Synthesis, physicochemical characterization and pharmacological investigation of indolacin-5-fluorouracil-1-ylmethyl ester

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Abstract: The objective of this work is to synthesize indolacin-5-fluorouracil-1-ylmethyl ester and the structure was confirmed by means of UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. The physicochemical parameters of melting point, solubility, apparent partition coefficient were investigated. S180 sarcoma, H22 hapatitic cancer and Lewis-transplanted mice were used to evaluate the anti-tumor activity of indolacini-5-fluorouracil-1-ylmethyl ester compared with 5-fluorouracil *in vivo*. Anti-inflammatory and analgesic activities were evaluated in mice. The inhibitory ratio of indolacini- 5-fluorouracil-1-ylmethyl ester is comparative to that of 5-fluorouracil. This study indicates that 5-fluorouracil-1-ylmethyl ester may represent a new anticancer predrug of 5-fluorouracil to produce a combined effect of indolacin and 5-fluorouracil for cancer therapy.

Keywords: 5-Fluorouracil, Indolacini-5-fluorouracil-1-ylmethyl ester, Anti-tumor; Anti-inflammatory; Analgesic activity; LD_{50} values

INTRODUCTION

The anticancer agent 5-fluorouracil (5-FU) is used to treat a wide range of solid tumors, particularly gastric, colorectal, and head cancers. Until now, 5-FU has been the only chemotherapeutic agent with significant activity in advanced colorectal cancers (Tebbutt *et al*., 2002). However, 5-FU is often administrated as continuous infusion or intravenous bolus, which resulted in gastrointestinal (GI) side effects. Oral administration of 5-FU alone demonstrates erratic and decreased absorption and nonlinear pharmacokinetics, because of the high activity of dihydropyrimidine dehydrogenase, a catabolizing enzyme of 5-FU, which causes rapid metabolism of 5-FU. This may be resolved by the co administration of other drugs orally that inhibit 5-FU degradation in the GI tract. Reports on the design of 5-FU prodrugs (i.e., tegafur, doxifluridine, or capecitabine) (Meropol, 1998; Sun *et al*., 2008; Saif *et al*., 2009; Lucietti *et al*., 1980) aimed at enhancing its oral absorption and reducing its first-pass metabolism. Indolacin (ILN), a nonsteroidal anti-inflammatory drug (NSAID) has been widely used to reduce inflammation and pain in patients (Lundholm *et al*., 1994).

In order to produce a combined effect of ILN and 5-FU for cancer therapy, a new prodrug of indolacini-5-fluorouracil-methyl ester (ILFM) was synthesized and characterized by determining its melting point, solubility, partition coefficients, ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectrometric (MS) spectra. S_{180} sarcoma, H_{22} Hapatitic cancer and Lewis-transplanted mice were used to estimate the antitumor activity of ILFM compared to 5-FU *in vivo*. Anti-inflammatory, analgesic activities and acute toxicity were evaluated in mice. A was also examined in mice.

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MATERIALS AND METHODS

5-FU (99.9%) was kindly supplied by Nantong Haiers Pharmaceutical Co., Ltd. (Jiangsu Province, China). ILN (99.5%) was purchased from Shenyang No.5 Pharaceutical Factory (Liaoning Province, China). *N*,*N*-Dicyclohexylcarbodiimide (DCC) was obtained from Shanghai Tianlian Fine Chemical Co., Ltd. (Shanghai, China). 4-Dimethylaminopyridine (DMAP) was obtained from Jiangsu Wuxi Bisheng Chemical Co., Ltd. (Jiangsu Province, China). Mice sarcoma S_{180} cell line, H22 Hepatocarcinoma cell line and Lewis lung carcinoma solid tumors were kindly supplied by the lab of Prof. Yingliang Wu (ShenYang Pharmaceutical University). Other reagents and solvents were of analytical/spectroscopic high-performance liquid chromatography (HPLC) grade as required.

Synthesis of 1, 3-dimethylol-5-fluorouracil

5-FU (1.30g, 10mmol) and formaldehyde (1.78g, 22m mol) solutions (37%) were added to a round bottom flask and heated on a water bath at 60-65°C until dissolved. The reaction solution was stirred for 50 minutes and concentrated under reduced pressure to remove excess water and formaldehyde. A white oily crude product was obtained as 1,3-dimethylol-5-fluorouracil.

