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Substitued (*E*)-β-(benzoyl)acrylic acids suppressed survival of neoplastic human HeLa cells

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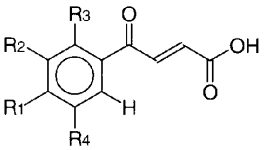
The bacteriostatic activity of some of alkyl substituted (*E*)-β-(benzoyl)acrylic acids was shown earlier. The aim of this study was to investigate the antiproliferative action of 19 alkyl-, or halogeno-, or methoxy-, or acetamido- substituted (*E*)-β-(benzoyl)acrylic acids, against human cervix carcinoma, HeLa, cells. Target HeLa cells were continuously treated with increasing concentrations of substituted (*E*)-β-(benzoyl)acrylic acids during two days. The MTT test was used for assessment of the antiproliferative action of this group of compounds. Treatment of HeLa cells with 4-methyl-, 4-fluoro-, 4-chloro-, 4-bromo- and 4-methoxy- derivatives of (*E*)-β-(benzoyl) acrylic acid leads to the expression of cytostatic activity against HeLa cells (IC50 were in the range from 31–40 μM). Their antiproliferative action was less than that of the basic compound (*E*)-β-(benzoyl)acrylic acid whose IC50 was 28.5 μM. The 3,4-dimethyl-, 2,4-dimethyl- and 2,5-dimethyl- derivatives as well as the 4-ethyl- and 3,4-dichloro- and 2,4-dichloro-derivatives, have stronger cytostatic activity than the correspoding monosubstituted and parent compound. Their IC50 were 18.5 μM; 17.5 μM; 17.0 μM; 17.5 μM; 22.0 μM and 18 μM, respectively. The 4-*iso*-propyl- and 4-*n*-butyl-derivatives exerted higher cytostatic activity than the compounds with a lower number of methylene $-CH_2-$ groups in the substitutent. Their IC50 were 14.5 μM and 6.5 μM respectively. The 2,5-di-*iso*-propyl- and 4-*tert*-butyl-derivatives expressed the most strong antiproliferative action against the investigated HeLa cells, IC50 being 4.5 μM and 5.5 μM, respectively. The investigated compounds affected the survival of HeLa cells, expressing a strong structure-activity relationship of the Hansch type.

Key words: benzoylacrylic acids, HeLa cells, cytotoxicity, QSAR.

In this study the cytostatic action of various (*E*)-β-(benzoyl)acrylic acids was investigated. The determination of the extent of the bacteriostatic activity of a similar group of compounds was performed earlier.[1] A marked increase in the activity of *para*-alkyl-substituted (benzoyl)acrylic acids against Gram-positive bacteria was found when the substituent group was changed from methyl to nonyl.

para-Methoxy- and ethoxy-derivatives had the same order of activity as the methyl- or ethyl-substituted compounds. *p*-Chloro- and *p*-bromobenzoyl acrylic acids were only moderately active. The found antibacterial activity of this group of compounds towards *Staphilococcus aureus*, *Escherichia coli* and *Kllebsiella* was ascribed to the presence of the highly conjugated benzoylacrylic system, which may react with biologically essential –SH groups. Bowden *et al.*[2] reported data on the bacteriostatic activity of a series of (*E*)-3-(aroyl)acrylic acids and their methyl esters. They found that the bacteriostatic activity measured as minimum inhibitory concentrations (C) are well correlated with a Hansch-type equation, where π is the partition substituent constant. Results reported by Bowden *et al.*[3] performed on a series of substituted (*E*)-3-(4-phenylbenzoyl)acrylic acids, indicated that nucleophilic addition at the ketovinyl double bond is not the mode of bacteriostatic action of this group of compounds. The receptor for bacteriostatic action appears to be related to the lipophilic region associated with the benzoylphenyl group.

TABLE I. Structures of substituted (*E*)-β-(aroyl)acrylic acids studied in this work



Compound	R_1	R_2	R_3	R_4
1	H	H	H	H
2	Me	Me	H	H
3	Me	H	Me	H
4	H	H	Me	Me
5	Me	H	H	H
6	Et	H	H	H
7	*iso*-Pr	H	H	H
8	H	H	*iso*-Pr	*iso*-Pr
9	*n*-Bu	H	H	H
10	*tert*-Bu	H	H	H
11	$-CH_2-CH_2-CH_2-CH_2-$		H	H
12	Cl	H	Me	H
13	Cl	H	H	H
14	Br	H	H	H
15	Cl	Cl	H	H
16	Cl	H	Cl	H
17	OMe	H	H	H
18	AcNH	H	H	H
19	F	H	H	H

On the other side, the cytostatic activity of a similar compound, the sodium salt of (*E*)-[β-bromo-β-(4-methoxy)benzoyl]acrylic acid, (*Z*)-Br,H(Cytembena) was extensively investigated using experimental models, as well as on patients with various malignancies.[4-7] The principal mode of antitumor action of this compound was ascribed to its inhibitory effect on tetrahydrofolate formylase. The compound without a halogen on the Cβ was found to be inactive in the inhibition of this enzyme.[6]

In this study, nineteen aroylacrylic acids were synthesized, and their cytostatic activity towards human cervix carcinoma, HeLa cells, was studied. The structures of the studied acids are given in Table I.

