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Short communication

Title

Synthesis and evaluation of thieno[3,2-*d*]pyrimidine derivatives as novel FMS inhibitors

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Abstract

Colony stimulating factor-1 receptor (CSF-1R or FMS) and it ligand, CSF-1, signaling regulates the differentiation and function of tumor-associated macrophages (TAMs) that play an important role in tumor progression. Derivatives of thieno[3,2-*d*]pyrimidine were synthesized and evaluated as kinase inhibitors of FMS. The most representative compound **21** showed strong activity (IC_{50} = 2 nM) against FMS kinase and served as candidate for proof of concept. Anti-tumor activity alone and/or in combination with paclitaxel was examined via a tumor cell growth inhibition assay and via an *in vitro* tumor invasion assay using human breast adenocarcinoma cells.

Keywords

FMS; CSF-1R; Colony stimulating factor-1; Tumor associated macrophages (TAMs); Anti-tumor activity; Human breast adenocarcinoma cells

Macrophages are characterized as classical M1- and alternative M2-subtypes depending on how they influence the immune response.[1] M1 macrophages influence the inflammatory response and anti-tumor immunity, whereas M2 macrophages are involved in immunosuppressive and tumor promoting activities. Tumor-associated macrophages (TAMs), which are classified as M2-polarized macrophages, are recruited by tumor cells and infiltrate into the stromal environment of malignant tumors. TAMs in the tumor microenvironment are known to play a crucial role in tumor progression, including tumor initiation and development, matrix remodeling and metastasis, and immune suppression.[2-3]

Colony stimulating factor-1 (known as CSF-1 or macrophage-colony stimulating factor, M-CSF) is a growth factor for monocyte and macrophage-derived cells. CSF-1 and its receptor, colony stimulating factor-1 receptor (CSF-1R or M-CSFR, also known as FMS) have been reported as having important roles in regulating the production, migration, differentiation, survival, and function of macrophages and their precursors.[4] Therefore, over-activation of CSF‐1/CSF-1R signaling in macrophages has been implicated in many disease states including osteoclast proliferation in bone osteolysis, and a number of inflammatory disorders as well as the growth/metastasis of cancer.[5-14] Especially, a number of CSF1/CSF1R-targeting agents such as Pexidartinib and ARRY-382 are undergoing clinical trials in several cancer including breast cancer, prostate cancer, and melanoma.[15]

A previous report showed that the transfer of the CSF1 gene into tumor cells induced TAM infiltration into syngeneic BALB/c mice.[16] High expressions of CSF-1 and CSF-1R in tumor and stromal cells and a high TAM density are known to be correlated with poor prognoses in many cancer types.[17] CSF-1 promotes metastates via the regulation of TAMs in the tumor microenvironment.[18] In human metastatic breast cancer, elevated CSF-1 levels were correlated with marked CSF-1R-positive macrophage infiltration into tumors and were associated with poor outcomes.[19] Another study also showed that macrophage-deficient/CSF-1-deficient mice rarely developed pulmonary metastases, despite the rapid growth of primary mammary tumors.[20]

Furthermore, some chemotherapies stimulate tumor cells to release CSF-1, which then recruits TAMs expressing CSF-1R. These TAMs in the microenvironment stimulate tumor progression by enhancing tumor resistance to chemotherapy. In combination therapy with paclitaxel and CSF-1R antagonists on primary and metastatic tumors of mammary tumor-bearing mice, inhibition of TAM infiltration via CSF-1R antagonists increased the anti-tumor efficacy of cytotoxic agents.[21] For these reasons, modulation of the CSF-1 signaling pathway in the microenvironment has been of great interest in chemotherapy research.

In this paper, we describe the identification of the potent FMS inhibitor compound **21** and evaluate its anti-tumor activities against breast cancer. By computational screening compounds from the internal small molecule compound bank of Hanmi Pharmaceutical, we identified 1*H*-indazol-4-yl)thieno[3,2-*d*]pyrimidine derivative **7** (Fig. 1) as a highly potent (IC_{50} = 13 nM) FMS inhibitor.

