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Original article

Nitrobenzyl esters as potential conjugated alkylating and differentiation promoting agents: antitumor effect *in vivo*

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Summary — A series of *ortho* and *para*-nitrobenzyl esters of short chain fatty acids (C_2 to C_5) and of the lipophilic aminoacid, L-valine, and some other *o*-nitrobenzylated products were prepared. Bioreductive or hydrolytic cleavages of these compounds could release both differentiation promoting agents such as butyric acid and electrophilic moieties able to alkylate DNA. The antitumor effect of these compounds, either alone and/or associated with an immunostimulating agent (corynebacterium parvum, CP) or interferon (IFN) treatment, was studied using the 180 TG Crocker Sarcoma grafted onto Swiss mice. Based on the mean survival time and the final survival rate, the most active compounds used alone were the *o*-nitrobenzyl acetate and the *o*-nitrobenzyl butyrate. Previous stimulation of the immune competent cells by CP before antitumor treatment increased the efficiency of most of the nitrobenzylated derivatives studied. The association with IFN did not significantly improve the antitumor effect.

Résumé — **Esters de nitro-benzyle, agents potentiels d'alkylation et de différenciation cellulaire: effect antitumoral *in vivo*.** *Une série d'esters d'*ortho *et de* para*-nitrobenzyle d'acides gras à courte chaîne (C_2 à C_5) et de la* L*-valine, ainsi que quelques autres produits* ortho*-nitrobenzylés, ont été préparés. Des coupures bioréductrice ou hydrolytique de ces composés pourraient libérer des agents pouvant intervenir dans la différenciation cellulaire tel que l'acide butyrique et des espèces électrophiles capables d'alkyler le DNA. Selon un protocole préalablement établi, l'effet antitumoral de ces composés (employés seuls ou associés à un traitement par un immuno-stimulant* Corynebacterium parvum, *CP, et/ou l'interféron) a été étudié chez la souris Swiss ayant subi la greffe de cellules de sarcome 180TG de Crocker. En considérant la moyenne de temps de survie ainsi que la survie finale, les plus actifs par eux-mêmes sont l'acétate d'*ortho*-nitrobenzyle et le butyrate d'*ortho*-nitrobenzyle. Une stimulation préalable du système immunitaire par le CP augmente la résistance antitumorale des animaux et accroît ainsi l'efficacité de la plupart des dérivés nitrobenzylés étudiés. L'association de l'interféron ne modifie pas, d'une façon significative, l'effet antitumoral induit par la plupart de ces composés.*

nitrobenzyl esters / antitumor effect

Introduction

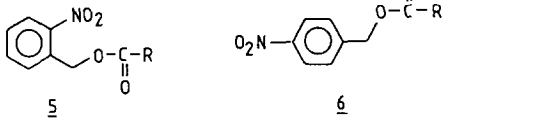
Covalent conjugation of 2 compounds acting by 2 different biochemical mechanisms could, in a favorable case, lead to synergism to obtain an antitumor effect. Bioactivation of a latent conjugated molecule could add the possibility of a simultaneous selective drug delivery of 2 activated moieties. For example, 3-*p*-nitrobenzyloxycarbonyl-5-fluorouracil **1a** (scheme 1) is a potential bioreductive alkylating agent [1–5] which displays slightly higher anticancer effects in EMT6 tumor cell culture and in mice bearing the P338 leukemia or sarcoma 180 than those produced by the 5-fluorouracil itself [6]. It was hypothesized that bioreduction [7, 8] of **1a** within the hypoxic region [8–12] of solid tumors could generate a *p*-aminobenzyloxycarbonyl-5-fluorouracil **2a** (scheme 1, eq 1) decomposing to give an electrophilic quinonimine methide **3a** and a carbamate anion. Further fragmentation [13, 14] of the anion **4a** could liberate the 5-fluorouracil anion and carbon dioxide (scheme 1, eq 2). Such a 1,6-elimination mechanism (eq 1, step j) is known [15]. For instance, selective reduction of *p*-nitrobenzyl esters **1b** frees carboxylic acids **4b** [16]. *Ortho*- and *para*-aminobenzyl halides are biological alkylating agents [17, 18] and *ortho*-nitrobenzyl chloride and carbamates are more toxic to hypoxic than to oxygenated cells in tissue cultures [19, 20]. However, no study was performed on animal models.

Instead of an antimetabolite, such as 5-fluorouracil, we concentrate in this study, on the possible release of a differentiation promoting agent. Among the compounds able to induce phenotypic reversion of malignant cells to cells with normal growth control [21–25], are the short chain fatty acids and the lipophilic aminoacids such as butyric acid [26–28] or valine.

