PREPARATION AND BIOLOGICAL PROPERTIES OF A COVALENT ANTITUMOR DRUG–ARM–CARRIER (DAC CONJUGATE)

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1. Introduction

Cancer chemotherapeutic agents are not selective in their action against cancer cells. Therefore, the possibility of using a macromolecule, such as an antibody molecule, as a specific carrier of antitumor drugs has attracted considerable attention [1,2]. For instance, daunorubicin was covalently linked to antibodies [3–6] or to Fab dimers [7] or to a lectin [8] by coupling methods using glutaraldehyde, carbodiimide and periodate oxidation of the drug. For technical reasons, and because the activity of a drug is partially or totally lost when it is substituted or chemically modified, we devised a spacer arm such that the drug–carrier conjugate is stable in serum and can be specifically split by lysosomal proteases leading to the free drug inside the target cells.

2. Materials and methods

2.1. *Materials*

Daunorubicin–HCl was kindly provided by Rhône-Poulenc, Vitry-sur-Seine. D-Glucose, *N*-acetylglucosamine, CB, trypsin and WGA, prepared as in [9] were purchased from IBF-Réactifs, Pharmindustrie, Villeneuve-la-Garenne. L-Arginyl–L-leucine was purchased from Serva. Iodoacetic acid, *N*-hydroxysuccinimide and dicyclohexylcarbodiimide were from Aldrich. Di-*N*-methylformamide, from Merck, was refluxed over *N*-carbobenzoxyglycine-*p*-nitrophenyl-ester and distilled before use.

Solvent mixtures: (A) chloroform/methanol/water, 80:30:3, (by vol.); (B) chloroform/methanol/acetic acid, 6:3:1 (by vol.); (C) *n*-butanol/ethanol/acetic acid/water, 5:2:1:2 (by vol.).

2.2. *Preparation of the spacer arm: Glc–S-Et–Arg–Leu (VI)*

2,3,4,6 Tetra-*O*-acetyl-β-D-glucopyranosyl isothiouronium (I) [10] was allowed to react with sodium iodoacetate to form 2-(2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosyl)–ethanoate (II). Compound II was de-*O*-acetylated by treatment with sodium methoxide leaving 2-(1-thio-β-D-glucopyranosyl–ethanoate (III) of which the hydroxysuccinimide ester (compound IV) was prepared. Finally, compound IV was reacted with the dipeptide L-arginyl–L-leucine leading to compound VI (fig.1). Complete data about the synthesis of compound VI will be described elsewhere.

2.3. *Preparation of the spacer arm–drug conjugate: Glc–S-Et–Arg–Leu–D (VIII)*

Compound VI (518 mg, 1 mmol) was dissoved in

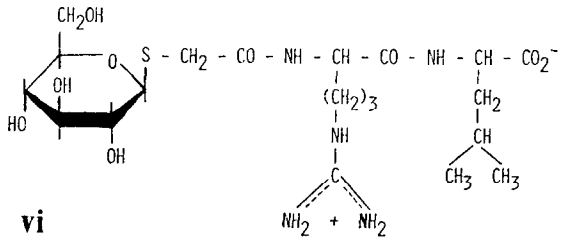


Fig.1. Structure of compound VI, 2-(1-thio-β-D-glucopyranosyl)–ethanoyl–L-arginyl–L-leucine (Glc–S-Et–Arg–Leu).

Abbreviations: A, spacer arm [2-(1-thio-β-D-glycopyranosyl)–ethanoyl–L-arginyl–L-leucine] (VI); D, daunorubicin; A–D, daunorubicin-substituted spacer arm; S-; succinyl; WGA, wheat germ agglutinin; SWGA, succinyl wheat germ agglutinin; CB, di-*N*-acetylchitobiose

di-*N*-methylformamide (4 ml) and *N*-hydroxysuccinimide (138 mg, 1.2 mmol) was added. The solution was cooled at 4°C and dicyclohexylcarbodiimide (250 mg, 1.2 mmol) was added. After 16 h, the precipitate of dicyclohexylurea was removed by filtration. To 1 ml solution of the hydroxysuccinimide ester (0.25 mmol) of compound (VI), was added daunorubicin–HCl (110 mg, 0.2 mmol) (VII) in di-*N*-methylformamide (1 ml); the solution was maintained at pH 9.0 by addition of triethanolamine. This solution was stirred at 25°C for 16 h and compound (VIII) was isolated by chromatography on a column (40 × 3 cm) of silica-gel using solvent mixture (A) (180 mg, 0.18 mmol, yield 90%).

