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Inhibition of prostaglandin E_2 release by salicylates, benzoates and phenols: a quantitative structure-activity study

J. HABICHT* AND K. BRUNE**

*Department of Pharmacology, Biocentre, University of Basel, Switzerland,
**Department of Pharmacology, University of Erlangen-Nürnberg, FRG

Concentrations inhibiting 50% of the prostaglandin E_2 release from phorbol ester-stimulated mouse peritoneal macrophages in-vitro were determined for 59 monosubstituted congeners of salicylic acid, benzoic acid and phenol. Twenty-seven further compounds, mainly benzoic acids, were found to be inactive. An attempt was made to establish a quantitative structure-activity relationship (QSAR) from our experimental data using literature or calculated values for the logarithmic n-octanol/water partition coefficients of the compounds, molar refractivity and sigma values of substituents as well as structural indicator variables. The equations found have moderate predictive power and must be considered as a first step in the investigation of factors determining the biological activity of salicylates, benzoates and phenols.

In 1971 Vane and other workers (Vane 1971; Smith & Willis 1971; Ferreira et al 1971) proposed that non-steroidal anti-inflammatory drugs (NSAID) exert their actions by inhibiting the enzyme cyclo-oxygenase which converts arachidonic acid to prostaglandins. Though experimental evidence in favour of this hypothesis has accumulated over the past decade, a detailed theory on the molecular mode(s) of action of NSAIDs has not yet been established. Also, few systematic investigations on quantitative structure-activity relationships (QSARs) which are increasingly applied in other areas of pharmacology have been performed with respect to the inhibition of cyclo-oxygenase and prostaglandin biosynthesis (e.g. Moser et al 1975; van den Berg et al 1975). In fact, only one short communication has so far been published reporting an attempt to correlate anti-inflammatory activity and lipophilicity of 27 ring-substituted derivatives of aspirin (Dearden & George 1979).

We here present data on the activity of 86 monosubstituted congeners of salicylic acid (SA), benzoic acid (BA) and phenol (Ph) as inhibitors of prostaglandin release from mouse peritoneal macrophages in-vitro. The experimental system is based on the assay of Glatt et al (1977) and Brune et al (1981) and has been refined to allow the more precise determination of median inhibitory concentrations (IC50's) from dose-response curves. Using data from the literature on n-octanol–water partition coefficients and two substituent parameters, namely molar refractivity and the sum of the Hammett sigma values of substituents, we have tried to obtain equations relating the compounds' biological activities to these physico-chemical properties. During the course of this analysis it was also found necessary to introduce indicator variables which help to describe structural features of the molecules rather than having direct physicochemical significance.

** Correspondence.

MATERIALS AND METHODS

Materials

All solvents and salts used were purchased from Merck, Darmstadt (FRG) and were of analytical grade. Sterile tissue culture materials were products of Falcon Plastics, Becton Dickinson AG, Basle, Switzerland.

Young male NMRI-mice (25–30 g) were obtained from the 'Süddeutsche Versuchstierfarm KG', D-72 Tuttlingen, FRG. Dulbecco's Modified Eagle's Medium (DMEM) and newborn calf serum (NCS) which was heat-inactivated were products of North American Biologicals Inc., Miami, Florida, U.S.A. DMEM was adjusted to pH 7·2 with 1 M hydrochloric acid.

Most of the compounds tested could be obtained from commercial sources or were gifts from chemical companies. If necessary, they were recrystallized or vacuum-distilled. The remaining compounds were synthesized by adaptation of known methods:

3-Fluoro- and 5-isopropylsalicylic acid (Parham & Anderson 1948); 4-fluorosalicylic acid (Kaeding & Collins 1965); 6-phenylsalicylic acid (Staedel 1895). 6-Methyl-SA was synthesized by esterification of 6-methyl-2-nitrobenzoic acid, catalytic reduction of the nitro group, followed by diazotation, hydrolysis of the diazonium salt by boiling, and ester hydrolysis with 10% potassium hydroxide in water. 3-Isopropyl-BA was made by treating monomethyl isophthalate with three equivalents of methyl magnesium chloride in dry tetrahydrofuran, followed by ammonium chloride hydrolysis and catalytic reduction of the obtained 3-(α-hydroxyisopropyl)-BA with hydrogen under standard pressure and temperature using a 10%-palladium–charcoal catalyst. The tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was a kind gift from Prof. E. Hecker, Deutsches Krebsforschungzentrum, Heidelberg, FRG.