Therefore, we propose the synthesis and study of a series of nitrobenzyl esters of these acids. The

selective toxicity of *ortho*-nitrobenzyl compounds being higher than that of their *para*-isomers [19, 20], this study was focussed on *ortho*-nitrobenzyl derivatives. However, some *para*-nitrated analogs were included for comparative purposes. Therefore, *ortho*-nitrobenzyl esters of acetic, propionic, butyric, valeric acids (**5a–d**) and L-valine (**5e**, *p*-toluene sulfonate) as well as *p*-nitrobenzyl acetate and butyrate (**6a**, **6b**) were prepared. *p*-Nitrobenzyl valinate hydrobromide **6c** was also studied.

Apart from the release of L-valine from its carboxyl end, liberation may occur from its amino-end by the fragmentation of a carbamate anion (scheme 1, eq 2). Thus *ortho*- and *para*-nitrobenzyloxycarbonyl-L-valine (**5f** and **6d**) were synthesized. To investigate the biological effect of the quinonimine moiety alone and the role of the leaving group in the reaction, other nitrobenzyl compounds were included in the study: *ortho*- and *para*-nitrobenzyl alcohols and halides (**7**, **8**, **9a** and **10**), *ortho*-nitrobenzyl succinate (**5g**), *ortho*-nitrobenzylphenyl ether (**11**) and *ortho*-nitrobenzyl pyridinium chloride (**12**).



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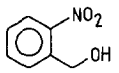
a: R = CH_3; b: R = C_2H_5
c: R = C_3H_7; d: R = C_4H_9
e: R = CH–NH2;
iPr
f: R = NH–CH–CO_2H;
iPr
g: R= $CH_2CH_2CO_2$H

a: R= CH_3; b: R = CH_{37};
c: R = CH–NH_2;
iPr
d: R = NH–CH–CO_2H
iPr



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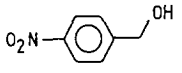

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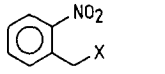

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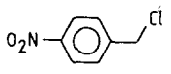
a: X=Cl
b: X=Br



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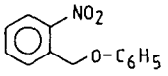

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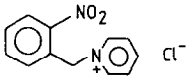

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Results

Synthesis

Ortho-nitrobenzyl esters of acetic, propionic, butyric and valeric acids (**5a–d**) and *para*-nitrobenzyl esters of acetic and butyric acids (**6a**, **6b**) were prepared from the *o*- or *p*-nitrobenzyl chloride and the corresponding carboxylic acid following a modified version of Schwartz's procedure for preparation of N^{α}-*Z*-aminoacid *p*-nitrobenzyl esters [29]. The *ortho*-nitrobenzyl esters are light-sensitive [30–31] and care was taken to protect them from a possible photochemical decomposition.

The reaction of succinic anhydride with *o*-nitrobenzyl alcohol catalyzed by 4-dimethylaminopyridine [32] yielded the *o*-nitrobenzyl succinate monoester **5g** soluble in water at pH 8. *o*-Nitrobenzyl *Z*-valine *p*-toluenesulfonate (**5e**) was obtained by esterification of the alcohol in the presence of *p*-toluenesulfonic acid in benzene with azeotropic removal of water, by a modified version of a method of preparation of aminoacid *p*-nitrobenzyl esters [33].

N-o-nitrobenzyloxycarbonyl-L-valine **5f** was prepared from L-valine and 1-methyl-3-*o*-nitrobenzyloxycarbonyl imidazolium chloride which was obtained by reacting *o*-nitrobenzylchloroformate [34] with N-methyl imidazole [35].

A good yield of *o*-nitrobenzyl phenyl ether (**11** [36]) was obtained by a phase-transfer catalyzed alkylation [37] of phenol. The water-soluble *N-o*-nitrobenzyl pyridinium chloride (**12**) was prepared by a slightly modified version of the procedure described in [38].

Antitumor therapeutic assays

Antitumor efficacy was studied as specified in the experimental section using the 180 TG Crocker sarcoma cells grafted onto young Swiss mice. The results were estimated by comparing different lots of mice randomly selected and treated with a potent immune stimulator (Coryne bacterium parvum extracts, CP [39]), alone or in association with interferon (IFN). The choice of this methodology was based on previous experiments showing that an important amplification in antitumor protection can be obtained by associating a single injection of this immune modulator prior to butyrate derivatives. Furthermore, we showed that mice grafted with malignant sarcoma cells can be affected by arginine butyrate treatment, which decreases the tumor mass and develops the cytoskeleton and extracellular matrix in these transformed cells. This is necessary for the expression of the IFN receptors associated to the cell membrane. The result is a significant improvement in the sensitivity of the malignant cells to IFN [27].