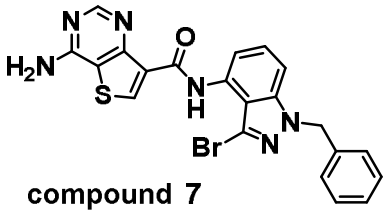
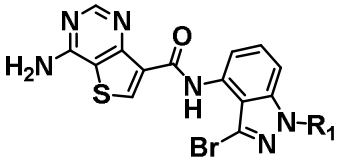
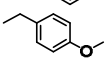
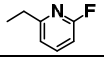


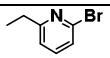
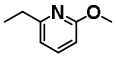
Figure 1. Hit compound **7**

To improve its enzyme activity and cellular activities on NFS-60 cells that express endogenous FMS,[22-23] structure-activity relationship (SAR) studies probed substituent effects at the N-1 position (R^1) and C-3 position of the indazole moiety (R^2). Initially, analogues were synthesized with substitutions at R_1 (Table 1). The replacement of the phenyl moiety with methoxy phenyl moiety (**8**) led to an almost two-fold loss of enzyme and cellular activity. In contrast, the replacement of the phenyl moiety with a pyridine moiety (**9-11**) showed good enzyme activity (IC_{50} < 10 nM). To further improve enzyme and cellular activity, we added an adopted substituent to the pyridine moiety. Halogen substituents (**12-13**) in the pyridine moiety had similar enzyme activities compared to regular pyridine moieties (**9-11**), and cellular activity did not dramatically improve. In contrast, alkyl substituents (**14-15**) on the pyridine moiety also showed improved cellular activity. In particular, a methyl substituent (**14**) on the pyridine moiety showed good cellular activity (GI_{50} = 27.5 nM) in an NFS-60 cell line whose growth depends on FMS.[22-23] Next, we tried to attach an alicyclic, heterocyclic, or alkynyl substituent (**16-18**) to the indazole moiety. Although all the resultant compounds showed good enzyme activities, they did not show sufficiently strong cellular activity.

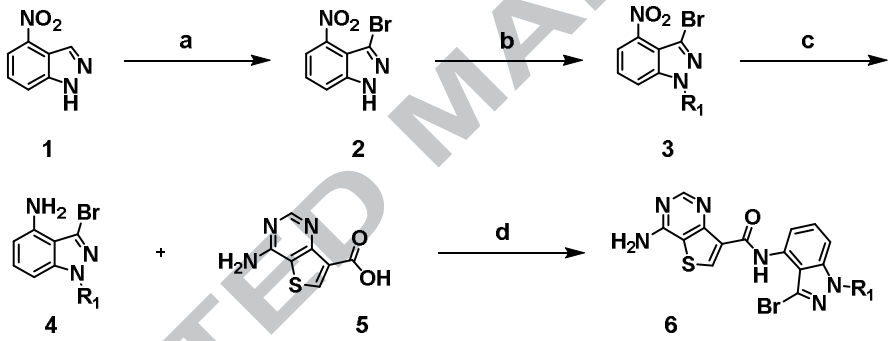
Table 1. FMS enzyme and cellular activity of compounds **7-18**



Compound	R^1	FMS IC_{50} (nM)	NFS-60 GI_{50} (nM)
GW-2580	-	3	94
7		13	1005
8		37	2390
9		9	2502
10		6	77
11		7	135
12		13	233

13		12	243
14		23	28
15		10	34
16		30	254
17		19	62
18		26	>10000

The synthetic route of R^1-changed derivatives is shown in **Scheme 1**. Compounds **7-18** were prepared from commercially available 4-nitro-1*H*-indazole (**1**). Bromination of **1** was performed using bromine gas, and gave an excellent yield (94%). The 1-substituted indazole of **2** was easily derived from an SN2 reaction using an alkyl-halide. Reduction of **3** was performed using Pd/C, and the amide coupling of **4** and **5** using commercially available HATU. Each step was isolated via column chromatography.