^{3}H-Labelled prostaglandin E_2 was obtained from the Radiochemical Centre Amersham, U.K. Unlabelled prostaglandin E_2 used for radioimmunoassay standard curves was purchased from Upjohn Diagnostics, Kalamazoo, Michigan, U.S.A. Anti PGE_2-antiserum was a generous gift from Prof. B. A. Peskar, Bochum, FRG.

Methods

The in-vitro assay used was based on the assay of Glatt et al (1977) and the findings of Brune et al (1978, 1981). Macrophages were obtained from young male NMRI-mice by peritoneal lavage, seeded into 35 mm petri dishes at 2×10^6 cells per dish and incubated with 2 ml DMEM containing 10% NCS. After a 16 h incubation at 37 °C in an atmosphere containing 10% CO_2, non-adherent cells were washed off and 2·0 ml DMEM added to each culture. Serum-free DMEM was used to avoid protein binding of drugs. Two groups of five cultures each were then treated with 10 μl of the solvent dimethyl sulfoxide (DMSO), while the remaining cultures were treated with drugs. This was done by applying 10 μl of drug dilutions in DMSO to a series of cultures to obtain a dose-dependent response. Usually ten dilutions of a compound were used lying within a concentration range of three decades. After 1 h the first group of five cultures was treated with 10 μl of DMSO as a solvent control group (DMSO-group). To obtain a measure for maximum PGE_2-release (TPA group) and to stimulate the drug-treated macrophage cultures, 10 μl of a 2×10^{-5} M solution of the tumour-promoting irritant TPA (final concentration: 10^{-7} M) were added to the remaining group of five DMSO-treated cultures and to the drug-treated cultures.

Two hours after TPA treatment the culture fluids were decanted into centrifuge tubes and spun at 250 g for 10 min. The viability of the cells was immediately assayed by the trypan blue exclusion method and the PGE_2 contents of the supernatants was measured by means of a specific radioimmunoassay (Liebig et al 1974) with a linear standard curve plotting PGE_2 standards against the free-to-bound ratio (Ekins 1974). Values for percent inhibition were obtained by comparing the PGE_2 release from drug-treated cultures with the DMSO-group (100% inhibition) and the TPA-group (0% inhibition). By this procedure a dose-response curve was obtained from the dilution series of a given drug.

The dose-response curves were then linearized by probit transformation (Finney 1964). All values between 5 and 95% inhibition inside the range of one logarithmic unit over and under the graphically estimated IC50 were included in the linear regression of log concentrations versus probits. As a rule, of percent inhibition values five to seven out of ten were found to fulfil these criteria. From the linearized dose-response curves corrected IC50 values and their 95%-confidence limits were obtained for each compound by a standard statistical method (Goldstein 1964).

QSARs were established by means of stepwise multiple regression analysis with the program BMDP2R of the 'Health Science Computing Facility', University of California. The dependent variable, pIC50, is the negative log IC50 which corresponds to log (1/IC50) frequently used in QSARs. The independent variables used in this investigation were log $P_{7.2}$ values of the compounds, and the molar refractivity (MR) and sum of Hammett sigma values (sum-sigma) of the substituents. Values for these variables were taken from the literature ('Pomona College Medicinal Chemistry Project', Seaver Chemistry Laboratory Claremont, California 91711; Hansch et al 1973; Norrington et al 1975). In some instances no literature values for log P were available. They were calculated by the additive method of Fujita et al (1964). All log P-values of SAs and BAs were corrected for dissociation at pH 7·2 as literature values (log P_0) are all based on the undissociated species. The log $P_{7.2}$ values were calculated by the following formula (Moser & Jäkel personal communciation): log $P_{7.2} \cong$ log P_0-(pH-pK). Since no strongly acidic Phs (e.g. dinitrophenols) were investigated, no pH corrections were necessary for phenols. pK values were taken from Rainsford

(1978) and standard handbooks of organic chemistry or were calculated by the method of Barlin & Perrin (1966). In SAs, sigma values were used with respect to the carboxyl group.

