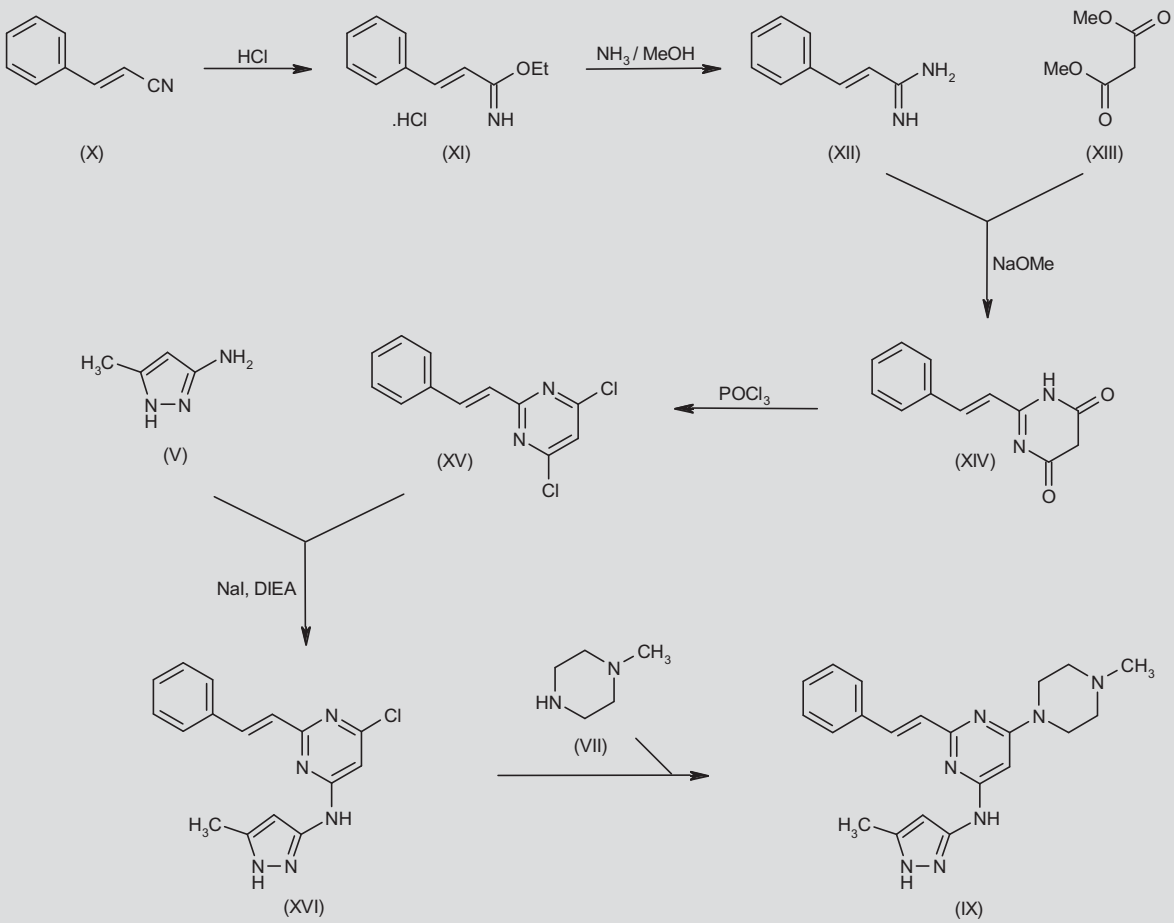
Scheme 1. Synthesis of ENMD-2076



BACKGROUND

Following the discovery of the human kinome, protein kinases have become important targets for anticancer therapy (2). The human genome encodes about 518 protein kinases, which play important roles in regulating the majority of cellular pathways, especially those involved in signal transduction (3). Protein kinases can modify other proteins by transferring the terminal phosphate of ATP to substrates that usually contain a serine, threonine or tyrosine residue. Protein kinases are involved in many cellular processes, including cell survival, proliferation, metastasis and angiogenesis, and their dysregulated activity has been implicated in many cancers (2). Four groups of protein kinases are generally recognized. First, the receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF-1 receptor), vascular endothelial growth factor receptor (VEGFR), basic fibroblast growth factor receptors FGFR-1, FGFR-3 and FGFR-4, tyrosine-protein kinase receptor FLT3 and proto-oncogene c-Kit (2, 3). Among these receptor tyrosine kinases, VEGFR and FGFR are important in promoting abnormal blood vessel formation, or angiogenesis, in tumors (4). Antiangiogenic drugs inhibit tumor growth by stopping blood vessel growth and then limiting the blood supply (5). A second group is comprised of the non-receptor tyrosine-protein kinases, including Src, ABL, JAK2, Yes and focal adhesion kinase (FADK) (2, 3,

Scheme 2. Synthesis of ENMD-2076



6). Src family kinases function as second messenger molecules in response to activated growth factor receptors and play an important role in regulating mitotic events (7). JAK2 transduces cytokine-mediated signals via the JAK2/signal transducer and activator of transcription (STAT) pathway (8). Third are the serine/threonine kinases, including proteins such as Akt, ATM, mTOR, PKCI, S6K, B-raf, LKB1 and cell cycle control kinases, including cyclin-dependent kinases (CDK), Aurora kinases and the Polo-like kinases (2). Fourth are the lipid kinases, such as phosphatidylinositol 3-kinase (PI3K) and SK1. A key downstream effector of PI3K is the serine/threonine kinase Akt, which phosphorylates and regulates the activity of a number of targets, including kinases, transcription factors and other regulatory molecules. The PI3K/Akt pathway plays a crucial role in cell growth and survival and is activated in various cancers (9).