As suggested, to analyze therapeutic effects in mice, we took into consideration the tumor incidence at 10 d after the tumor graft, the mean survival time (MST) for an observation period of 100 d and the final survival rate. The results are summarized in table I. The different short chain fatty acids covalently

conjugated with the *ortho-* or *para*-nitrobenzyl group were classed according to the growing number of carbon residues, ranging from C_1 to C_5. As controls, the fatty acid residues were replaced by alcohol, halides, valine, succinate or phenyl ether group. The *o-* and *p*-nitrobenzyloxycarbonyl-L-valine (**5f** and **6d**) proved to be insoluble in saline medium and the *o*-nitrobenzyl pyridinium chloride (**12**) too toxic to be studied. It is of interest that the association of interferon did not significantly modify the development of antitumor resistance. In contrast, the single immunostimulatory shot delivered prior to treatment leads to an improvement of the results.

Those of the conjugates which induced the antitumor effect were identified and grouped according to increasing statistically significant efficacy, and to whether they were applied alone or in association with CP (table II).

Discussion

Reduction of the electron-withdrawing nitro group ($\sigma_p^+ = + 0.79$) gives the strong electron-donating amino substituent ($\sigma_p^+ = - 1.47$) which can stabilize an incipient benzyl cation in the transition state of a

Table I. Antitumor assay as described in experimental section and based on the study of number of tumors at 10 d, mean survival time (Student's test) and final survival rate (Chi square test). $^1P < 0.001$, $^2P < 0.01$ when compared to control 180TG; $^3P < 0.001$, $^4P < 0.01$, $^5P < 0.02$ when compared to IFN; $^6P < 0.001$, $^7P < 0.01$, $^8P < 0.02$ and $^9 0.01 < P < 0.02$ when compared to CP.

Experimentals series	*No of animals bearing tumors at 10 d*	*Mean survival time (MST)*	*Δ % MST (increase versus) (control 180TG)*	*Survival rate*	*Total of animals / experimental series*
Control 180TG	45	18.8 ± 1.4		0	45
IFN	24	28.8 ± 4.7	53.2	2	45
CP	37	34 ± 5	80.8	2	45
CP + IFN	14	52.2 ± 10.4	193.6	17	45
CP + *o*-NB Acetate + IFN	ND	ND	ND	ND	ND
CP + *o*-NB Acetate	11	73.5[6] ± 10	290.9	27[6]	45
o-NB Acetate + IFN	ND	ND	ND	ND	ND
o-NB Acetate **5a**	8	54.3[1,3] ± 10.8	188.8	17[1,3]	45
CP + *p*-NB Acetate + IFN	ND	ND	ND	ND	ND
CP + *p*-NB Acetate	2	72.9[6] ± 17.8	287.7	9[6]	15
p-NB Acetate + IFN	ND	ND	ND	ND	ND
p-NB Acetate **6a**	4	41.3[2] ± 15.7	119.7	3	15
CP + *o*-NB Propionate + IFN	2	59.9[3,6] ± 18	218.6	6[2]	15
CP + *o*-NB Propionate	2	73.6[6] ± 17.3	291.5	9[6]	15
o-NB Propionate + IFN	2	49[5] ± 16.8	160.6	4	15
o-NB Propionate **5b**	2	31.7[2] ± 10	68.6	1	15
CP + *o*-NB Butyrate + IFN	10	68.5[6] ± 13.7	264.4	17[6]	30
CP + *o*-NB Butyrate	48	69.5[6] ± 7	269.7	59[6]	105
o-NB Butyrate + IFN	19	31.1[4] ± 10	65.4	4	30
o-NB Butyrate **5c**	62	33.9[2] ± 6.4	80.3	19[4]	105
CP + *p*-NB Butyrate + IFN	9	61.6[3] ± 20	227.6	7	15
CP + *p*-NB Butyrate	10	66.5[6] ± 12.6	253.7	15	30
p-NB Butyrate + IFN	8	22 ± 2.5	17	0	15
p-NB Butyrate **6b**	17	26.1[2] ± 9.5	38.8	3	30
CP + *o*-NB Valerate + IFN	8	65[3] ± 20.2	245.7	8[6]	15
CP + *o*-NB Valerate	5	60.4[7] ± 17.8	221.3	6[6]	15
o-NB Valerate + IFN	4	26.6 ± 2.2	41.5	0	15
o-NB Valerate **5d**	5	24.8[3] ± 1.2	31.9	0	15
CP + *o*-NB Valinate + IFN	1	75[6] ± 18.9	298.9	10[6]	15
CP + *o*-NB Valinate	10	66[6] ± 13.8	251	15[6]	30
o-NB Valinate + IFN	11	21.3 ± 3	13.3	0	15
o-NB Valinate **5e**	24	25.3[2] ± 5.8	34.6	0	30