Synthesis of indolacin-5-fluorouracil-methyl ester

The above intermediate product was transferred to a round bottom flask, to which 150mL anhydrous acetonitrile, indolacin0 (4.2g, 12mmol), DDC (2.47g, 14

mmol) and DMAP (0.08g) were then added. The mixture was stirred for 72 hours at room temperature and the progress of the reaction was monitored by thin-layer chromatography. The mixture was then filtered and evaporated under vacuum. The obtained residue was dissolved in ethyl acetate, washed with dilute hydrochloric acid, saturated sodium bicarbonate solution, and distilled water. The organic phase was dried over anhydrous sodium sulfate, filtered, and purified by column chromatography using acetone/dichloromethane (1:9, v/v) as the eluting system to obtain the purified pale yellow crystals. The overall actual yield was 73%.

The melting point was determined in an open capillary tube on a Sonar melting point apparatus. The structures of the synthesized compounds were confirmed by spectral (UV, IR, NMR, and MS) data analysis. A 4802H double beam spectrophotometer was used for UV absorption determination of the compounds. The UV spectra were recorded on UV-4802 double beam UV spectrophotometer (Shanghai Unique Company). The IR spectra were recorded using KBr disks on a Bruker Is-55 infrared spectrometer. The ^{1}H-NMR spectra were obtained on a Bruker-ARX 300 spectrometer operating at 300 MHz in dimethyl sulfoxide-d6 (DMSO-d_6). Chemical shifts were reported in parts per million downfield from the internal standard, tetramethylsilane. MS were recorded on a GCMS-QP5050A analyzer in positive mode.. The ^{13}C-NMR spectra were recorded on a Bruker BioSpin GmbH operating at 150 MHz in DMSO-d_6.

HPLC analysis

The free 5-FU and ILFM were assayed by reversed-phase HPLC. The chromatographic mobile phase consisted of methanol: water: 36% acetic acid (3:96.9:0.1) and methanol: 25mM ammonium acetate (70:30) for 5-FU and ILFM, respectively. The flow rates were 1.0mL/min at 260 nm. The operating temperature was ambient and the injected volume was 20 μL. The HPLC system consisted of a HITACHI L-7100 pump, a UV-Vis L-7420 detector, and a Diamonsil C_{18} (200 × 4.6mm, 5μm) column.

Solubility studies

The solubility of ILFM was determined at room temperature in a variety of solvents. An excess of ILFM was equilibrated with each solvent in a screw-top vial with frequent shaking (180rpm), vortexing and sonicating. The saturated solution was passed through a 0.25μm Millipore filter, and the filtrate was analyzed by HPLC after appropriate dilution with mobile phase.

Apparent partition coefficient studies

Classic shake-flask method was applied (Leo *et al*., 1971). A known concentration (C_0) of ILFM in 1-octanol saturated with buffer solution was vortexed for 2 hours with an equal volume of buffer solutions (pH 1.2 and 7.4, respectively) in a screw-top vial. The equilibrium concentrations of ILFM in 1-octanol (C) were determined by HPLC and each experiment was performed in triplicate. The apparent partition coefficients were calculated using the following equation.

$$P_{\text{app}} = \frac{C}{C_0 - C}$$

Where C_o and C represent the initial and equilibrium concentrations of ILFM in the octanol phase.

Antitumour activity of ILFM in vivo

S_{180} (Mice sarcoma cell line), H_{22} (Hepatocarcinoma cell line) and Lewis (lung carcinoma cell line) cells were resuscitated *in vitro* respectively. Those grow in exponential phase were collected, centrifuged (1000 r/min), then washed twice with PILFMS. Supernatant was transferred and diluted with sterile normal saline to 2×10^7cells/mL. Select healthy mice randomly; each was inoculated with 0.2mL of cells suspension (i.p.). About one week after inoculation, there was a significant increase of abdomen in inoculated mice. The milky ascites were drawn into sterile tubes, diluted once with sterile normal saline (the concentration of tumor cells was 2×10^7cells/mL (S_{180}, H_{22}) or 4×10^7cells/mL (Lewis). In the disinfected armpit, mice models were made by transplanting the diluted tumor cells subcutaneously (s.c.). Each tumor cell of animal experiment was repeated three times; calculate the tumor inhibition rate respectively.

Tumor-bearing mice were randomly divided into 7 groups with 12 mice in each group, including tumor-bearing group, low, medium and high dose of 5-Fu (10 , 20 , 40 mg·kg^{-1}) groups, and low, medium and high dose of ILFM (38.5 , 77 , 154mg·kg^{-1}) groups (equivalent to 5-FU equimolar dose). From the second day of modeling, mice were dosed with corresponding drugs for 7 d (i.p.), while control group were given the same amount of 1% CMC-Na per day. The weight of each mouse was recorded before dosing and after administration drugs. After 24 h of the last administration, cervical dislocation was used to kill the mice. Tumor was stripped and weighed. Calculate tumour inhibition rate using the following formula

$$\textit{Inhibition ratio}(\%) = \frac{C - T}{C} \times 100\%$$

Where C is the average weight of tumor in the control group and T is the average weight in experimental group.