During the course of the analysis it was found necessary to introduce indicator variables characterizing structural features of the molecules to obtain a higher degree of explanation of the experimental data. The indicator variable I_1 equals 1 for BAs and 0 for all other compounds. Likewise, I_2, I_3, I_4 and I_5 designate phenols, amino- and hydroxy-substituted phenols, SAs with a 'lipophilic' substituent (i.e. Methyl-SAs, etc.) and SAs substituted in the 4- or 6-position, respectively.

RESULTS AND DISCUSSION

The corrected IC50-values and their 95%-confidence limits obtained from the linearized dose-response curves as well as the number of points (n) from which the curves were constructed are summarized in Table 1.

Although various structure-activity studies on NSAIDs have been performed (e.g. Moser et al 1975), only one systematic QSAR-investigation has

Table 1. Log IC50-values and 95%-confidence limits (c.l.) for salicylic acids, benzoic acids and phenols. (n) Designates the number of points from which the dose-response curves were constructed. All benzoic acids with amino-, hydroxy- (except SA), fluoro-, chloro-, methyl- and ethyl substituents were found to be inactive (log IC50 > −3).

Compound	log IC50 ± 95%-c.l.	(n)
Salicylic acid	−3·33 ± 0·31	4
3-Amino-SA	−3·72 ± 0·08	6
4-Amino-SA	−2·09 ± 0·44	5
5-Amino-SA	−2·94 ± 0·25	6
3-Hydroxy-SA	−4·43 ± 0·19	7
4-Hydroxy-SA	−3·02 ± 0·20	7
5-Hydroxy-SA	−4·61 ± 0·36	6
6-Hydroxy-SA	>−3	–
3-Fluoro-SA	−3·82 ± 0·64	4
4-Fluoro-SA	>−3	–
5-Fluoro-SA	−3·82 ± 0·35	6
6-Fluoro-SA	>−3	–
3-Chloro-SA	−3·89 ± 0·30	7
4-Chloro-SA	−3·31 ± 0·19	5
5-Chloro-SA	−4·06 ± 0·32	6
6-Chloro-SA	−2·76 ± 0·87	5
5-Bromo-SA	−4·19 ± 0·09	7
3-Methyl-SA	−2·71 ± 0·14	5
4-Methyl-SA	−2·37 ± 0·56	5
5-Methyl-SA	−3·12 ± 0·19	5
6-Methyl-SA	−2·62 ± 0·09	5
3-Isopropyl-SA	−3·92 ± 0·24	7
4-Isopropyl-SA	−3·29 ± 0·13	4
5-Isopropyl-SA	−4·12 ± 0·54	7
6-Isopropyl-SA	>−3	–
3-Cyclohexyl-SA	−4·90 ± 0·08	6
5-Cyclohexyl-SA	−5·90 ± 0·65	6
3-Phenyl-SA	−4·37 ± 0·14	6
4-Phenyl-SA	−4·82 ± 0·08	7
5-Phenyl-SA	−5·31 ± 0·12	7
6-Phenyl-SA	>−3	–
4-n-Propyl-BA	−2·98 ± 0·26	4
4-t-Butyl-BA	−2·69 ± 0·44	4
4-Cyclohexyl-BA	−3·86 ± 0·13	6
2-Isopropyl-BA	−2·82 ± 0·16	8
3-Isopropyl-BA	−3·01 ± 0·07	5
4-Isopropyl-BA	−2·93 ± 0·14	7
2-Phenyl-BA	>−3	–
3-Phenyl-BA	−4·46 ± 0·16	8
4-Phenyl-BA	−4·34 ± 0·09	8
Phenol	−3·54 ± 0·16	4
2-Amino-Ph	−4·99 ± 0·14	7
3-Amino-Ph	−4·09 ± 0·07	7
4-Amino-Ph	−3·81 ± 0·45	4
2-Hydroxy-Ph	−5·34 ± 0·16	5
3-Hydroxy-Ph	−5·15 ± 0·11	7
4-Hydroxy-Ph	−4·43 ± 0·12	4
2-Fluoro-Ph	−3·57 ± 0·26	5
3-Fluoro-Ph	−4·05 ± 0·79	5
4-Fluoro-Ph	−3·98 ± 0·22	7
2-Chloro-Ph	−4·62± 0·19	7
3-Chloro-Ph	−4·61 ± 0·14	6
4-Chloro-Ph	−4·86 ± 0·09	6
2-Methyl-Ph	−5·16 ± 0·17	6
3-Methyl-Ph	−4·74 ± 0·21	7
4-Methyl-Ph	−5·26 ± 0·14	6
2-Ethyl-Ph	−5·88 ± 0·17	7
3-Ethyl-Ph	−5·26 ± 0·16	7
4-Ethyl-Ph	−5·61 ± 0·16	5
2-Isopropyl-Ph	−4·81 ± 0·06	5
3-Isopropyl-Ph	−4·41 ± 0·33	4
4-Isopropyl-Ph	−5·48 ± 0·15	6
2-Phenyl-Ph	−5·61 ± 0·12	7
3-Phenyl-Ph	−5·46 ± 0·07	5
4-Phenyl-Ph	−6·04 ± 0·13	6