Table I. *Continued*

Experimentals series	*No of animals bearing tumors at 10 d*	*Mean survival time (MST)*	*Δ % MST (increase versus) (control 180TG)*	*Survival rate*	*Total of animals/ experimental series*
CP + *p*-NB Valinate + IFN	3	73.3^{6} ± 17.9	289.9	9^{6}	15
CP + *p*-NB Valinate	11	65.7^{6} ± 12	249.5	14^{6}	30
p-NB Valinate + IFN	12	27 ± 11	43.6	1	15
p-NB Valinate **6c**	21	28.4^{2} ± 7.5	51	2	30
CP + *o*-NB Succinate + IFN	10	64^{6} ± 20.8	240.4	8^{6}	15
CP + *o*-NB Succinate	4	81.2^{6} ± 16.8	331.9	11^{6}	15
o-NB Succinate + IFN	5	41.6^{4} ± 15.7	121.3	3	15
o-NB Succinate **5g**	11	20.5 ± 2.2	9	0	15
CP + *o*-NB Ether + IFN	2	70.6^{6} ± 19.5	275.5	9^{6}	15
CP + *o*-NB Ether	3	66^{6} ± 18	251	8^{6}	15
o-NB Ether + IFN	3	$52^{3,9}$ ± 18.3	176.6	5	15
o-NB Ether **11**	8	22.1 ± 2.2	17.5	0	15
CP + *o*-NB Chloride + IFN	ND	ND	ND	ND	ND
CP + *o*-NB Chloride	2	66.4^{6} ± 19.4	253.2	8^{6}	15
o-NB Chloride + IFN	ND	ND	ND	ND	ND
o-NB Chloride **9a**	3	33^{2} ± 10	75.5	1	15
CP + *p*-NB Chloride + IFN	ND	ND	ND	ND	ND
CP + *p*-NB Chloride	8	69^{6} ± 20	267	9^{6}	15
p-NB Chloride + IFN	ND	ND	ND	ND	ND
p-NB Chloride **10**	4	25.6^{2} ± 5	36.1	0	15
CP + *o*-NB Alcohol + IFN	ND	ND	ND	ND	ND
CP + *o*-NB Alcohol	9	56.3^{8} ± 19	199.5	6	15
o-NB Alcohol + IFN	ND	ND	ND	ND	ND
o-NB Alcohol **7**	10	25.3^{2} ± 11.3	34.6	1	15
CP + *p*-NB Alcohol + IFN	ND	ND	ND	ND	ND
CP + *p*-NB Alcohol	6	57.9^{8} ± 18.6	207.9	6	15
p-NB Alcohol + IFN	ND	ND	ND	ND	ND
p-NB Alcohol **8**	8	26.5^{2} ± 10.8	40.9	1	15
CP + arginine butyrate + IFN	5	80.6 ± 11.6	328.7	33	75
CP + arginine butyrate	15	77.8 ± 11.3	313.8	15	75
arginine butyrate + IFN	12	36 ± 6	91.5	12	75
arginine butyrate	42	22.5 ± 5.9	19.7	2	75

reaction. Therefore, *ortho-* and *para*-aminobenzyl compounds possessing a good leaving group are very reactive and the benzylic substitution generally occurs by an elimination-addition mechanism involving a very electrophilic quinonimine methide intermediate [15]*. Complete 6-electron reduction to the amino level may not be necessary to activate a molecule of type **1** or **5**. Apart from the mechanism shown on scheme 1, other mechanisms must be considered because the reduction of an aromatic nitro compound is a stepwise process with at least 4 well-recognized intermediate species in the pathway [4, 7, 8, 41–43]. A 1-electron reduction leading to the radical anion **13** which fragments (scheme 2, path a) is sufficient to expel a good leaving group such as Cl^- but not a carbamate anion [20, 44] (however see [45]). Microsomal reduction of the *p*-nitrobenzyl chloride *via* the nitrobenzyl radical anion has been demonstrated [44]. Other intermediates, such as the hydroxylamine **15**, may also fragment [46] (scheme 2, path b)**.

Obviously, spontaneous or enzymatic hydrolysis [48] of **5** (scheme 2, path d; or their *para*-analogues)

*These 1,4- and 1,6-eliminations (scheme 1, step b) do not require the abstraction of a proton to form a nitrogenated anion of pK about 25 [20]. The neutral anilino substituent (pK about 4.60) donates enough for the reaction at 25°C near neutral pH to take place [18, 40].