Anti-inflammatory activity

Anti-inflammatory was evaluated by ear swollen in mice. Animals (n=70) were randomly divided into seven groups. Mice in control groups were given 0.5% CMC-saline by P.O.; Mice in ILN groups were given at dose of 5, 10, 20 mg/kg by P.O. once a day, for 3 days; Mice in IFM groups were given P.O. at dose of 7, 14, 28mg/kg once a day, for 3 days prior to testing. Paint xylene on both side of right ear of mouse (0.1 ml) 1 h after the last administration and sacrifice the mouse 1h later. Punched

of 10 mm (diameter) wafer with puncher at the same position of two ears and weighing on analytic balance. Swollen extent defined weight of right ear minus weight of left ear. Values are expressed as mean ± standard deviation ($x \pm SD$, $n = 10$).

Analgesic activity

The analgesic activity was valuated using acetic acid writhing text in mice compared to ILN**.** Groups and administration were the same as above. The mouse was given 0.6% acetic acid solution (10 ml/kg) by intra-peritoneal injection 1h after the last administration. The writhing number of each mouse was counted during a period of 5 to 15min after the acetic acid injection. The inhibitory rate was calculated.

Acute toxicity

The acute toxicity (LD_{50}) was determined by probit test using a death percent verse log versus of doses. For determination of LD_{50} value, various groups of mice weighting from 18-22 g were given single oral doses of various drugs and doses. These animals were under close observation for 14 days after dosing. The number of animals which died during this interval was expressed as a percentile.

STATISTICAL ANALYSIS

Statistical significance between groups was determined by one-way analysis of variance (ANOVA). Data analysis was performed using the SPSS 10.0 (SPSS Inc., Chicago, IL, USA) statistical package program. $P<0.05$ was accepted as the level of statistically significant.

RESULTS

Synthesis and characterization of indolacini -5-fluorouracil-methyl ester

We report herein the synthesis of indolacini -5-fluorouracil-methyl ester. fig. 1 represents the reaction between ILN and 5-FU. First, 1, 3-dimethylol-5-fluorouracil was synthesized from formaldehyde and 5-FU to form a hydroxyl-containing intermediate product. In the presence of *N*, *N*-Dicyclohexylcarbodiimide and DMAP, the carboxyl group of indolacin reacted with the hydroxyl group of the intermediate product to form a new ester.

The structure of the compound ILFM was confirmed by UV, IR, ^{1}H-NMR, ^{13} C-NMR and mass spectral data as described in experimental section.

Water solubility and apparent partition coefficient studies

The results of the solubility studies of ILFM are presented in table 1. The data show that ILFM is more soluble in organic solvents especially acetonitrile and tetra-hydrofuran, compared with water. ILFM was found it was not soluble in water and buffer solutions with the assay method that we used.

The apparent partition coefficient of ILFM in 1-octanol/phosphate-buffered saline (pH 1.2 and 7.4) was found to be 271.3 and 375.6 respectively. The partition coefficient study of ILFM showed that the major fraction of ILFM partitioned into the organic phase, which indicates an increased lipophilicity compared to that of parent compound 5-FU as reported previously (Buur *et al*., 1996). Furthermore, the different log P_{app} values demonstrated the pH dependence of ILFM.

Pharmacological activity

Table 2 and 4 showed the anti-tumor activity by the inhibition tumor growth effect of ILFM against Hepatocarcinoma22 (H_{22}), Sarcoma180 (S_{180}) and Lewis lung cancer of tumor-bearing mice compared to 5-FU. The result showed that the anti-tumor activity of ILFM was comparable to 5-FU. table 5 showed the anti-inflammatory activity. table 6 showed the analgesic activity of ILFM and ILN.

Acute toxicity

The acute toxicity results were shown in table 7. LD_{50} of ILFM was the biggest among 5-FU, ILN, ILFM and the mixture of ILN and 5-FU (1:1 mol/mol) and was 22 fold more than that of 5-FU. This shows that the acute toxicity of ILFM was the lowest among the four.

DISCUSSION

Although 5-FU is one of the most commonly drugs to treat many types of cancer, oral administration of 5-FU results in variable bioavailability. Many prodrugs of 5-FU have been synthesized to alter the physicochemical property and hence the absorption of 5-FU. ILN is a typical NSAID widely used for indications ranging from inflammation and pain to cardiovascular and genitourinary diseases. However, GI side effects often occur. Such compounds have long been known to adversely injure the mucosa of the GI tract. In this study, to take advantages of the synergic effect of two drugs for cancer therapy and minimize the GI tract disturbance induced by alone administration, we synthesized a novel combination of 5-FU and indolacin, which is an ester.