been published, relating the activity of acetylsalicylic acid derivatives in the carageenan-induced rat paw oedema to log P and the Verloop-steric parameters (Dearden & George 1979).

In our study we have investigated a large number of systematically ring-substituted derivatives of SA, BA and Ph with the aim of gaining information on the effects that a given substituent might exert in all possible positions of substitution. The basic structures, SA, BA and Ph, were chosen for three reasons. Firstly, powerful antiphlogistics and (or) analgesics may be found in all three classes of compounds (e.g. aspirin, fenamates, paracetamol). Secondly, they are structurally closely related, since SA contains the structural elements of both BA and Ph, and thirdly, the molecules are simple enough to allow quantum chemical calculations (Mehler et al 1982).

From the biological data we obtained, the following qualitative observations can be made. When comparing the groups of SAs, BAs and Phs with the same substituent it is found that the Ph-group is most potent, followed by the corresponding SA- and BA-group. The BAs are always least active and, in fact, the log IC50s of most of the BAs investigated could not be determined accurately, since they were considerably larger than -3, causing experimental difficulties (solubility, pH, cytotoxicity).

Within each of the three substance classes fluoro-substitution slightly enhances, and amino-, hydroxy- and chloro-substitution clearly enhance the potency of the parent compounds. This is in agreement with the suggestion that the higher in-vivo activity of SA may be due to partial metabolization to 5-hydroxy-SA (Flower 1974). Also, the desacylated metabolite of paracetamol, 4-amino-Ph, has recently been found to be more potent as an inhibitor of PG-biosynthesis than its parent compound (see Bruchhausen & Baumann 1982).

In general it is also found that potency increases from methyl- to phenyl-substituents with growing side chain. Also, for all SAs investigated, with the exception of 4-phenyl-SA, 3- and 5-substituted SAs were found to be more active than the 4- and 6-substituted isomers. The lower potency of the 4- compared with the 5-derivatives of SA has been interpreted as a sterical effect by Dearden & George (1979). It seems plausible though, that electronic as well as steric effects may play a role in enhancing or decreasing activity. For instance, a mixture of electronic (pi-conjugation) and steric effects (planarity) may be responsible for the lower activity of 3- and 5-phenyl-SA compared with the cyclohexyl-SAs. We suggest that the general observation that 3-substituted SAs are less active than 5-substituted SAs (especially for substituents $-CH_3$, $-iC_3H_7$, $-cC_6H_{11}$ and -phenyl) and that 6-substituted SAs are less active than 4-substituted SAs may also be explained by steric effects. Since the 3- and 6-positions are nearest to the *o*-hydroxy-carboxyl moiety, substituents in these positions may interfere with molecular processes such as chelate formation. We have also found the rank order of potencies for fluoro- and phenyl-SAs to be largely in agreement with the in-vivo-findings of Hannah et al (1978), though no conclusive correlation between the results from these two experimental systems could be found (due to the small sample size of common compounds).

The starting point for the QSAR calculations was a correlation of the experimental data as a whole versus log $P_{7.2}$. This correlation (not shown) proved highly unsatisfactory since it had a very low r^2 value. By introducing further parameters, indicator variables (Table 2) based on the observations discussed before and, finally, by dividing the data up into groups and sub-groups, improved equations were obtained which are summarized in Table 3.