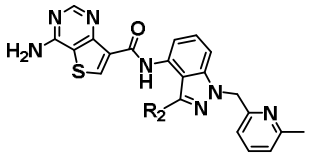


Scheme 1. Preparation of compounds **7-18**. Reagents and conditions: (a) Br_2, NaOAc, AcOH/$CHCl_3$(1:1), 20°C; (b) R^1CH_2Cl, K_2CO_3, DMF, rt; (c) H_2, Pd/C, THF/MeOH(1:1), rt; (d) HATU, DIPEA, DMF, rt.

Compound **14** was the most potent compound of the R^1 changed-derivatives, based on cellular activity. This compound also showed good oral bioavailability (37%) (Supplementary data).

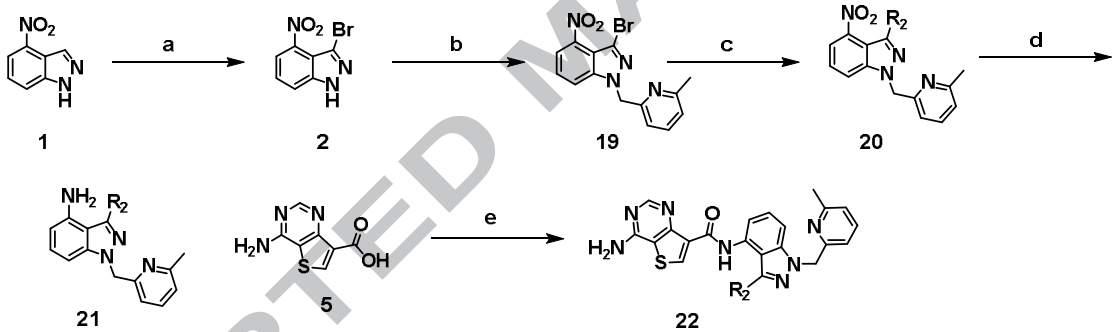
Next, we fixed R^1 as 2-methylpyridine and replaced R^2 with the C-3 position of an indazole moiety (compound **14:** Br). We then tried halogen (**23-24**) and alkyl (**25-27**) substituents at R^2 (Table 2). Compound **23** with a halogen moiety showed more potent cellular activity (3.4 fold) than compound **14**, and compound **25** with an alkyl moiety had the most potent enzyme activity (IC_{50} = 2 nM). Compound **25** showed more stable liver metabolism than compound **23** in microsomal stability test using human hepatocytes. Remaining value of parent molecule at 60 minutes of compound **25** and **23** were 87% and 66%, respectively.

Table 2. FMS enzyme and cellular activity of compounds **23-27**



Compound	R^2	FMS IC_{50} (nM)	NFS-60 GI_{50} (nM)
23	Cl	20	8
24	I	9	30
25	Me	2	80
26	Et	9	>10000
27	Vinyl	12	4458

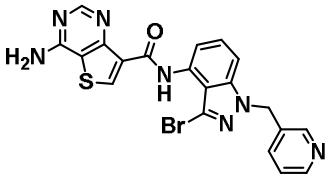
The synthetic route of R^2-changed derivatives is shown in **Scheme 2**. Compounds **25-27** were prepared from commercially available 4-nitro-1*H*-indazole (**1**). After same two steps with scheme 1, the N-substituted indazole of **19** was produced using the Suzuki reaction in a good yield (85%). Reduction of **20** was performed using Pd/C, and the amide coupling of **21** and **5** using commercially available HATU. Each step was isolated via column chromatography.



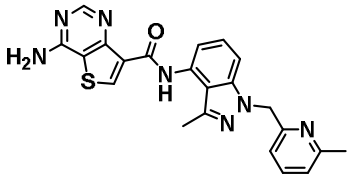
Scheme 2. Preparation of compounds **25-27**. Reagents and conditions: (a) $R_2B(OH)_2$, K_3PO_4, $Pd(OAc)_2$, PhMe/H_2O, 100°C; (b) H_2, Pd/C, THF/MeOH(1:1), rt; (c) HATU, DIPEA, DMF, rt.