EXPERIMENTAL

Synthesis of aroylacrylic acids

(*E*)-β-(Aroyl)acrylic acids were prepared, according to the method of Papa *et al.*,[8] using modification of the Frieded-Crafts reaction, by adding an aromatic substrate to a solution of maleic anhydride and anhydrous aluminum trichloride (molar ratio 1:2) in 1,1,2,2-tetrachloroethane. Instead of tetrachloroethane, we used 1,2-dichloroethane and moderately higher yields were obtained.[9]

Typical experimental procedure

In a 100 ml two-necked flask, equipped with a magnetic stirrer, reflux condenser and dropping funnel, 6.125 g (62.5 mmol) of maleic anhydride was suspended in 25 ml of dry 1,2-dichloroethane. After 10 min, 15.5 g (125 mmmol) of powdered anhydrous aluminum trichloride was added and the reaction mixture was stirred for another 20 min, until a homogeneous yellow suspension was obtained. An aromatic substrate (62.5 mmol) was added at such a rate to keep the temperature below 50 °C and foaming under control. The reaction mixture was stirred for 9 h at 20 °C; 0.5 h at 60 °C; refluxed for a further 0.5 h and then poured into 200 g of ice/water mixture (1:1) containing 20 ml concentrated hydrochloric acid. The dichloroethane was removed by steam distillation. The crude acid was collected by filtration, dissolved at 20 °C in water containing sodium carbonate at pH 8.5–9.0 and the traces of aluminum hydroxide were filtered off. The mother liquor was acidified with hydrochloric acid to pH 1.0 and the pure acid was collected by filtration, washed with water and dried in open air. The yields, recrystallization solvents, and melting points are given in Table II.

Stock solutions of the examined compound were made in dimethylsufoxide, in the concentration range 42–62 μM and then various dilutions were prepared in nutrient medium, (RPMI 1640 medium supplemented with L-glutamine (3 mmol/L), streptomycin (100 μg/mL) and penicilin (100 IU/mL), 10% heat inactivated fetal bovine serum, FBS, and 25 mM Hepes, adjusted to pH 7.2 (with bicarbonate solution) to various final concentrations (between 1.3–80 or 142 μM). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MMT) was purchased from Sigma Chemicals (St. Luis, MO, U.S.A.). The MTT was dissolved, 5 mg/ml in phosphate buffer saline pH 7.2, and filtered through a Millipore filter, 0.22 μm, before use. RPMI 1640 cell culture medium and FBS were products of ICN Pharmaceuticals Co, USA.

Treatment of HeLa cells

HeLa cells were seeded into 96-well microtiter plates, 2000 cells per well. After 20 h, to one series of wells both with and without HeLa cells, five different concentrations, of the to be investigated substituted (*E*)-β-(benzoyl)acrylic acid derivative were applied to the wells to various final concentrations, except to the control wells, *i.e.,* wells with cells grown in a nutrient medium only. All concentraitons were set up in triplicate. Nutrient medium with the corresponding concentrations of the

investigated agent, but without cells, was used as blank, in triplicate too.

TABLE II. Crystallization solvent, melting points and yields of the investigated compounds (**1**–**19**)

Compound	Solvent	M.p. °C	Yield (%)
(**1**) H	C_6H_6	98–99	74
(**2**) 3,4-dimethyl	EtOH	123	75
(**3**) 2,4-dimethyl	EtOH:H_2O	113–114	70
(**4**) 2,5-dimethyl	EtOH:H_2O	89–90	95
(**5**) 4-methyl	C_6H_6	139–140	85
(**6**) 4-ethyl	C_6H_6	105–106	94
(**7**) 4-*iso*-propyl	C_6H_6	103–103.5	72
(**8**) 2,5-di-*iso*-propyl	cyclohexane:hexane	96–99	71
(**9**) 4-*n*-butyl	C_6H_6	90–91	72
(**10**) 4-*tert*-butyl	C_6H_6	125–127	75
(**11**) 4-tetralinoyl	EtOH	147–149	72
(**12**) 4-chloro-2-methyl	H_2O	107–110	89
(**13**) 4-chloro	H_2O	154–155	89
(**14**) 4-bromo	H_2O	159–160	82
(**15**) 3,4-dichloro	C_6H_6	143	66
(**16**) 2,4-dichloro	H_2O	190	22
(**17**) 4-methoxy	C_6H_6	138–139	72
(**18**) 4-acetamido	EtOH	240	35
(**19**) 4-fluoro	C_6H_6	142	91