The melting point was 217~219°C. The purified ILFM had UV absorbance maxima at 211 (fig. 2) and 315 nm in acetonitrile. IR (KBr) cm^{-1}: 3437.5, 3199.7, 2830, 1732.3, 1238, 1709 (fig. 3). ^{1}H-NMR (DMSO-d_6): δ: 2.53 (s, 3H, $-CH_3$), 3.78 (s, 3H), 3.85 (s, 2H,), 5.62 (s, 2H), 6.86 (dd, 1H, J=8.920), 7.01 (d, 1H, J=2.0), 7.42 (d, 1H, J=15.8), 7.48 (m, 4H), 7.73 (d, 1H, J=8.9), 7.80 (d, 1H, J=15.8), 7.83 (m, 2H), 8.12 (d, 1H, J=6.5), 12.02 (s, 1H) (fig. 5). EI-MS (*m*/*z*): $[M]^+$ =491.5 (fig. 5). ^{13}C-NMR (150 MHz, DMSO-d_6): δ 170.34, 165.68, 157.44, 157.27, 155.60, 149.20, 144.81, 140.18, 138.65, 135.02, 134.30, 130.76, 130.43, 129.47, 129.15, 128.69, 121.75, 111.63, 111.60, 101.64, 70.82, 55.34, 29.11, 13.36 (fig. 6).

Table 1: The solubility of ILFM in different solvents

Solvent	Solubility (μg/mL)	Solvent	Solubility (μg/mL)
Ethanol	68.34	Chloroform	54.27
Isopropanol	28.47	THF	2412
Acetonitrile	3002	N-octyl alcohol	24.78
Ether	49.90	Benzene	58.12

Table 2: Inhibitory effects of ILFM on H22 hepatitic cancer mice (mean ± SD)

Group	Dose (mg/kg/d)	Number of animals		body /g		Tumor /g	*I*/ %
		0 d	8 d	0 d	8 d		
Control	0.2 mL/d	12	10	20.39 ± 1.54	24.70 ± 2.14	1.55 ± 0.52	—
5-FU	10	12	12	20.11 ± 1.78	23.62 ± 1.60	0.88 ± 0.26*	43.2
	20	12	11	20.03 ± 1.83	23.93 ± 2.72	0.83 ± 0.29*	46.4
	40	12	11	19.62 ± 1.47	21.98 ± 1.85	0.74 ±0.35**	52.2
ILFM	38.5	12	12	21.32 ± 1.43	24.11 ±1.92	0.86 ± 0.23*	44.5
	77	12	10	20.99 ± 1.52	23.77 ± 1.62	0.78 ± 0.20*	49.7
	154	12	11	21.22 ± 1.44	24.00 ± 1.97	0.70 ±0.33**	54.8

Table 3: Inhibitory effects of ILFM on S_{180} sarcoma-transplanted mice (mean ± SD)

Group	Dose (mg/kg/d)	Number of animals		Body/g		Tumor/g	*I* / %
		0 d	8 d	0 d	8 d		
Control	0.2 mL/d	12	8	22.12 ± 1.68	24.16 ± 1.97	1.32 ± 0.46	—
5-FU	10	12	11	20.70 ± 1.53	23.12 ± 2.12	0.77 ± 0.34*	41.7
	20	12	11	21.77 ± 1.87	23.78 ± 2.94	0.73 ± 0.39*	44.7
	40	12	10	21.89 ± 1.94	24.45 ± 1.79	0.64 ± 0.28**	51.5
ILFM	38.5	12	11	19.34 ± 1.83	21.83 ± 2.06	0.70 ± 0.22*	47.0
	77	12	12	21.23 ± 1.99	23.09 ± 1.56	0.67 ± 0.25*	49.2
	154	12	11	22.12 ± 1.67	24.88 ± 2.02	0.62 ± 0.18**	53.0

Table 4: Inhibitory effects of ILFM on Lewis-transplanted mice (mean ± SD)