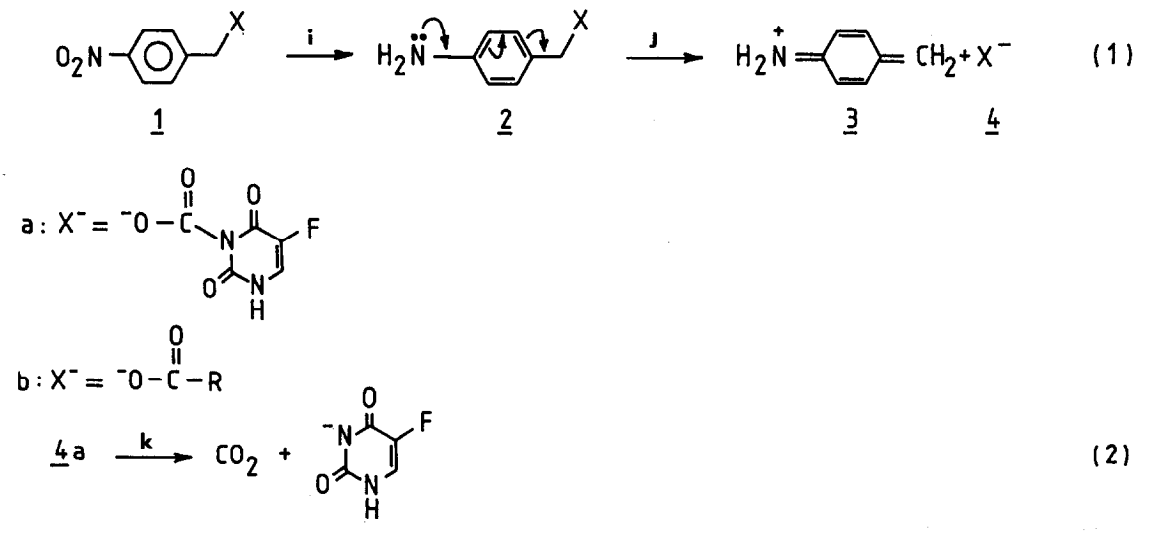
**Like the amino group, the hydroxylamino substituent is electron donating (compare for instance $\sigma_{pNHOH} = -0.34$ and $\sigma_{pNH2} = -0.66$ [47]). Therefore a large amount of the molecule will not be diverted at the hydroxylamine stage (four electron reduction) [4].

can also liberate the carboxylic acid. Therefore, a variety of mechanisms combine to the release of the differentiation promoting agent in a particular case of drug latentiation [49–51]. The reductive mechanisms also lead to strong electrophiles such as **3**, **17**, **18** and **19** which can alkylate DNA or damage other essential biomolecules.

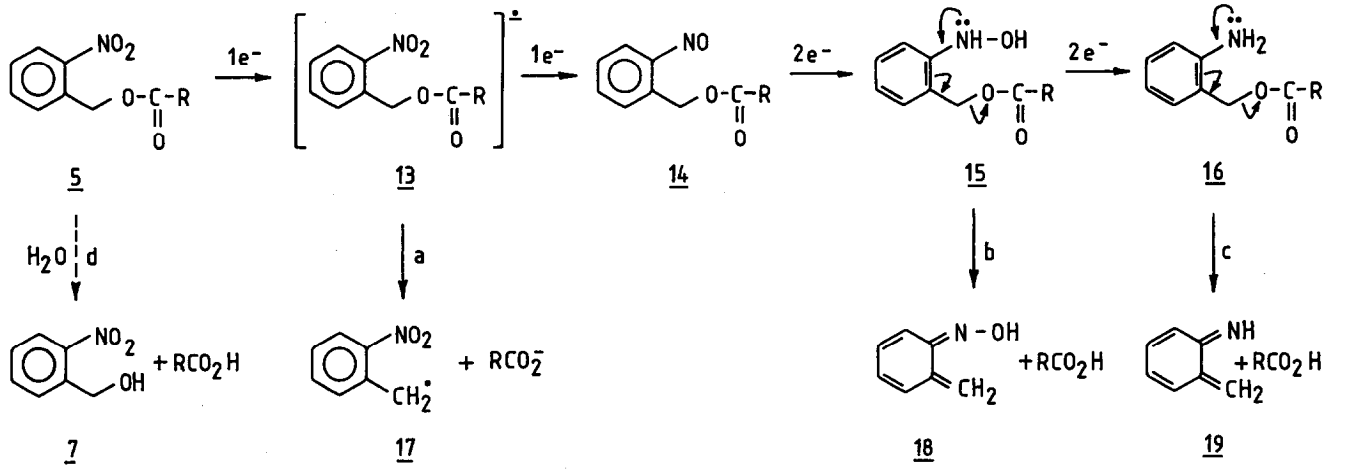
Antitumoral protection obtained after treatment with different compounds can be estimated considering both the MST and the final survival rate of grafted animals. On both parameters, the most active of the studied molecules is the *o*-NB acetate (**5a**) for which the MST, compared to the controls, increased from 18.8 to 54.3 d ($P < 0.001$) and for