2.4. *Preparation of a daunorubicin–arm–succinylated wheat germ agglutinin*

Compound VIII (180 mg, 0.18 mmol) was dissolved in 3 ml 0.2 M sodium acetate buffer (pH 5.0). Sodium periodate (128 mg, 0.6 mmol) was added and the solution stirred at 0°C, in the dark, for 6 h. KCl (100 mg, 0.57 mmol) was added, and the precipitate of potassium periodate removed by centrifugation.

The supernatant (850 μl) was added to a solution of wheat germ agglutinin (36 mg, 1 μmol) in 2.5 ml saturated sodium carbonate (pH 9.5). The solution was kept at 25°C for 6 h and then sodium borohydride (4 mg, 0.1 mmol) was added, and the solution was kept at 25°C for 1 h. The solution was brought to pH 4.5 by addition of acetic acid. The DAC was purified by affinity chromatography on immobilized *p*-aminophenyl-1-(thio-*N*-acetyl-β-D-glucosaminide) [9].

The DAC was succinylated twice under the conditions in [11] and the SWGA–A–D was dialized against distilled water and freeze dried.

2.5. *Effect of D–A–SWGA on L 1210 cells*

Mouse leukemia L 1210 cells, derived from in vivo passaged ascites tumor lines in DBA/2 mice, were grown in vitro in RPMI 1640 medium (Gibco) supplemented with L-glutamine and 10% heat-inactivated foetal calf serum (Rehatuin, IBF-Réactifs, Pharmindustrie) and 1% antibiotic and antimycotic solution (Gibco). The doubling time of exponentially-growing cells was 12 h.

2.5.1. Cytotoxic activity

Cells were suspended in RPMI medium supplemented with foetal calf serum at 8×10^5 cells/ml and dispensed into the wells of a flat bottom microtiter plate in 50 μl aliquots. The drug solution in the growth medium (50 μl) was added and the plates were incubated at 37°C in a 5% CO_2 atmosphere incubator for 4 h or 24 h. The cytotoxicity was estimated by erythrosin or trypan blue uptake and exclusion.

2.5.3. Fate of the SWGA–A–D

Cells (2×10^6) in 100 μl phosphate-buffered saline were incubated at 37°C in the presence of daunorubicin and of the various peptide and protein derivatives for 30 min, 1 h, 2 h, 4 h or 8 h. At the end of the incubation, the supernatant was collected and cells were washed 3 times with PBS (1 ml). The aqueous supernatant was extracted with 0.3 ml *n*-butanol, 3 times. The organic extracts were concentrated to dryness, redissolved in methanol and analyzed by thin-layer chromatography (solvents A,C). The spots are localized under UV light, and were scraped off from the plate. The compounds were extracted from the silica-gel using the solvent mixture (B). The various organic extracts were concentrated to dryness and the residues were dissolved in methanol (1 ml). The concentration of daunorubicin was assessed by spectrofluorimetry, λ_{exc} = 495 nm, λ_{em} = 595 nm.

The cell pellets were extracted with 1 ml chloroform/methanol (2:1, v/v), 3 times. The organic compounds were separated and analyzed as above.

Trypsin hydrolysis was carried out at pH 8.5 in 0.05 M Tris–HCl, 0.15 M NaCl buffer (2 μg trypsin/ml).

2.6. *Fluorescence microscopy*

L 1210 cells (5×10^6 cells/ml) were incubated with daunorubicin or its derivatives (3 μg free or bound daunorubicin/ml) for 30 min, 1 h, 2 h and 4h at 4°C and at 37°C. Cells were washed 3 times with cold PBS. The cells were examined with a Zeiss microscope equipped with epifluorescent illumination and filters setting for rhodamine fluorescence.