Group	Dose (mg/kg/d)	Number of animals		Body /g		Tumor /g	*I* /%
		0 d	8 d	0 d	8 d		
Control	0.2 mL/d	12	11	22.12 ± 1.68	24.16 ± 1.97	1.63 ± 0.41	—
5-FU	10	12	11	20.70 ± 1.53	23.12 ± 2.12	0.90 ± 0.30*	44.8
	20	12	11	21.77 ± 1.87	23.78 ± 2.94	0.84 ± 0.27*	48.5
	40	12	10	21.89 ± 1.94	24.45 ± 1.79	0.75 ± 0.24**	54.0
ILFM	38.5	12	12	19.34 ± 1.83	21.83 ± 2.06	0.88 ± 0.20*	46.0
	77	12	11	21.23 ± 1.99	23.09 ± 1.56	0.81 ± 0.22*	50.3
	154	12	10	22.12 ± 1.67	24.88 ± 2.02	0.71 ± 0.26*	56.4

*$P < 0.05$, ** $P < 0.01$. Compared with control

Table 5: Effect of ILFM, ILN on xylene-induced ear swelling in mice (mean ± SD, n = 10)

Group	Dose (mg/kg)	Swelling extent (mg)	Inhibition ratio (%)
Control	–	0.018 ± 0.027	–
ILFM	28	0.015 ± 0.002*	11.8
ILFM	14	0.015 ± 0.003*	11.8
ILFM	7	0.017 ± 0.004	0
ILN	20	0.012 ± 0.002*	29.4
ILN	10	0.015 ± 0.001*	11.8
ILN	5	0.016 ± 0.002	5.9

*$P < 0.05$

Table 6: Effect of ILFM, ILN on acetic acid writhing in mice (mean ± SD, n =10)

Group	Dose (mg/kg)	Writhing times	Inhibition ratio (%)
Control	–	24.1 ± 16.7	–
ILFM	28	3.6 ± 4.1*	86.0
ILFM	14	5.4 ± 7.0*	76.1
ILFM	7	7.8 ± 12.7*	67.4
ILN	20	4.3 ± 8.7*	81.2
ILN	10	6.3 ± 7.9*	74.7
ILN	5	8.5 ± 9.7*	64.1

*P<0.05

Table 7: Results of LD_{50} and 95% confidence limits of different drugs

Drug	LD_{50} (mg/kg)	95% confidence limits (mg/kg)
5-FU	90.16	72.7 – 114.3
ILN	270.2	197.1 – 372.9
5-FU/ILN(1:1, mol/mol)	210.6	170.5 – 260.1
ILFM	1960.4	1475.3 – 2624.8

High lipophilicity is often required because it can affect the absorption, distribution, metabolism, and excretion properties of drugs in biological systems. To obtain a better understanding of the overall properties of the compound, its lipophilicity, expressed as the 1-octanol/water partition coefficient, was determined under two different pH conditions. The apparent partition coefficients represent the distribution of both ionized and nonionized drug molecules between the two phases. As is to be expected, the apparent partition coefficient varies with the pH of the aqueous solution. For ionizing compound, it is pH dependent.

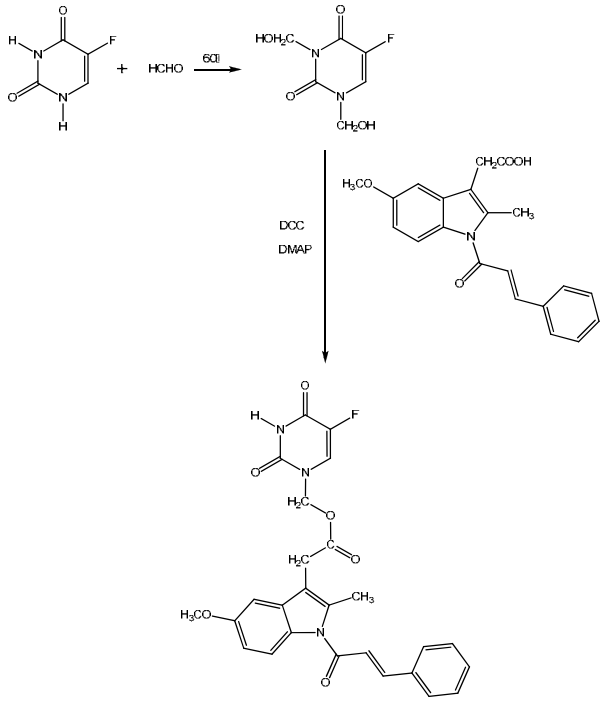

Fig. 1: Synthesis of indolacini-5-fluorouracil-1-ylmethyl ester

Table 5 showed that ILFM had not anti-inflammatory activity at low dose, but had anti-inflammatory activity at higher dose and comparable to ILN at middle dose. The anti-inflammatory activity of ILFM was not dependent on dose.