Determination of cell survival

HeLa cell survival was determined by the MTT test, according to Mosmann[10] modified by Ohno and Abe,[11] 44 h after addition of the drug, as was described previously.[12] Briefly, 20 μL of MTT solution (5 mg/ml PBS) were added to each well. The samples were incubated for a further four hours at 37 °C in a 5% CO_2 humidified air atmosphere. Then, 100 μL of 10% SDS in 0.01 M HCl were added to the wells. The optical density (OD) at 570 nm was measured the next day. The percent change of the OD was used as a measure of cell survival (%). Hence, the OD of a sample with cells, grown in the presence of a certain concentration of the substituted (*E*)-β-(benzoyl)acrylic acid derivative, OD, was divided by the control optical density, ODc, (the OD of the cells grown only in nutrient medium) × 100. (It is obvious that the OD of the corresponding blank must always be substracted from the OD of the corresponding sample with target cells).

RESULTS

The concentrations of the examined agents that induced a 50% decrease in cell survival (IC50), determined under exactly the same experimental conditions, are given in Table III.

It can be seen that the 4-methyl-, 4-fluoro-, 4-chloro-, 4-bromo- and 4-methoxy-derivatives of (*E*)-β-(benzoyl)acrylic acid expressed similar cytostatic activity against HeLa cells (IC50 were 40; 35; 40; 31 and 36 μM, respectively). This

antiproliferative action was less than that of the basic compound, (*E*)-β-(benzoyl)acrylic acid, whose IC50 was 28.5 μM.

TABLE III. IC50 values of the studied compounds

Compound	IC50 [μM]	Compound	IC50 [μM]	Compound	IC50 [μM]	Compound	IC50 [μM]
1	28.5	**6**	17.5	**11**	8.5	**16**	18
2	18.5	**7**	14.5	**12**	31.5	**17**	36
3	17.5	**8**	4.5	**13**	40	**18**	>117
4	17	**9**	6.5	**14**	31	**19**	35
5	40	**10**	5.5	**15**	22		

3,4-Dimethyl-, 2,4-dimethyl- and 2,5-dimethyl-substituted (*E*)-β-(benzoyl) acrylic acid, as well as the 4-ethyl- then 3,4-dichloro- and 2,4-dichloro-derivatives have stronger cytostatic activity than the corresponding monosubstituted and parent compound: their IC50 were: 18.5; 17.5; 17.0; 17.5; 22.0 and 18 μM, respectively.

The 4-*iso*-propyl-, then 4-*n*-butyl-derivatives extered higher cytostatic activity than compounds with a lower number of methylene $-CH_2-$ groups in the substituent. Their IC50 were 14.5 and 6.5 μM, respectively.

2,5-Di-*iso*-propyl- and 4-*tert*-butyl- derivatives expressed the strongest antiproliferative action against the investigated HeLa cells their IC50 being 4.5 and 5.5 μM, respectively.

The IC50 values show a very good correlation with the lipophilicity of the

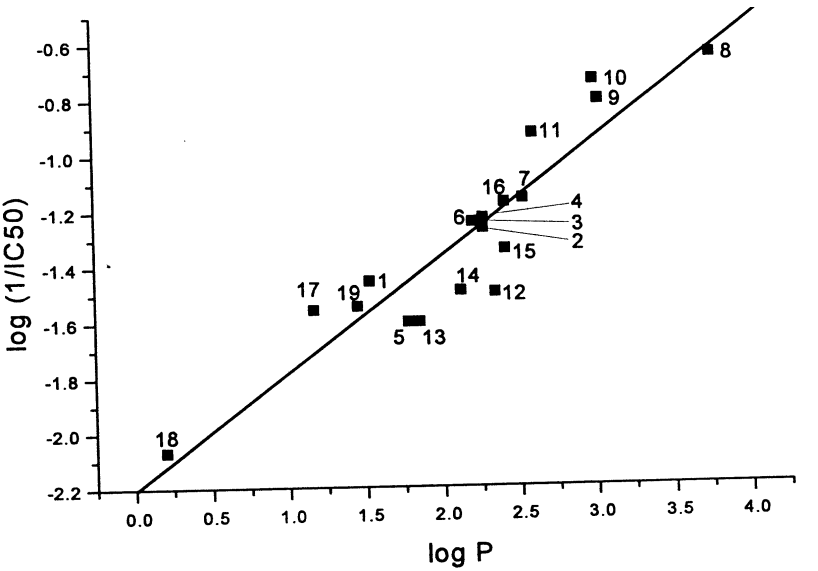


Fig. 1. Correlation between the lipophilicity constant (log *P*) and –log (IC50).

studied compounds.