From equations 1 to 4 it becomes evident that, apart from the lipophilicity of the compounds, the molar refractivity of the substitutents contributes strongly to the biological activity of the compounds. In fact, of the non-structural variables only MR was found to contribute significantly in equation 1. It has been suggested by Hansch (1978) that a positive MR coefficient may indicate essential conformational changes to be taking place. Unfortunately this finding is as yet not firmly established because collinearity, r^2, between log $P_{7.2}$ and MR, which is low in equation 1 ($r^2 = 0{\cdot}139$), increases to $r^2 = 0{\cdot}749$, $0{\cdot}805$ and $0{\cdot}589$ in equations 2, 3 and 4. This obscures the roles of log $P_{7.2}$ and MR since they are no longer independent if the structural variables are introduced. Selectively including further substituents in such a way that collinearity of all variables will be low (Hansch 1976) can help to overcome these problems in further investigations. Also, a pilot study on the role of quantum chemical parameters in QSARs has yielded promising results for the correlation of biological activity of eighteen SAs, BAs and Phs with π-Highest Occupied Molecular Orbital (π-HOMO) energies (Mehler et al 1982), so that quantum chemical parameters such as orbital energies and dipole moments will be able to be included in future QSAR studies of these compounds.

Like in many other investigations of this kind, the QSARs reported here must be regarded as being a

Table 2. Input-data of independent variables for stepwise regression with BMDP2R.

Compound	log P	pK	log $P_{7.2}$	MR	Sum-sigma	Compound	log P	pK	log $P_{7.2}$	MR	Sum-sigma
SA	2·27	2·97	−1·96	2·85	1·22	4-n-Propyl-BA	3·42	4·32	0·54	14·96	−0·15
3-Amino-SA	1·04	3·13	−3·03	5·42	1·06	4-t-Butyl-BA	3·85	4·40	1·05	19·62	−0·20
4-Amino-SA	1·32	3·52	−2·36	5·42	0·56	4-Cyclohexyl-BA	4·38	4·41	1·59	26·69	−0·22
5-Amino-SA	1·04	5·84	−0·32	5·42	1·06	2-Isopropyl-BA	3·40	3·64	−0·16	14·98	−0·15
3-Hydroxy-SA	1·60	3·79	−1·81	2·85	1·34	3-Isopropyl-BA	3·40	4·26	0·46	14·98	−0·07
4-Hydroxy-SA	1·54	3·22	−2·44	2·85	0·85	4-Isopropyl-BA	3·40	4·35	0·55	14·98	−0·15
5-Hydroxy-SA	1·60	2·97	−2·63	2·85	1·34	3-Phenyl-BA	3·83	4·13	0·76	25·36	0·06
3-Fluoro-SA	2·41	2·63	−2·16	0·92	1·56	4-Phenyl-BA	3·83	4·20	0·83	25·36	−0·0
5-Fluoro-SA	2·41	2·63	−2·16	0·92	1·56	Phenol	1·48	9·89	1·48	1·03	0·0
3-Chloro-SA	2·98	2·60	−1·62	6·03	1·59	2-Amino-Ph	0·57	9·71	0·57	5·42	0·0
4-Chloro-SA	2·98	2·74	−1·48	6·03	1·45	3-Amino-Ph	0·16	9·87	0·16	5·42	−0·16
5-Chloro-SA	3·09	2·80	−1·31	6·03	1·59	4-Amino-Ph	0·04	8·46	0·04	5·42	−0·15
6-Chloro-SA	2·98	2·63	−1·59	6·03	2·50	2-Hydroxy-Ph	0·95	9·85	0·95	2·85	−0·09
5-Bromo-SA	3·05	2·57	−1·40	8·88	1·61	3-Hydroxy-Ph	0·78	9·81	0·78	2·85	0·12
3-Methyl-SA	2·83	2·90	−1·47	5·65	1·15	4-Hydroxy-Ph	0·56	10·85	0·56	2·85	−0·37
4-Methyl-SA	2·99	3·15	−1·06	5·65	1·05	2-Fluoro-Ph	1·68	8·82	1·68	0·92	0·54
5-Methyl-SA	2·78	3·40	−1·02	5·65	1·15	3-Fluoro-Ph	1·93	9·36	1·93	0·92	0·34
6-Methyl-SA	2·83	3·32	−1·05	5·65	1·51	4-Fluoro-Ph	1·79	9·92	1·79	0·92	0·06
3-Isopropyl-SA	3·80	3·30	−0·10	14·98	1·15	2-Chloro-Ph	2·16	8·48	2·16	6·03	0·68
4-Isopropyl-SA	3·80	3·20	−0·20	14·98	1·07	3-Chloro-Ph	2·50	9·08	2·50	6·03	0·37
5-Isopropyl-SA	3·80	3·04	−0·36	14·98	1·15	4-Chloro-Ph	2·40	9·38	2·40	6·03	0·23
3-Cyclohexyl-SA	4·78	3·05	0·63	26·69	1·14	2-Methyl-Ph	1·99	10·20	1·99	5·65	−0·13
5-Cyclohexyl-SA	4·78	3·05	0·63	26·69	1·14	3-Methyl-Ph	1·97	10·01	1·97	5·65	−0·07
3-Phenyl-SA	4·23	4·30	1·33	25·36	1·16	4-Methyl-Ph	1·94	10·17	1·94	5·65	−0·17
4-Phenyl-SA	4·23	2·98	0·01	25·36	1·21	2-Ethyl-Ph	2·47	10·20	2·47	10–30	−0·15
5-Phenyl-SA	4·23	2·91	−0·06	25·36	1·28	3-Ethyl-Ph	2·40	9·90	2·40	10·30	−0·07
						4-Ethyl-Ph	2·26	10·01	2·26	10·30	−0·15
						2-Isopropyl-Ph	2·88	10·20	2·88	14·98	−0·23
						3-Isopropyl-Ph	2·70	9·90	2·70	14·98	−0·07
						4-Isopropyl-Ph	2·56	10·01	2·56	14·98	−0·15
						2-Phenyl-Ph	3·09	10·01	3·09	25·36	0·0
						3-Phenyl-Ph	3·23	9·64	3·23	25·36	0·06
						4-Phenyl-Ph	3·20	9·51	3·20	25·36	−0·01