Having successfully achieved good enzyme and/or cellular activities, the drug metabolism and pharmacokinetic profiles for the more potent compounds (**10** and **25**) were evaluated. To understand the potential drug-drug interaction liabilities of these compounds, a CYP (Cytochromes P450) reversible inhibition assay using human liver microsomes was conducted. In this test, compound **25** showed weak inhibition of five major CYP enzymes, whereas compound **10** inhibited four CYP enzymes either strongly or moderately (IC_{50} range from < 0.25 to 2.02 μM) except CYP2D6 (Table 3). These results indicate that compound **25** has a lower risk of drug-drug interactions than compound **10**. Moreover, compound **25** showed both good oral bioavailability (F = 36%) in mice (Table 4) and also an IC_{50} value > 50 μM for the binding capacity to the hERG (Human ether-a-go-go-related gene) membrane. Based on these results, compound **25** was chosen for further study.

Table 3. CYP enzyme inhibition assay for compounds **10** and **25**



compound 10



compound 25

	IC_{50} (μM)				
Compound	**CYP1A2**	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4**
10	< 0.25	1.38	2.02	> 20.00	0.31
25	> 20.00	> 20.00	> 20.00	> 20.00	> 20.00

CYP enzyme inhibition assay used the HLM with LC/MS/MS analysis method.

Table 4. Pharmacokinetic parameters for compound **25**

Compound	**Route**	**$t_{1/2}$ (hr)**	**C_{max} (ng/ml)**	**T_{max} (h)**	**$AUC_{0\text{-}7h}$ (ng·hr/ml)**	**V_d (L/kg)**	**Cl (L/hr/kg)**	**BA (%)**
25[a]	IV	1.6	2546.5	NA	3367.2	2.0	0.9	NA
	PO	2.6	900	2.0	4037.8	7.5	2.0	36

Male CD-1 mice treated with 3 mg/kg IV and 10 mg/kg PO.

[a] 5 % DMSO in 30 % PEG400 solution for IV and 0.5 % HPMC in 0.05 % Tween80 solution for PO

To determine the biochemical selectivity for compound **25**, twenty eight kinases were tested.[24] As summarized in Table 5, compound **25** was highly selective against FMS and inhibited FMS with an IC_{50} of 2 nM. It also inhibited the receptor tyrosine kinases c-RAF and DDR2 (IC_{50} < 100 nM for both) and FGFR1, KDR, MEKL, and Tie2 (IC_{50} 100 ~ 600 nM). However, the other twenty one kinases were inhibited only at much higher concentrations of **25**.

Table 5. Kinase selectivity of compound **25**

Kinase	**IC_{50} (nM)**
FMS	2
DDR2	64
c-RAF	80
FGFR1	145

KDR	527
MELK	258
Tie2	208

IC_{50} values were measured at the respective ATP Km for each kinase.

The inhibition potential of compound **25** on the migration of tumor cells by TAMs was evaluated via an invasion assay using MDA-MB-231 cells (human breast adenocarcinoma cells) and RAW264.7 cells (mouse macrophage cells). Compound **25** inhibited the migration of MDA-MB-231 cells in a dose-dependent manner; the inhibitory effect after treatment with 1 μM was 67%. (Fig. 2)

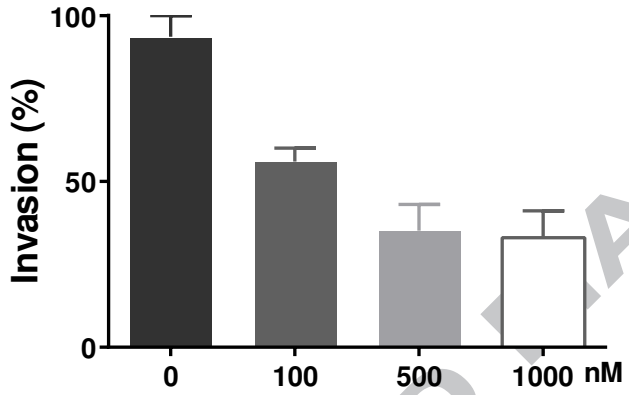
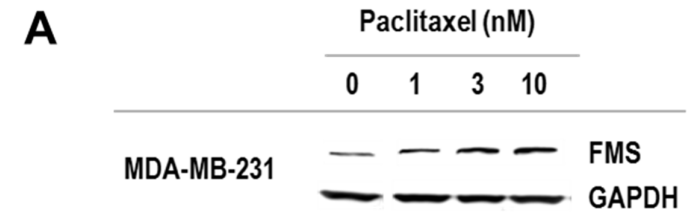