The logarithm of the partition coefficient [*n*-octanol/water] log(*P*)=log(K_{ow}) was estimated by the Crippen fragmentation method.[13] For the unsubstituted compound **1**, the literature experimental value of log K_{ow} = 1.530 was used.[14] The plot of –log(IC50) *versus* log(*P*)is shown in Fig. 1.

The regression equation is:

$$\log(1/IC50) = 0.418(\pm 0.042)\times\log(P) - 2.202(\pm 0.096) \quad (1)$$

with a regression coefficient 0.924.

DISCUSSION

The results obtained in this work showed that (*E*)-β-(benzoyl)acrylic acids expressed cytostatic activity toward HeLa cells *in vitro*. A marked increase in the cytostatic activity of the 4-alkyl- substituted (*E*)-β-(benzoyl)acrylic acids was observed in going from the methyl- to ethyl-, *iso*-propyl-, *n*-butyl-, and *tert*-butyl substituent.

The similar increase in the bacteriostatic activity against Gram-positive bacteria was observed with *para*-alkyl substituted (benzoyl)acrylic acids with the substituent group ranging from methyl to nonyl.[1] The *para*-methoxy-derivative had the the same order of magnitude of bacteriostatic activity as the methyl compound.

Changes in the structure of the substituent can also lead to a suppression of the cytotoxic activity, as it was found for (*E*)-β-(4-acetamido)benzoylacrylic acid.

The cytostatic activity of (*E*)-β-(aroyl)acrylic acids is not connected with the inhibition of tetrahydrofolate formylase, as it was found[6] that compounds without a halogen on the Cβ are inactive in the inhibition of this enzyme.

In conclusion, the investigated substituted (*E*)-β-(aroyl)acrylic acids showed antiproliferative activity against human carcinoma cells *in vitro*. A sample Hansch-type correlation of IC50 with log *P* was found. The linear regression has a high correlation coefficient (0.924), which means that the biological activity is directly correlated with lipophilicity, and no additional effects need to be considered. Based on this result, the synthesis of more lipophilic (*E*)-β-(benzoyl)acrylic acids is in progress.

List of abbreviations:

IC50 – concentraiton of agent that induces a 50% decrease in cell survival
FBS – fetal bovine serum
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
HeLa cells – human cervix carcinoma cells
RPMI 1640 – standard nutrient medium

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И З В О Д

УМАЊЕНО ПРЕЖИВЉАВАЊЕ НЕОПЛАСТИЧКИХ ХУМАНИХ HeLa ЋЕЛИЈА СУПСТИТУИСАНИМ (*E*)-β-(БЕНЗОИЛ)АКРИЛНИМ КИСЕЛИНИМА

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Још раније је показана бактериостатска активност неких алкил супституисаних (*E*)-β-(бензоил)акрилних киселина. Циљ овог рада је био проучавање антипролиферативног дејства 19 (*E*)-β-(бензоил)акрилних киселина, алкил-, или халогено-, или метокси-, или ацетамидо-супституисnаих, на ћелије хумног карцинома грлића материце, HeLa ћелије. Цињне HeLa ћелије, су континуално третиране растућим концентрацијама супституисаних (*E*)-β-(бензоил)акрилних киселина током два дана. MTT тест је коришћен за утврђивање антипролиферативног дејства овог једињења. Третирање HeLa ћелија 4-метил-, 4-флуоро, 4-хлоро-, 4-бромо- и 4-метокси-дериватима (*E*)-β-(бензоил)акрилне киселине довело је до испољавања цитостатске активности према HeLa ћелијама (IC50 су између 31–40 μM). Њихово антипролиферативно дејство је било мање него код основног једињења, (*E*)-β-(бензоил)акрилне киселине, чије IC50 је било 28,5 μM. 3,4-Диметил-, 2,4-диметил- и 2,5-диметил- супституисани, као и 4-етил- те 3,4-дихлоро-и 2,4- дихлоро- деривати, имају јачу цитостатску активност од одговарајућег моносупституисаног и основног једињења. Њихове IC50 вредности су 18,5 μM; 17,5 μM, 17,0 μM; 17,5 μM; 22,0 μM и 18 μM, у наведеном редоследу. 4-*iso*-Пропил- и 4-*n*-бутил-деривати показују вишу цитостатску активност од једињења са мањим бројем метиленских –CH_2– група у супституенту. Њихове IC50 вредности су 14,5 μM односно 6,5 μM. 2,5-Ди-*iso*-пропил- и 4-*tert*-бутил- деривати испољавају најјаче антипролиферативно дејство према испитиваним HeLa ћелијама, IC50 су 4,5 μM и 5,5 μM у наведеном редоследу. Проучавана једињења утичу на преживљавање HeLa ћелија, испољавајући изразиту релацију Hansch-овог типа између структуре и биолошке активности.

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