Aurora kinases are essential for cell proliferation, regulating mitotic entry, the formation of mitotic spindle, centrosome maturation and separation, and cytokinesis (10-12). There are three human homologues of Aurora kinases, A, B and C, which have specific cellular localization and functions, despite significant sequence homology. Aurora kinase A localizes to the centrosome during centrosome duplication through mitotic exit, and functions in the entry into mitosis, centrosome maturation and division, bipolar spindle assembly, chromosome alignment and cytokinesis (11, 13, 14). The kinase activity of Aurora kinase A is tightly regulated throughout the cell cycle. Aurora kinase A activity depends on the phosphorylation status of a threonine residue (T288) in the activation loop (15, 16). Aurora kinase A is considered an oncogene based on a number of observations. Overexpression of wild-type Aurora kinase A is oncogenic in murine models (17, 18). Also, the gene encoding for Aurora kinase A, *AURKA*, is located at chromosome position 20q13, a site that is amplified in a number of tumor types, including breast (19, 20), colorectal (18, 21), glioma (22, 23), bladder (24), and head and neck cancers (25), and is associated with a poor prognosis. In addition, overexpression of Aurora kinase A occurs independently of gene amplification in a wide range of tumor types compared with essentially nonproliferating matched normal tissue, although this may be related to rapid cell division rather than a cause of the malignant phenotype (26-28).

Aurora kinase B is the catalytic component of the chromosomal passenger complex, which consists of three additional noncatalytic subunits that direct its activity: survivin, borealin and INCEP (29, 30). Aurora kinase B localizes to the inner centromeric region from prophase to metaphase, and then to the spindle midzone and midbody from anaphase through cytokinesis (31). Aurora kinase B is involved in accurate chromosomal segregation, cytokinesis, correct microtubule–kinetochore attachment, and regulation of the mitotic checkpoint. During mitosis, Aurora kinase B phosphorylates histone H3 on Ser10, which has proven to be an important pharmacodynamic endpoint in the preclinical development of Aurora kinase B inhibitors. Aurora kinase B is overexpressed in a variety of human tumor types (27).

The role of Aurora kinase C is less well understood. The expression of Aurora kinase C is restricted to the testicular tissue, and possesses overlapping functions with Aurora kinase B (32, 33). The potential role of Aurora kinases in tumorigenesis and their overexpression in a variety of tumor types has made them an attractive therapeutic target for the development of small-molecule inhibitors.

Herein we describe the novel, orally active Aurora kinase and multiple tyrosine kinase inhibitor ENMD-2076. ENMD-2076 inhibits Aurora kinase A and B and multiple tyrosine kinases in vitro, including FLT3, c-Kit, VEGFR, macrophage colony-stimulating factor 1 receptor (CSF-1-R) and FGFR, at nanomolar and submicromolar concentrations (34, 35). ENMD-2076 induced cell cycle arrest and apoptosis in multiple cultured tumor cell lines and demonstrated potent antitumor activity in mice bearing cancer cell lines or human tumor xenografts (34-37). As discussed below, ENMD-2076 has potential activity against solid and hematologic cancers in vitro and in vivo, and is currently being investigated clinically in ovarian cancer, multiple myeloma (MM) and acute myeloid leukemia (AML).

ENMD-2076 is the L-(+)-tartrate salt of ENMD-981693, which was discovered in the course of screening for novel inhibitors of Aurora kinases. ENMD-981693 was found to be relatively selective for Aurora kinase A (34). Also, when evaluated against a panel of 100 recombinant kinases, ENMD-981693 was shown to have potent activity against oncogenic tyrosine kinases, including FLT3, c-Kit and CSF-1-R, involved in the pathogenesis of a number of hematologic cancers, as well as VEGFR-2 and FGFR-1, which play important roles in angiogenesis. In vitro, ENMD-981693 has been shown to inhibit the growth of human leukemia and MM cell lines, inducing cell cycle arrest and apoptosis. Furthermore, in mouse models ENMD-981693 demonstrated inhibition of xenografts derived from leukemia, colon, breast cancer and myeloma cell lines, with minimal toxicity (34, 37). ENMD-981693 also exhibited antiangiogenic activity in xenograft models by preventing the formation of new blood vessels and inducing regression of formed vessels at well-tolerated doses (34).

In screening for a soluble form of ENMD-981693, the tartrate salt ENMD-2076 was selected for development because it had similar pharmacokinetic properties to the free base but characteristics better suited for large-scale manufacturing (35). Similar to the free base, ENMD-2076 inhibited multiple tyrosine kinases, including FLT3, Src, VEGFR-2, FGFR-1 and c-Kit, with IC_{50} values of 1.86, 20.2, 58.2, 92.7 and 120 nmol/L, respectively (Table I). As an Aurora kinase inhibitor, ENMD-2076 is relatively selective for Aurora kinase A, with an IC_{50} of 14 nmol/L compared to an IC_{50} of 350 nmol/L for Aurora kinase B (35, 36).