Figure 2. Tumor invasion assay in MDA-MB-231/Luc cells

The anti-tumor effects of compound **25** against breast cancer in combination with cytotoxic agents was evaluated using an *in vitro* tumor cell growth inhibition assay. As shown in Figure 3, paclitaxel dose- dependently induced FMS expression in MDA-MB-231 cells, and compound **25** effectively reduced paclitaxel-induced FMS expression.



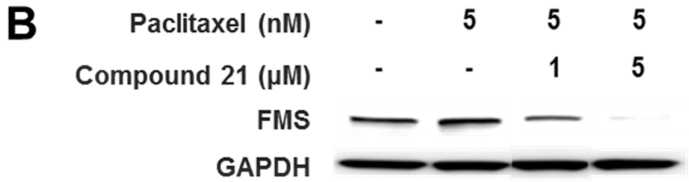


Figure 3. (A) FMS expression levels after treatment with paclitaxel in MDA-MB-231 cells was increased. (B) Compound **25** dose-dependently inhibited overexpressed FMS by paclitaxel treatment in MDA-MB-231 cells. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

In a combination study with paclitaxel and compound **25** in MDA-MB-231 cells, treatment with compound **25** (5 μM) alone showed a reduction in cell growth by 20%, whereas treatment with paclitaxel in combination with compound **25** showed a synergistic growth inhibition effect (CI < 0.1) compared with treatment with paclitaxel alone. (Fig. 4)

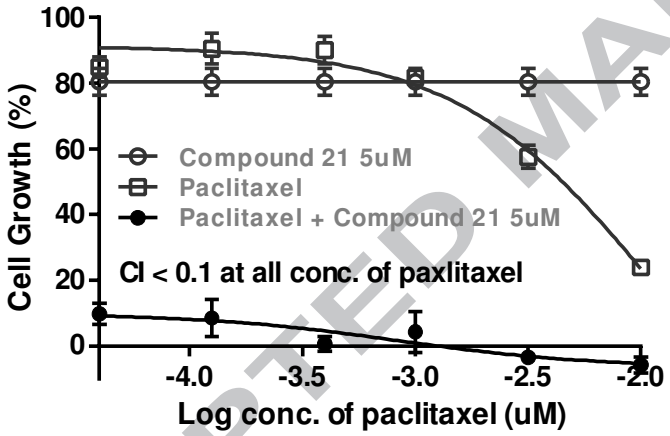


Figure 4. *In vitro* combination study of compound **25** and/or paclitaxel on cell growth inhibition in MDA-MB-231 cells. The synergistic effect was determined by calculating the combination index (CI) using the Calcusyn program (Cambridge.UK). The CI value was considered statistically significant when it was less than 1.0.

In summary, we identified and optimized a novel and potent class of FMS kinase inhibitors based on a thieno[3,2-*d*]pyrimidine core. One of these, compound **25**, was employed in an *in vitro* efficacy study to demonstrate its potential benefit in the treatment of cancer. The inhibition of FMS signaling by compound **25** was efficacious in reducing the migration of cancer cells and showed a synergistic anti-tumor activity against breast cancer in combination with a cytotoxic agent.

Declarations of interest: none.

Funding: not applicable

A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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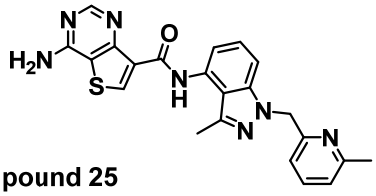
Graphic Abstract



compound 25
FMS (IC_{50}): 2nM
NFS-60 (GI_{50}): 79.7nM
Anti-tumor activity in tumor cell inhibition assay
and in vitro tumor invasion assay