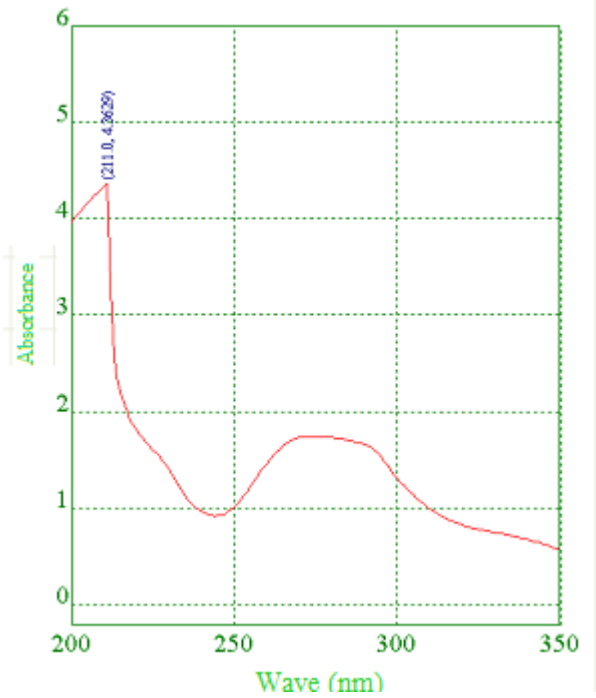

Fig. 2: UV spectrum of indolacini-5-fluorouracil-1-ylmethyl ester.

The analgesic activity of ILFM was a little better than ILN and was dependent on dose. LD_{50} values of indolacini-5-fluorouracil-1-ylmethyl ester, 5-fluorouracil, indolacin, and the mixture of 5-fluorouracil: indolacin (1mol: 1mol) were 1960.43, 90.16, 270.19 and 210.66 mg/kg, respectively.