The indicator variables $I_1 - I_5$ are described in the text.

Table 3. QSAR-equations.

Equation No./ group of data	Variables used	Equation obtained
1. All data	log $P_{7.2}$, log $P_{7.2}^2$ MR, I_1, I_2, I_3, I_4, I_5 $n = 59, r^2 = 0.744, s = 0.528$	$pIC50 = 3.489 + 0.078\,(\pm 0.010)\,MR - 1.632\,(\pm 0.287)I_1 + 0.669\,(\pm 0.192)I_2 - 0.516\,(+0.244)I_4 - 0.898\,(\pm 0.226)I_5$ $F_{5,53} = 30.86\,(P < 0.001)$
2. Salicylic acids	log $P_{7.2}$, log $P_{7.2}^2$, MR, sum-sigma I_4, I_5 $n = 26, r^2 = 0.774, s = 0.484$	$pIC50 = 1.576 - 0.495\,(\pm 0.174)\log P_{7.2} + 0.12\,(\pm 0.022)\,MR + 0.535\,(\pm 0.282)$ sum-sigma $- 0.968\,(\pm 0.207)\,I_5$ $F_{4,21} = 17.99\,(P < 0.001)$
3. 'Lipophilic' salicylic acids	log $P_{7.2}$, log $P_{7.2}^2$ MR, sum-sigma, I_5 $n = 12, r^2 = 0.933, s = 0.347$	$pIC50 = 1.517 - 0.513\,(\pm 0.289)\log P_{7.2} + 0.151\,(+0.026)\,MR - 0.491\,(\pm 0.223)\,I_5$ $F_{3,8} = 37.35\,(P < 0.001)$
4. 'Lipophilic' benzoic acids	log $P_{7.2}$, log $P_{7.2}^2$, MR, sum-sigma $n = 8, r^2 = 0.934, s = 0.219$	$pIC50 = 1.866 + 0.097\,(\pm 0.016)\,MR + 3.361\,(\pm 0.901)$ sum-sigma $F_{2,5} = 18.31\,(P < 0.005)$
5. Phenols	log $P_{7.2}$, log $P_{7.2}^2$, I_3 $n = 25, r^2 = 0.591, s = 0.485$	$pIC50 = 1.236 + 2.164\,(\pm 0.722)\log P_{7.2} - 0.248\,(\pm 0.158)\log P_{7.2}^2 + 2.385\,(\pm 0.611)\,I_3$ $F_{3,21} = 10.09\,(P < 0.001)$

tool to study the influence of various parameters rather than having predictive character in the search for improved drugs.

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