Table I. *Inhibitory activity of ENMD-2076 against cellular kinases.*

Kinase	Recombinant protein IC_{50} (nmol/L)	Cellular IC_{50} (nmol/L)
Aurora kinase A	14	130
Aurora kinase B	350	2,400
VEGFR-2	58.2	80
FGFR-1	92.7	600
FGFR-3	n/a	850
PDGF-R-α	56	1,000-5,000
FLT3	1.86	20
c-Kit	120	40
Src	20.2	100
JAK2	120	300
ABL	295	> 25,000

n/a, not available.

PRECLINICAL PHARMACOLOGY

ENMD-2076 and its free base ENMD-981693 show significant antitumor activity both in vitro and in vivo. Early studies demonstrated that ENMD-981693 had significant cytotoxic activity against a broad range of tumor cell lines. Following 48-h exposure, ENMD-981693 was cytotoxic to a variety of human lymphoblastic and myeloid leukemia cell lines, with IC_{50} values in the range of 0.02-7 μM, and to primary leukemic blasts, with IC_{50} values of 0.2-6.0 μM. ENMD-981693 was cytotoxic to solid tumor cell lines at 96 h in the submicromolar range (IC_{50} = 0.12-0.6 μM) (34). ENMD-2076 has similar antiproliferative activity to ENMD-981693. In a 4-day assay, the IC_{50} of ENMD-2076 was between 0.12 and 0.7 μM against seven solid tumor cell lines. Against 10 human leukemia cell lines the IC_{50} values were 0.025-0.53 μM (35). Using a panel of human MM cell lines, including IM-9, ARH-77, U266, RPMI 8226, MM.1S and MM.1R, we have recently shown that ENMD-2076 inhibits MM cell growth in a concentration- and time-dependent manner. The mean (± standard deviation) IC_{50} of ENMD-2076 was 6.90 ± 2.36 μmol/L after 24 h and 2.99 ± 0.67 μmol/L at 72 h. ENMD-2076 also had significant cytotoxicity against primary MM cells, with an IC_{50} of 7.06 ± 1.29 μmol/L at 24 h. Notably, however, normal hematopoietic progenitor cells (purified $CD34^+$ cells) were more resistant, with a mean IC_{50} of 16.37 ± 1.44 μmol/L at 24 h (37). The four- to fivefold difference in IC_{50} between tumor cells and normal hematopoietic progenitors suggests that hematopoietic toxicity is likely to be tolerable in vivo.

The antitumor activity of ENMD-2076 has also been demonstrated in vivo in murine tumor xenograft models. In athymic nude mice bearing human colorectal cancer (CRC) HT-29 xenografts, ENMD-2076 dosed at 100 or 200 mg/kg/day orally for 28 days significantly inhibited tumor growth relative to controls. Furthermore, 100 mg/kg ENMD-2076 inhibited the growth of three patient-derived CRC xenografts (36), providing a rationale for clinical testing of this drug in colorectal cancer. Similarly, ENMD-2076 also suppressed the growth of plasmacytoma xenografts in NOD/SCID mice. ENMD-2076 was administered orally at 50, 100 or 200 mg/kg/day for a period of 46 days, and significantly inhibited the growth of H929

plasmacytomas in a dose-dependent manner, with minimal growth observed with 200 mg/kg/day (Fig. 1A). In addition to inhibiting growth, ENMD-2076 also induced regression of established H929 plasmacytomas (Fig. 1B) (37). Similar in vivo antitumor activity has also been demonstrated in xenograft models of human tumors derived from leukemia, breast cancer and melanoma (35, 38).

In the above studies, ENMD-2076 induced multiple effects in vivo, inhibiting proliferation, inducing apoptosis and reducing tumor metabolic activity. Ki-76 is a cellular marker for proliferation. In both MM and CRC murine xenografts, Ki-76 expression is significantly reduced with ENMD-2076 treatment, indicating a significant antiproliferative effect (36, 37). Significant apoptosis, as shown by immunohistochemical staining for caspase-3 activation, was demonstrated in plasmacytoma xenografts following ENMD-2076 treatment. Similarly, phosphorylation of histone H3 Ser10 was decreased by ENMD-2076 in myeloma xenografts, indirectly showing Aurora kinase inhibition (37). Finally, a reduction in metabolic activity of tumors was also demonstrated in CRC HT-29 xenografts by ^{18}FDG-positron emission tomography (^{18}FDG-PET) (36).