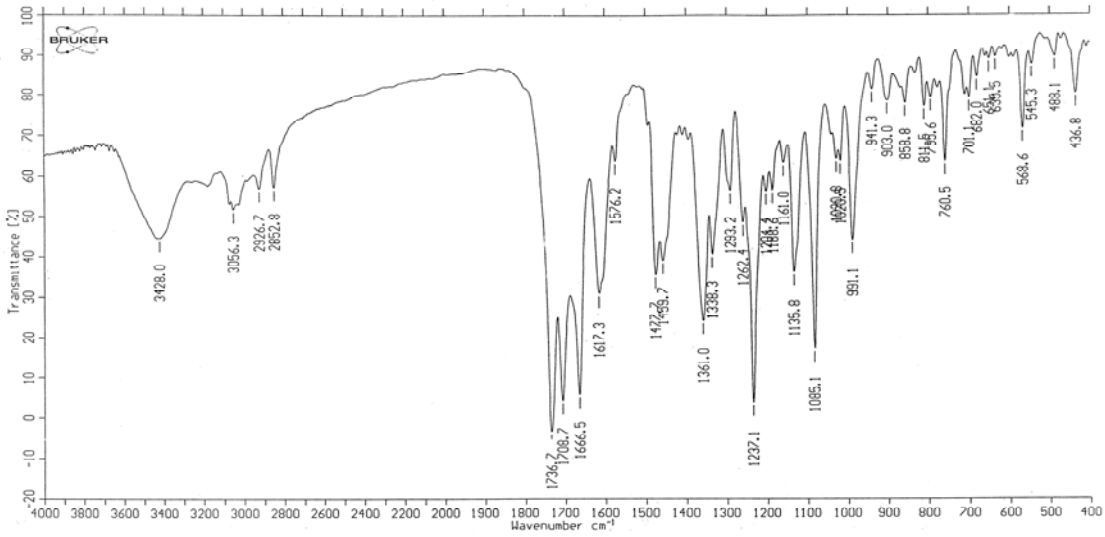

Fig. 3: IR spectrum of indolacini-5-fluorouracil-1-ylmethyl ester

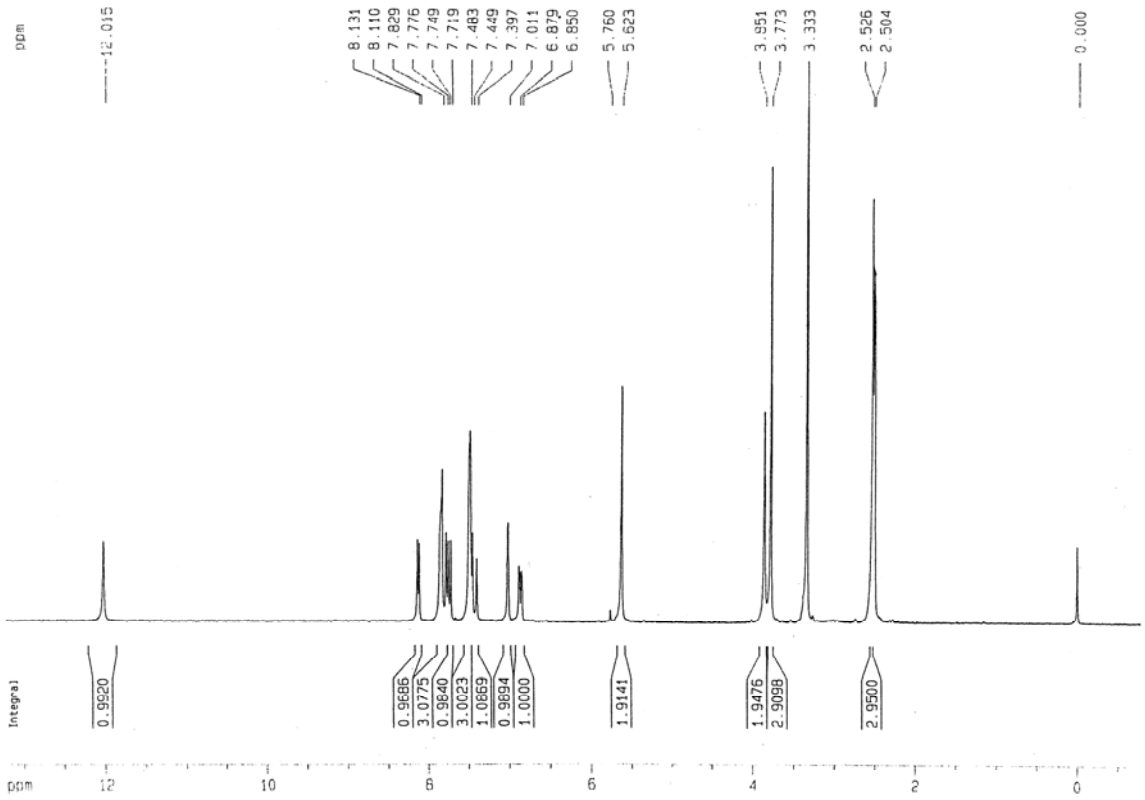

Fig. 4: ^{1}H-NMR spectrum (300 MHz, DMSO-d_6) of indolacini-5-fluorouracil-1-ylmethyl ester.

CONCLUSION

ILFM was synthesized and confirmed by means of UV, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectrometry for the first time. Physicochemical properties of the new compound demonstrated the formation of ILFM. Experiments showed that ILFM possessed analgesic and anti-inflammatory activity, as well as anti-tumor activity. Acute toxicity of ILFM was decreased compared to 5-FU, ILN, and the mixture of 5-FU and ILN (1mol/1mol). All about are very important for increasing life quality of cancer patients. ILFM would be a promising anti-cancer drug.

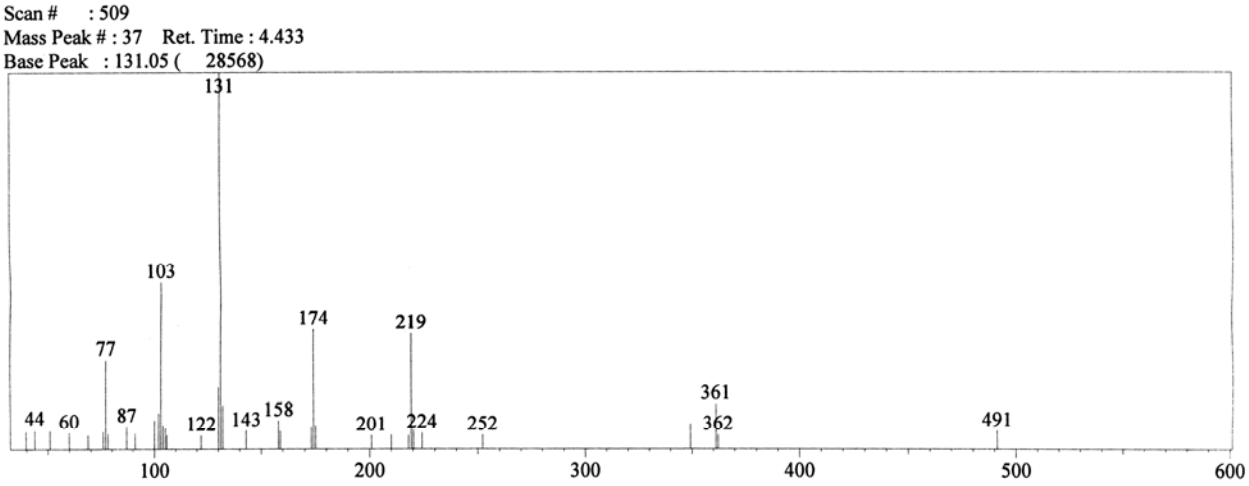

Fig. 5: Mass spectrum of indolacini-5-fluorouracil-1-ylmethyl ester (ILFM)