Table II. Summary of statistical analysis listed in order of increasingly significant *P* values.

Compared to	*Mean survival time (MST)*			*Survival rate*	
	P *0.001*	P *0.01*	P *0.02*	P *0.001*	P *0.05*
180TG	*o*-NB Acetate **5a**	*p*-NB Acetate **6a** *o*-NB Propionate **5d** *o*-NB Butyrate **5c** *p*-NB Butyrate **6b** *o*-NB Valerate **5d** *o*-NB Chloride **9a**		*o*-NB Acetate **5a**	*p*-NB Acetate **6a** *o*-NB Butyrate **5c** *p*-NB Butyrate **6b**
CP + 180TG	*o*-NB Acetate **5a** *p*-NB Acetate **6a** *o*-NB Propionate **5b** *o*-NB Butyrate **5c** *p*-NB Butyrate **6b** *o*-NB Valinate **5d** *p*-NB Valinate **6c** *o*-NB Succinate **5g** *o*-NB Ether **11** *o*-NB Chloride **9a** *p*-NB Chloride **10**	*o*-NB Valerate **5d**	*o*-NB Alcohol **7** *p*-NB Alcohol **8**	*o*-NB Acetate **5a** *p*-NB Acetate **6a** *o*-NB Propionate **5b** *o*-NB Butyrate **5c** *p*-NB Butyrate **6b** *o*-NB Valerate **5d** *o*-NB Valinate **5e** *p*-NB Valinate **5c** *o*-NB Succinate **5g** *o*-NB Ether **11** *o*-NB Chloride **9a** *p*-NB Chloride **10** *o*-NB Alcohol **7** *p*-NB Alcohol **8**	
	arginine butyrate			arginine butyrate	



Scheme 1. Reductive fragmentation of *p*-nitrobenzyl carbamates and esters. i: 6-electron reduction, j: 1,6-elimination, k: further fragmentation of a carbamate anion.



Scheme 2. Reduction and hydrolysis of *o*-nitrobenzyl esters. Four intermediates (**13**, **14**, **15** and **16**) are shown, 3 of which may easily fragmentate.

which 17 of the 45 starting mice definitively survived ($P < 0.001$). The MST is also significantly increased by treatment with *o*-NB propionate (**5b**) *o*-NB butyrate (**5c**) *p*-NB butyrate and *o*-NB valerate (**5d**) ($P < 0.01$). However, considering the survival rate, only *o*-NB acetate and *o*-NB butyrate, and to a lesser extent their *para*-analogs, **6a** and **6b**, have a statistically significant effect: 19/105 for **5c** and only 3/15 (**6a**) and 3/30 (**6b**) mice were alive after 100 d (tables I and II). The butyrate ester (*o*-NB) is significantly more active alone when compared to corresponding arginine salts. Prior immunostimulation further amplifies these results for both compounds, particularly for the survival rate.

When a single injection of immune modulation precedes antitumoral treatment, compounds other than those listed above significantly increase the antitumor activity (table II).

Generally, the products belonging to the *ortho*-series were more potent than those in the *para* one, when compared to the control population. The nitrobenzyl halides, having better leaving groups were less potent than the corresponding esters.

It is presently unknown whether the anti-tumor effects detected here could be attributed to a hydrolysis of the ester bond or to bioreductive cleavage of the molecule. Preliminary experiments, not documented here, studying the DNA replicative cycle incorporating ^{3}H-thymidine into mouse 180TG or human U937 macrophage cell suspensions in the presence of *o*-nitrobenzyl butyrate have shown an arrest persisting longer than 96 h. Such cells, when reinjected in mice, lost at least partially their capacity to induce tumors in mice.

Antitumor resistance was principally obtained by either *o*-NB acetate or *o*-NB butyrate, for which the survival rate of grafted mice was increased when compared to their parent acids. It can thus be concluded that the covalent linkage between the short chain fatty acids and *o*-nitrobenzyl alcohol leads to a real improvement of the antitumoral effect.