Significantly, in multiple tumor xenograft models, including colon cancer, myeloma and AML, treatment with ENMD-2076 did not result in any significant weight loss or outward morbidity in the

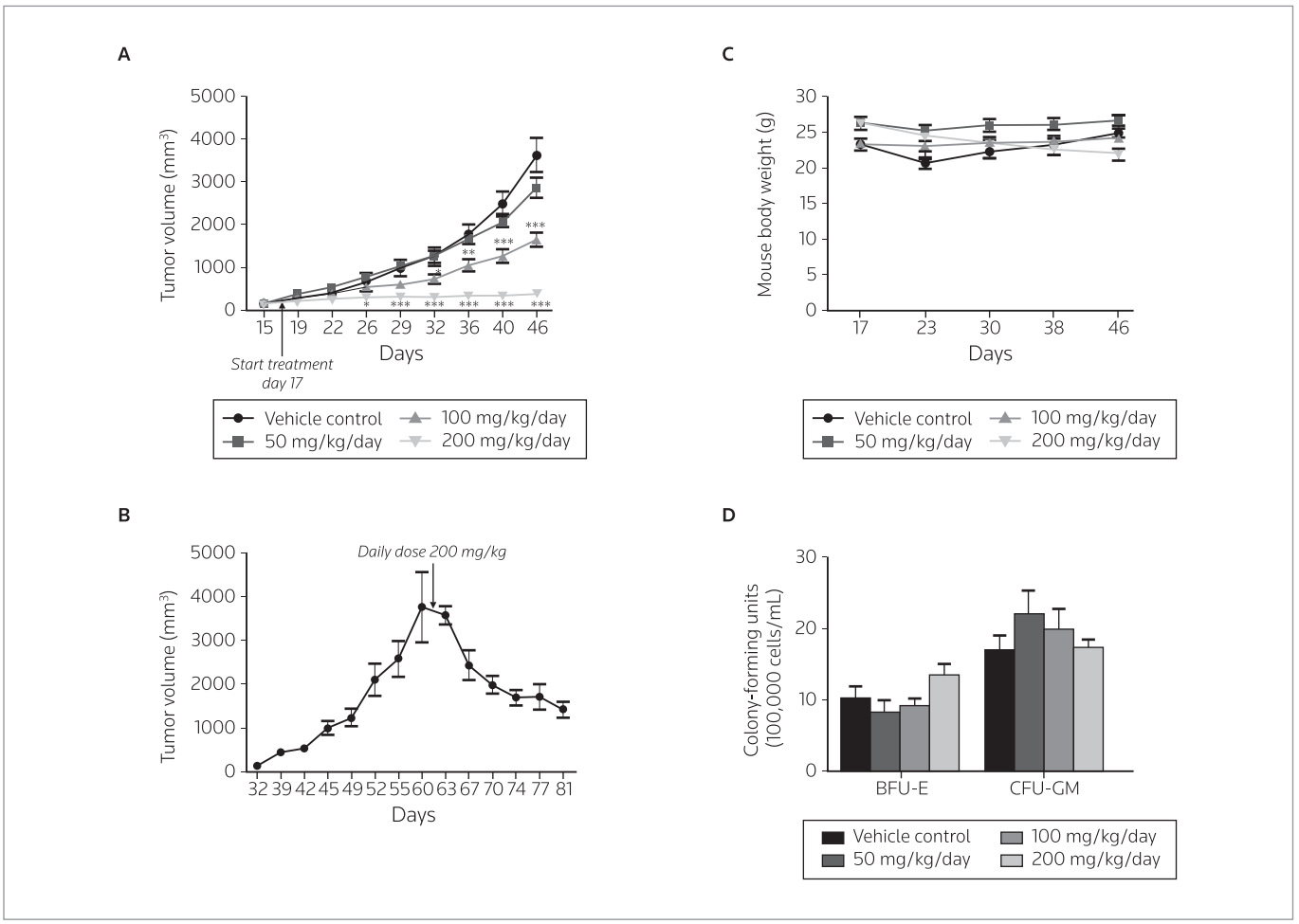


Figure 1. Activity of ENMD-2076 in vivo in an H929 plasmacytoma xenograft model. (**A**) Dose-dependent inhibition of tumor growth by ENMD-2076. Human H929 myeloma cells were implanted s.c. in 6- to 8-week-old NOD/SCID mice and mice were assigned to three ENMD-2076 treatment groups (50, 100 and 200 mg/kg/day) or sterile water when plasmacytomas reached a volume of 100-200 mm^3. ***P < 0.001, **P < 0.01, *P < 0.05 vs. vehicle control; n = 13 mice for vehicle control and n = 19 mice for other groups. (**B**) Regression of established plasmacytoma with ENMD-2076 treatment (200 mg/kg/day) with > 50% reduction in tumor volume within 10 days. (**C**) Weight of mice treated with ENMD-2076. At all dose levels tested, no significant change in weight was observed. n = 13 mice for vehicle control and n = 19 mice for other groups. (**D**) Effect of in vivo treatment with ENMD-2076 on mouse bone marrow erythroid (BFU-E) and myeloid (CFU-GM) hematopoietic progenitors. No significant reduction in either CFU-GM or BFU-E with 4 weeks of ENMD-2076 treatment was observed. n = 6 mice per group. Adapted with permission from Wang, X. Sinn, A.L., Pollok, K., Sandusky, G., Zhang, S., Chen, L., Liang, J., Crean, C.D., Suvannasankha, A., Abonour, R., Sidor, C., Bray, M.R., Farag, S.S. *Preclinical activity of a novel multiple tyrosine kinase and aurora kinase inhibitor, ENMD-2076, against multiple myeloma*. Br J Haematol 2010, 150(3): 313-25, © 2010, Wiley.

treated animals, indicating that doses of 50-200 mg/kg/day are well within the tolerability limits for ENMD-2076 in mice (Fig. 1C) (35-37). Furthermore, in one study where the effect on hematopoietic progenitor cells was assessed, ENMD-2076 did not result in significant suppression of erythroid or myeloid colony formation by normal bone marrow cells obtained from mice sacrificed after 30 days of treatment, indicating minimal toxicity to hematopoietic progenitors in vivo (Fig. 1D) (37). The above results provide a rationale for the clinical development of ENMD-2076 in a variety of hematopoietic and solid tumors, as discussed below.