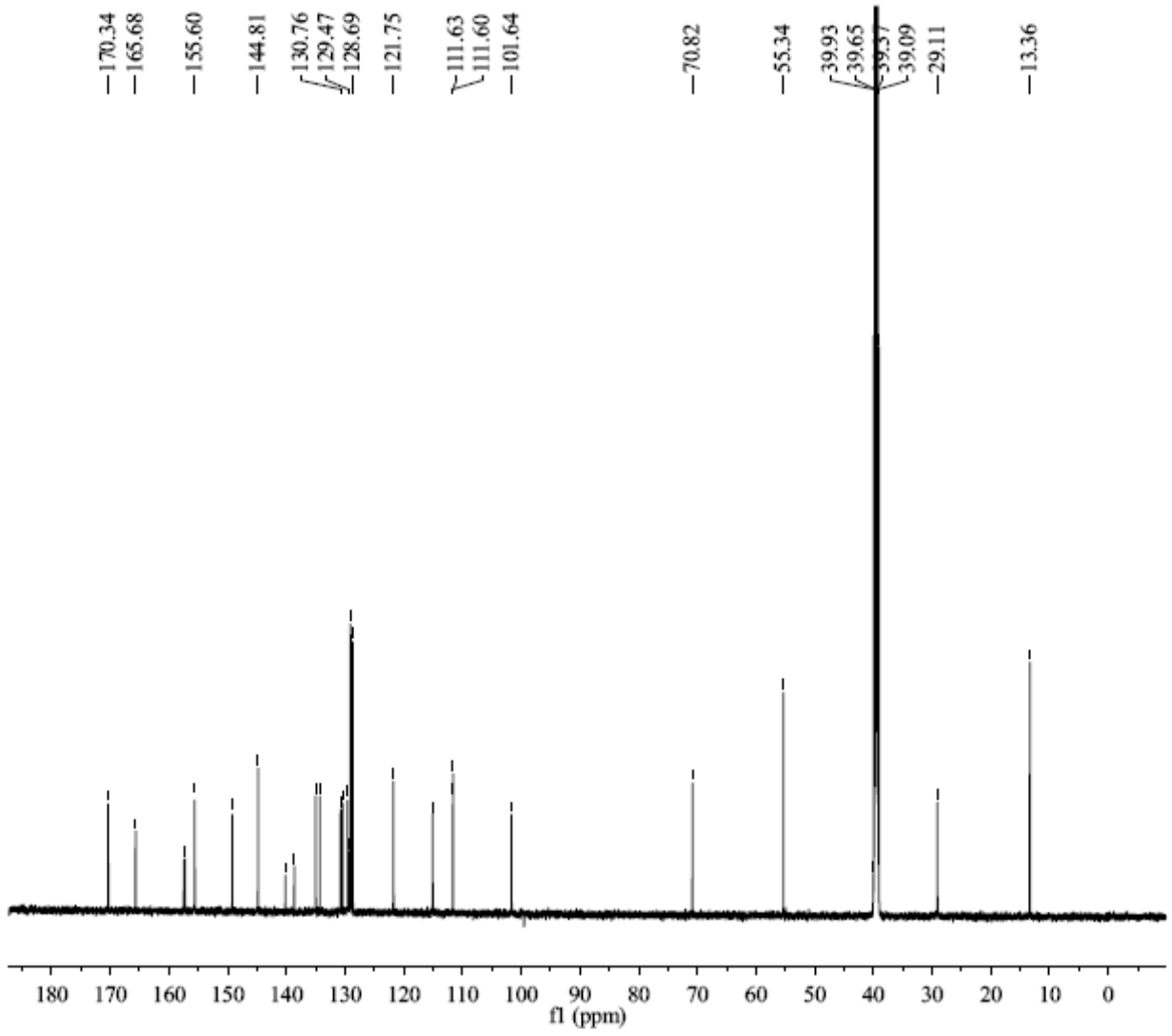

Fig. 6: ^{13}C-NMR spectrum (300 MHz, DMSO-d_6) of indolacini-5-fluorouracil-1-ylmethyl ester

ACKNOWLEDGEMENTS

This work was supported by grants from Liaoning province education administration (No.L2012364) and the young teacher career development support foundation of Shenyang Pharmaceutical University.

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