Experimental protocols

Melting points were determined on a Mettler FP61 apparatus and are uncorrected. Infra-red spectra were recorded on a Perkin–Elmer 1420 spectrophotometer and NMR spectra on a Brucker WH90 MS apparatus. Valine *p*-nitrobenzyl ester hydrobromide was obtained from Bachem.

*Nitrobenzyl esters **5** and **6** . General procedure*

To a solution of *o*- or *p*-nitrobenzyl chloride (172 mg, 1 mmol), the corresponding carboxylic acid (2 mmol) and dry triethylamine (202 mg, 2 mmol) in 5 ml of dry ethyl acetate was added sodium iodide (30 mg, 0.2 mmol). After heating for 20 h in darkness, the reaction mixture was filtered hot to remove triethylamine hydrochloride. To the cooled filtrate 0.25 ml of methanol was added. Washing with water, 1 N HCl, water, $NaHCO_3$, then drying over Na_2SO_4 and evaporation gave a residue which was purified by flash chromatography (SiO_2, CH_2Cl_2-pentane, column protected from light). Yields, m ps and analytical data of these esters are reported in table III.

Mono ortho-*nitrobenzyl succinate **5g***

To a stirred solution of *o*-nitrobenzyl alcohol (306 mg, 2 mmol), succinic anhydride (100 mg, 1 mmol) and triethylamine (101 mg, 1 mmol) in dry EtOAc (10 ml) 4-dimethylaminopyridine (2 mg) were added. After being

stirred overnight at room temperature, the reaction mixture was washed twice with 10% sodium carbonate. The aqueous phase was combined, acidified till pH 2 with chilled concentrated HCl and extracted with ethyl acetate. Drying over magnesium sulfate and evaporation of the solvent *in vacuo* gave the title compound as a white solid. 450 mg (89%). mp = 63.5°C. IR (CH_2Cl_2) 1710, 1740. NMR ($CDCl_3$) 2.85 (s, 4H, CH_2CH_2); 5.70 (s, 2H, $ArCH_2$); 7.5–8.4 (m, 4H, ArH). Anal Calc for $C_{11}H_{11}NO_6$: C, 52.22; H, 4.38; O, 37.94; found: C, 52.36; H, 4.40; O, 37.97.

Ortho-*nitrobenzyloxycarbonyl-L-valine* ***5f***

Ortho-nitrobenzyl chloroformate [34] (216 mg, 1 mmol) in dry ethyl ether (20 ml) was added at 0°C to a stirred solution of N-methyl imidazole (328 mg, 2 mmol) in dry ether (10 ml). The precipitate of 1-methyl-3-*o*-nitrobenzyloxycarbonylimidazolium chloride was rapidly filtered and added to a stirred solution of L-valine (118 mg, 1 mmol) in 1 N NaOH (10 ml). The mixture was stirred for 10 min, filtered, acidified to pH 3 and extracted with ethyl acetate. Drying over magnesium sulfate and evaporation gave a white solid which was recrystallized from benzene. 165 mg (66.5%), mp = 108.9°C. IR (CH_2Cl_2) 1700–1750 (CO), 1380, 1520. NMR ($CDCl_3$) 0.95 (m, 6H, CH_3); 2.3 (m, 1H, $CH(CH_3)_2$) 4.3 (m, 1H, CH); 5.45 (s, 1H, NH); 5.55 (s, 2H, CH_2); 7.3–8.2 (m, 4H, ArH); 9.9 (s, 1H, CO_2H); $[\alpha]^{25}$ + 4.3 (C, EtOH). Anal Calc for $C_{13}H_{16}N_2O_6$: C, 52.70; H, 5.44; N, 9.46. Found: C, 52.44; H, 5.48; N, 9.03.

Para-*nitrobenzyloxycarbonyl-L-valine* ***6d*** *[55–57]*

From *p*-nitrobenzyl chloroformate, N-methyl imidazole and L-valine as for the *ortho*-nitro isomer, mp = 73.6°C.