The mechanism by which ENMD-2076 (or its free base) is cytotoxic to hematopoietic and solid cancer cells is not completely understood, but is likely mediated via a number of different cellular pathways. To date, the pathways involved in ENMD-2076-mediated cytotoxicity have only been studied in MM cells (37). In MM cell lines, ENMD-2076 induces rapid apoptosis via activation of both the intrinsic and extrinsic pathways. Activation of caspase-9, -8 and -3, together with cleavage of poly(ADP-ribose)polymerase (PARP), and loss of mitochondrial membrane potential occur as early as 6 h after exposure to drug, indicating that mechanisms other than Aurora kinase inhibition are operating.

ENMD-2076-induced tumor cell killing is also associated with significant changes in the expression of antiapoptotic and proapoptotic protein levels, which have been shown to be important for the survival of cancer cells and their resistance to chemotherapeutic agents (39, 40). In myeloma cells, ENMD-2076 induces the cleavage of the antiapoptotic protein Mcl-1 as early as 6 h following treatment, but without significant changes in Bcl-2 and Bcl-xL. Similarly, ENMD-2076 downregulates the expression of the proapoptotic proteins of the inhibitor of apoptosis (IAP) family, survivin and XIAP (X-linked inhibitor of apoptosis) at 6 h (37). On the other hand, levels of the proapoptotic proteins of the Bcl-2 family, including BAX, BAD and Bcl2-L-11 remain unaffected, even with prolonged exposure to drug.

Intracellular signaling through the PI3K/Akt, JAK2/STAT and Ras/Raf/MEK/extracellular signal-regulated kinase (ERK) pathways contributes to the survival, growth, proliferation and chemotherapy resistance of cancer cells (41, 42). We have recently shown that ENMD-2076 inhibits the PI3K/Akt pathway. In MM cell lines, ENMD-2076 decreases Akt downstream targets, including phosphorylated BAD (pBAD), phosphorylated glycogen synthase kinase-3 beta (pGSK-3β) and phosphorylated forkhead box protein O1A, as well as IL-6-induced phosphorylation of Akt, P70S6K, 4E-BP1, BAD, forkhead box protein O1A and GSK-3β. Consistent with this effect, IL-6 does not protect MM cells from ENMD-2076-induced apoptosis. In contrast, ENMD-2076 has no effect on the JAK2/STAT3 or MAPK/ERK pathways in MM.1R cell lines (37).

Immunofluorescent detection of Aurora kinase A autophosphorylation on T288 (pT288) reflects the activity of the kinase in cells (15, 43). In MM cells, ENMD-2076 inhibits the activity of Aurora kinase A and B. However, although inhibition of Aurora kinase occurs at concentrations that cause MM cell death, this is observed at later time points. For example, while apoptosis of myeloma cells is induced by ENMD-2076 as early as 6 h, inhibition of Aurora kinase A autophosphorylation on T288 is observed only after 24-48 h of exposure to drug, with no effect apparent at 6 h, even at concentrations of 1-10 μmol/L (37). Similarly, inhibition of Aurora kinase B, reflected in the inhibition of histone H3 phosphorylation on Ser10, was also observed at ENMD-2076 concentrations of 5-10 μmol/L, but only after 24-48 h of exposure. As noted above, these data suggest that while ENMD-2076 inhibits Aurora kinases, other mechanisms are likely to also be operative in drug-induced tumor cell killing. Whether these changes also occur in other tumor cell types or are specific to myeloma cells is currently unknown. Indeed, it is possible that the dominant mechanism of cell killing may be different in different types of tumor cells.

Cell cycle arrest in the G_2/M phase is a characteristic effect of Aurora kinase inhibition (44, 45). In MM cells, we have shown that ENMD-2076 induces cell cycle arrest in the G_2/M phase, with the effect pronounced at the later time points of 24 and 48 h, corresponding to the time of maximal inhibitory effect on Aurora kinase A and B (37). Consistent with its effect on the cell cycle, ENMD-2076 induces a significant reduction in the expression of cyclins A and B, with no effect on cyclin D1 or p21 in MM cells. Similarly, in leukemia cells, ENMD-981693 also induced G_2/M cell cycle arrest. In both myeloma and leukemia cells, however, no induction of the endo-reduplication phenotype (> 4N DNA content), which is usually associated with Aurora kinase B inhibitors, is observed (34, 37), confirming a more dominant effect on Aurora kinase A.

FLT3 and Kit are closely related receptors of the platelet-derived growth factor receptor (PDGFR) subfamily of receptor tyrosine kinases. In the human AML THP-1 cell line, ENMD-2076 inhibited cellular FLT3 ligand-induced FLT3 autophosphorylation with an IC_{50} value of 28 nM. MO7e is a human megakaryocytic leukemia cell line that expresses wild-type Kit and is dependent on Kit signaling for cell growth and survival. ENMD-2076 has an IC_{50} of 40 nM for inhibition of stem cell factor (SCF)-induced Kit autophosphorylation in MO7e cells. Macrophage colony-stimulating factor CSF-1-R is another receptor tyrosine kinase that is overexpressed in various cancers. The AML cell line MV-4-11 expresses the CSF-1-R protein and the FLT3/ITD mutation and is dependent on FLT3 activity for survival. In vitro, ENMD-2076 inhibited CSF-stimulated CSF-1-R signaling with an IC_{50} of 600 nM in MV-4-11 cells. In an MV-4-11 xenograft model, the phosphorylation of both FLT3 and the FLT3 substrate STAT5 was decreased by ENMD-2076 at a dose of 45 mg/kg (35).

Fibroblast growth factors (FGFs) represent a large family of polypeptides that are potent regulators of cell proliferation, migration and differentiation (46). Five distinct genes encode for high-affinity receptors for FGFs (*FGFR1, FGFR2, FGFR3, FGFR4* and *FGFR5*), which belong to the immunoglobulin-like family of tyrosine kinases (47). Ligand-mediated receptor activation of FGFR triggers a signal transduction cascade from the cell surface to the nucleus (48) that is translated into a variety of processes related to cell growth and differentiation via Ras/MAPK and STAT1/p21 signaling pathways (46, 49, 50). Activating mutations in *FGFR3* have been shown to play an oncogenic role in tumorigenesis because they lead to ligand-independent dimerization and constitutive activation of the receptor (51), and have recently been found to be present in patients with uterine cervical carcinoma and urothelial carcinoma (52, 53). Also, in approximately 15% of cases of MM, ectopic expression of functional FGFR-3 in plasma cells occurs as a result of the chromosomal translocation t(4;14) (54-56), and is associated with a particularly

poor outcome, even with high-dose chemotherapy and stem cell transplant (57, 58). Ectopic expression of FGFR-3 on myeloma cells stimulates cell proliferation and prevents apoptosis (59), and myeloma cells ectopically expressing FGFR-3 are sensitive to the inhibitory and proapoptotic effects of specific FGFR inhibitors (60), suggesting that FGFR-3 may be a good therapeutic target in this subset of MM.

As noted above, the free base ENMD-981693 inhibits recombinant FGFR-3 kinase activity with an IC_{50} of 0.5 μM in cell-free in vitro assays (34). Although the activity of ENMD-2076 (or its free base) against urothelial and cervical cancer cells has not been reported, significant inhibitory activity against H929 myeloma cells with ectopic expression of FGFR-3 has been demonstrated in vitro, with an IC_{50} of 0.8 μM (61), and in vivo, as demonstrated by downregulation of pFGFR-3 in plasmacytoma xenografts excised from mice treated for 30 days with drug at doses of 100 and 200 mg/kg/day (37). In another study, the level of pFGFR-3 immunoprecipitated from lysates of H929 tumor xenografts showed a significant reduction following ENMD-2076 treatment, with no change in total FGFR-3 (61). These studies provide a strong rationale for the clinical investigation of ENMD-2076 in FGFR-3-expressing MM, and potentially also urothelial and cervical cancers that harbor activating mutations in *FGFR3*, although less preclinical data are available for efficacy in these tumors.

In a pancreatic carcinoma MIA PaCa-2 xenograft model, pFGFR-1 was inhibited as early as 4 h following doses of 75-225 mg/kg. The *FGFR2* gene was amplified in gastric carcinoma KATO III cells and a dose of 200 mg/kg could inhibit pFGFR-2 at 4 h in mice bearing KATO III xenografts, with decreases at 24-48 h with 50, 100 or 200 mg/kg (35).

Over the past decade, preclinical testing has indicated that targeting of the VEGF-A/VEGFR-2 pathway results in significant inhibition of neovascularization and tumor growth in various murine tumor xenograft models (27-31), and has resulted in the clinical investigation of a number of antiangiogenic drugs (62). Furthermore, in some tumors such as MM, VEGF may act directly as a growth and survival factor independent of new vessel formation (63, 64). As anticipated from the profile of kinase inhibition described above, ENMD-2076 has been shown to have significant antiangiogenic activity in vivo in preclinical models. In murine plasmacytoma models, we have recently shown that microvascular density, as assessed by CD34 immunohistochemical staining, is significantly reduced in tumors following treatment of mice with ENMD-2076 at 50-200 mg/kg/day (37). Similarly, in a human melanoma A-375 xenograft model, treatment with ENMD-2076 reduced VEGFR-2 activation (38). Inhibition of angiogenesis has also been demonstrated in HT-29 xenografts using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). ENMD-2076 decreased tumor vascular permeability and vascular perfusion as early as 7 days, and had a dramatic effect by 21 days, which was confirmed by pathologic examination of tumors from sacrificed mice (36). It should be acknowledged, however, that while ENMD-2076 has significant antiangiogenic activity, the relative contribution of this effect to the observed antitumor activity remains uncertain.

CLINICAL STUDIES

The preclinical studies summarized above provided the rationale for the clinical investigation of ENMD-2076, and paved the way for a number of clinical trials in patients with advanced solid tumors, ovarian cancer, MM and hematologic malignancies (Table II).