o-*nitrobenzyl L-valine* p-*toluene sulfonate* ***5e***

Valine (117 mg, 1 mmol), *o*-nitrobenzyl alcohol (765 mg, 2.6 mmol) and *p*-toluenesulfonic acid monohydrate (570 mg, 3 mmol) were suspended in 10 ml of dry benzene in darkness and refluxed for 24 h in a Dean Stark apparatus with continuous removal of water. The black solution was evaporated, the solid residue washed with dry CCl_4 (to remove excess of nitrobenzyl alcohol) and dissolved in a minimum volume of dry methanol. Precipitation with dry ether gave a crude title compound which cristallized with a molar excess of pTSA and was purified by ebullition with Norit in dry ethanol and filtration of the hot solution to give a white solid. 136 mg (23%), mp 171.9°C. Rf 0.61 (Al_2O_3, EtOAc–MeOH:9–1, ninhydrine positive). IR (CH_2Cl_2) 1760 NMR (CD_3OD) 0.99 (d, J = 0.2 Hz, 3H), 1.05 (d, J = 0.2 Hz, 3H), 2.25 (m, 1H, $CH(CH_3)_2$), 2.30 (s, 6H, $ArCH_3$) 4.0 (d, J = 4 Hz, CH), 4.82 (s, 4H, NH_3^+ + H^+) 5.60 (s, 2H, CH_2), 7.1–8.1 (m, 12H, ArH). $[\alpha]^{25}$ + 24.5 (C 0.71, pyr). Anal calc for $C_{26}H_{32}N_2O_{10}S_2$: C, 52.33; H, 5.40. Found: C, 52.37; H, 5.37.

o-*nitrobenzyl phenyl ether* ***11***

A mixture of 15 ml of dichloromethane, 15 ml of water, 858 mg (5 mmol) of *o*-nitrobenzyl chloride 941 mg (10 mmol) of phenol, 400 mg (10 mmol) of sodium hydroxide, 200 mg of aliquat and 150 mg of potassium iodide was efficiently stirred at room temperature for 24 h. The organic layer was then separated and the aqueous layer extracted twice with 15 ml portions of dichloromethane. The combined organic extract was washed twice with 2 N sodium hydroxide and water. After drying with sodium sulfate, the solvent was evaporated and the residual solid was recristallized from ethanol. 710 mg (62%), mp = 62.5°C (lit 63 [58]; 58–59 [36]).

o-*nitrobenzyl pyridinium chloride* ***12***

A solution of *o*-nitrobenzyl chloride (343 mg, 2 mmol) and dry pyridine (174 mg, 2.2 mmol) in dry acetone (3 ml) were heated to reflux for 5 h. After cooling, the crystalline material was filtered, washed with dry ether and recrystallized from EtOH–acetone to give a hygroscopic white solid. 278 mg (55.7%) mp 183.3°C (lit [38] mp 183–184°C). NMR (CD_3OD), 4.85 (s, 2H, CH_2), 7.5–9.3 (m, 9H, ArH).

Antitumor assays

Male Swiss mice, average weight 25 g, were routinely carried in our laboratory. Crocker Sarcoma 180 tumor cells were inoculated (10^6/0.5 ml) ip. At 10 d, all animals had tumors and the mean survival time was 18.8 ± 1.4 d, and none of them survived longer than 28–30 d. *Corynebacterium parvum* (CP, Mérieux, Lyon, France) was used at a dose of 200 µg/mouse administered ip (0.1 ml/animal). Mouse interferon was prepared according to a method described by Dusseix *et al* [59] with a specific activity of 10^6 IU/mg of protein, was administered ip (0.5 ml containing 20 000 IU/mouse). The different compounds were injected by the IP route (0.5 ml of a 6×10^{-3} M/l solution per mouse).

At this low concentration, far from the lethal dose, no toxicity and no loss of weight were observed, except for the *o*-nitrobenzyl pyridinium chloride **12** which induced diarrhea and bristling of fur. The study period was 100 d following the graft of the 180TG cells and the mean survival time was

Table III. Nitrobenzyl esters.

	mp	*(mp lit)* °C	*Yield* %	*NMR* $CH_2(CDCl_3)$	*Analysis: found (calc)* C	H	O
5a	37.8	(35–36 [52])	69.5	5.45			
5b		oil	86	5.55			
5c		oil	87	5.55	59.34(59.24)	5.98(5.88)	28.45(28.57)
5d		oil	70		60.70(60.81)	6.25(6.38)	27.04(27.00)
1b'	78.5	(78 [53, 54])	40				
1b''		(35 [53])		5.20			

calculated accordingly. The chi-square test (with Yate's modification) was used to estimate the significance of the survival rate. Student's *t*-test was used to calculate the significance of the mean survival time (± 95% confidence interval).

Using 50 mice, we also studied the dose response per group of *o*-nitrobenzyl butyrate, which was 1 of the 2 compounds which gave maximal anti-tumor effect in the absence of any associated treatment. The mean survival time with 100 mmol was 24.2 ± 2.2 d; with 50 mmol, 22.5 ± 1.25 d, with 25 mmol, 22.8 ± 1.9 d, with 12 mmol, 22.3 ± 2 d and finally, with 6 mmol, 30 ± 0.2 d, thus showing that a selected concentration of 6 mmol was optimal.

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