In a first-in-human phase I clinical trial in patients with advanced solid tumors, the maximum tolerated dose (MTD) of daily oral ENMD-2076 was defined as 160 mg/m^2 (65). In this trial, ENMD-2076 was administered orally on 28-day cycles at 5 dose levels: 60, 80, 120, 200 and 160 mg/m^2/day. Twenty-nine patients were treated in a dose-escalation phase, with dose-limiting grade 3 hypertension and neutropenia seen in 2 and 1 of 7 patients, respectively, treated at 200 mg/m^2. At the MTD of 160 mg/m^2/day, an additional 38 patients were treated in an expansion phase of the study. The most common drug-related adverse events at the MTD were grade 3-4 hypertension (n = 7), fatigue (n = 2), elevation of hepatic transaminases (n = 2) or alkaline phosphatase (n = 1), congestive cardiac failure (n = 1), electrolyte disturbances (n = 2) and dyspnea (n = 1). Dose delays were reported in 24 patients (63%) and dose reductions were required in 14 (37%) of the 38 patients treated at 160 mg/m^2/day, most commonly due to fatigue and hypertension. Among disease types included in the trial, ovarian cancer appeared to be most responsive to ENMD-2076 (Fig. 2). Of 58 patients evaluable for tumor response, 2 patients with platinum-resistant ovarian cancer treated at 60 and 160 mg/m^2/day, respectively, achieved partial responses (PR). Overall, 12 of 20 patients with ovarian cancer achieved either PR or stable disease (SD), and 9 patients had at least a 50% reduction in CA125. Furthermore, 5 of 19 patients with colorectal cancer achieved SD for at least 12 weeks. Plasma soluble VEGFR-2 concentrations significantly decreased from a mean of 8,419 pg/mL at baseline to 5,600 pg/mL after 28 days of treatment ($P < 0.001$), although changes did not appear to correlate with responses in disease markers. The results suggest that ENMD-2076 has acceptable toxicity at 160 mg/m^2/day, with evidence of antitumor activity in patients with ovarian and colorectal cancer. A single-agent phase II study of ENMD-2076 in platinum-resistant ovarian cancer is currently ongoing.

Table II. *Clinical trials of ENMD-2076.*

Phase	Disease	ClinicalTrials.gov Identifier	Ref.
Phase I	Advanced solid tumors (epithelial ovarian, colorectal, others)	NCT00658671	65
Phase I	Multiple myeloma	NCT00806065	67
Phase I	Hematological malignancies (AML, ALL, CLL, CML in blast crisis, high-risk MDS, agnogenic myeloid metaplasia)	NCT00904787	66
Phase II	Platinum-resistant ovarian cancer	NCT01104675	–

AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome.

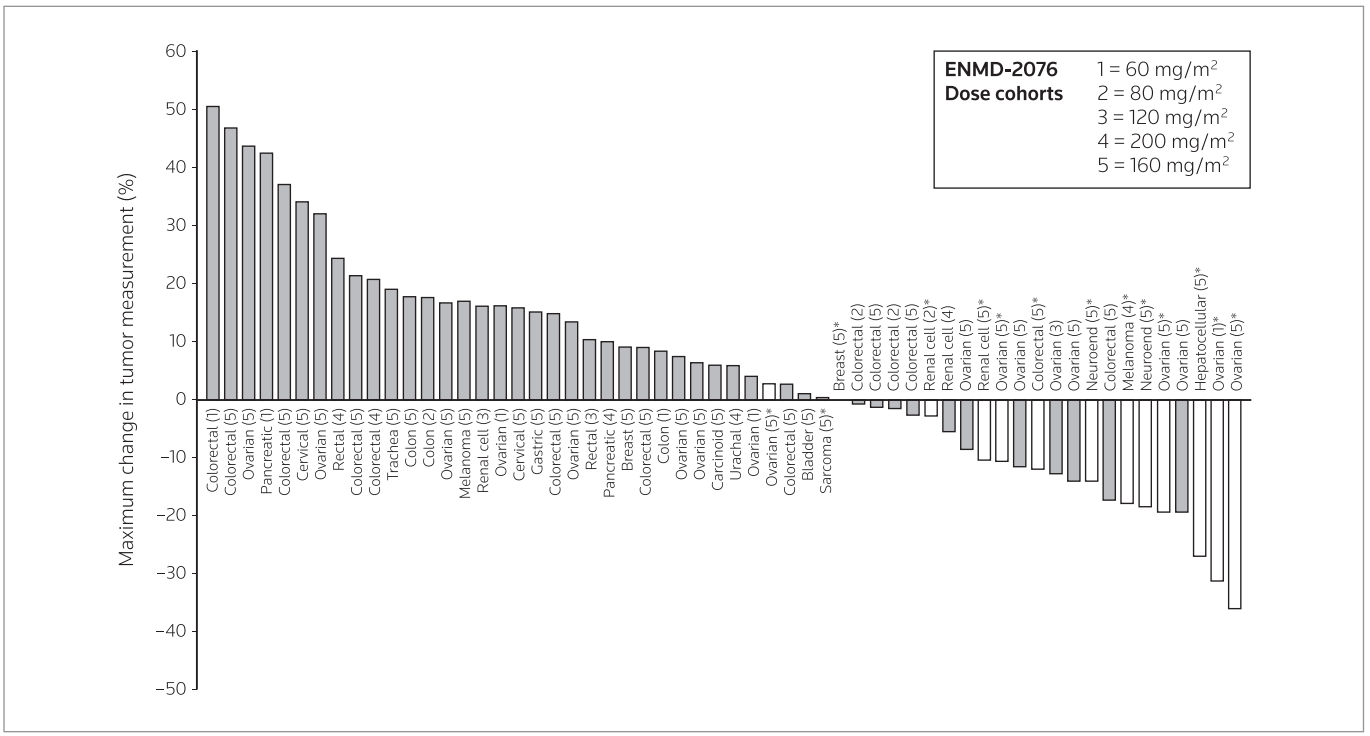


Figure 2. Clinical responses in solid tumor patients treated in a phase I trial of ENMD-2076. Waterfall plot shows the change in the sum of the longest diameters of target lesions observed at the time of best response. Dose cohorts are shown in parentheses. White bars and/or *s represent patients who remained on ENMD-2076 for at least 6 months. Adapted and reprinted from the American Association for Cancer Research: J.R. Diamond, B.R. Bastos, R.J. Hansen et al. *Phase I safety, pharmacokinetic and pharmacodynamic study of ENMD-2076, a novel angiogenic and aurora kinase inhibitor, in patients with advanced solid tumors*, Clin Cancer Res, 2011, 17(4): 849-60.

The preliminary results of a phase I study of ENMD-2076 in patients with relapsed or refractory hematologic malignancies were recently presented (66). Fifteen patients with AML were treated at dose levels of 225, 325 and 375 mg/day of ENMD-2076 administered orally. ENMD-2076-related side effects included grade 1-2 dizziness, petechiae, hypertension, nausea, fatigue, diarrhea and esophageal reflux. At the highest dose level of 375 mg/day, dose-limiting toxicity (DLT) consisting of grade 3 fatigue was observed in 2 patients. No grade 4 toxicities or treatment-related deaths occurred. Of 13 patients evaluable for response, 1 patient achieved a morphologic leukemia-free state with platelet transfusion independence and 2 other patients had a 12% and 14% reduction, respectively, in bone marrow blast counts, indicating that ENMD-2076 has clinical activity in this heavily pretreated group of AML patients. Given the inhibitory activity of ENMD-2076 against FLT3 kinase, it will be important to establish whether responses will correlate with FLT3-positive AML.

In an ongoing phase I trial in patients with relapsed and refractory MM at our institution, 9 patients have thus far received ENMD-2076 at dose levels of 150, 225 and 325 mg/day on 28-day cycles (67). The most commonly observed toxicities included grade 1-2 anorexia, nausea, diarrhea, fatigue, asymptomatic elevation of amylase and/or lipase, leukopenia and proteinuria. Grade 3 toxicities included hypertension (n = 1), asymptomatic elevation of lipase (n = 2) and thrombocytopenia (n = 1). No DLT was observed, with all toxicities resolving promptly upon interruption or discontinuation of dosing. Two patients treated with 325 mg/day had a 21% and 19% reduction, respectively, in serum M-protein after the first cycle. The results so far indicate that ENMD-2076 has acceptable toxicity with clinical anti-myeloma activity demonstrable at higher doses.

Although clinical trials with ENMD-2076 have so far targeted patients with MM, ovarian cancer and AML, it is likely that ENMD-2076 will have clinical activity in other cancers based on its profile of kinase activity and the preclinical data reviewed above, including colon, urothelial and cervical cancer. Furthermore, while signals of clinical activity have been demonstrated in ovarian and colon cancer, MM and AML, further investigation in phase II trials is required to better assess the single-agent efficacy of ENMD-2076 in these diseases. Emerging preclinical data, however, suggest that the optimum use of this promising drug will be in combination with other anticancer drugs. For example, ENMD-2076 may exhibit synergy with cisplatin in triple-negative breast cancer cells and with lenalidomide against MM (68), indicating that such a line of investigation is likely to be fruitful.

PERSPECTIVES

In summary, ENMD-2076 is a novel anticancer drug that targets multiple kinases and shows promising activity in early phase I trials in ovarian and colon cancer, as well as hematologic malignancies,

including MM and AML. Overall, however, preclinical studies indicate that it is likely to be active in other cancers where it has not yet been tested. Important to the further rational development of ENMD-2076 will be the determination of the primary cellular mechanisms responsible for its antitumor activity. ENMD-2076 appears to exert multiple and complex effects on tumor cells, inducing early caspase-dependent apoptosis, inhibiting Aurora kinases and cell cycle progression, inhibiting signaling via the PI3K/Akt pathway, FGFR-3 and VEGF receptors. The relative contribution of these mechanisms is currently unknown and may vary according to the tumor target. Data from preclinical studies provide for the development of biomarkers assessing each of these antitumor effects in clinical trials. Correlation of clinical responses with changes in biomarkers may shed light on the relative importance of these mechanisms for antitumor activity in different cancers, and is likely to provide a better understanding of the drug's action for the rational design of combination therapy, where optimal use of ENMD-2076 is likely to be found.

SOURCE

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