3. Results

Compound (VI) 2-(1-thio-β-D-glucopyranosyl)–ethanoyl–L-arginyl–L-leucine] (fig.1) is readily soluble in aqueous buffer. Compound VI on silica-gel thin-layer chromatography is detectable with Saka-

guchi's reagent showing the presence of an arginine residue, and, after treatment with periodic acid, with a benzidine reagent showing the presence of the sugar residue. Compound VI was found to be very easily hydrolyzed and the free leucine and 2-(1-thio-β-D-glucopyranosyl)–ethanoyl–L-arginine were detected as the only products by thin-layer chromatography.

Compound VIII which is readily soluble in aqueous buffer was shown to be homogeneous by thin-layer chromatography with solvents A–C.

Upon treatment with trypsin, compound VIII was split into 2-(1-thio-β-D-glucopyranosyl)–ethanoyl–L-arginine and L-leucyl–daunorubicin which were identified, using controls, by thin-layer chromatography.

After periodate treatment the oxidized compound VIII reacted with WGA and the reaction was monitored by thin-layer chromatography. The WGA–D purified by affinity chromatography, was found to contain 1.3 ± 0.2 daunorubicin molecule/protein molecule. This result was obtained by using the equation:

$$n = \frac{2.3 \times A_{495}}{A_{250} - 4.75\, A_{495}} \qquad (1)$$

where n is the number of daunorubicin molecules bound/protein molecule, 2.3 is the ratio of the molar absorbances of daunorubicin at 495 nm ($\epsilon^{D}_{495} = 10\,200$) and of wheat germ agglutinin at 250 nm ($\epsilon^{M}_{250} = 23\,400$) and 4.75 is the ratio of the molar absorbances of daunorubicin at 250 nm and at 495 nm. When the daunorubicin was >1.5 mol/mol the substituted protein was insoluble.

WGA–A–D as well as its succinylated derivative bind to immobilized *N*-acetylgluocosaminide and agglutinate human red blood cells and L 1210 cells at as low as 10 μg/ml.

The binding and the uptake of SWGA–A–D (100 μg SWGA–A–D containing 3 μg bound daunorubicin) was monitored by fluorescence microscopy. After 30 min at 4°C or at 37°C, cells were membrane labelled. After 1 h at 37°C, the fluorescence was mainly present as clusters. After 2 h, cells became leaky. The binding and the uptake of SWGA–A–D was totally prevented by incubation in the presence of 0.1 M di-*N*-acetylchitobiose. Cells incubated in the presence of Glc–S-Et–Arg–Leu–D (3 μg bound daunorubicin/ml) were very weakly labelled; cells incubated with free daunorubicin (3 μg/ml) were slowly labelled; the fluorescence intensity reached a plateau after an incubation of several hours.

These observations are in good agreement with the quantitative measurements of the uptake of SWGA–A–D, of daunorubicin and of its derivatives (tables 1,2). As shown in table 1, the concentration of free daunorubicin present in the supernatant, estimated

Table 1
Time course analysis of total daunorubicin concentration in the supernatant of L 1210 cells incubates, expressed as % of the initial value

Drug	Incubation (h)					
	0	0.5	1	2	4	8
D	–	53	47	25	14	4
A-D	–	100	90	88	53	40
SWGA–A–D(a)	100	57	47	50	50	90
(b)	0	2	4	8	45	90

(a) Total concentration of daunorubicin derivatives; (b) Concentration of daunorubicin derivatives extractible by *n*-butanol; L 1210 cells (1 × 10^6 cells/ml) were incubated in the presence of 3 μg free or bound daunorubicin/ml. Results are mean values from 3 individual expt.

Table 2
Relative composition of daunorubicin and daunorubicin derivatives present in the cell pellets as a function of the incubation time

Drug	R_F	Incubation (h)				
		0.5	1	2	4	8
D	0.25	40	50	70	80	90
	0.7	0	0	0	0	10
A–D	0.25	0	0	0	0	0
	0.65	1	5	10	40	50
	0.75	0	0	0	0	20
SWGA–A–D	0.25	20	25	30	20	20
	0.65	24	40	34	20	14
	0.70	0	0	0	2	4
	0.75	0	0	4	8	20

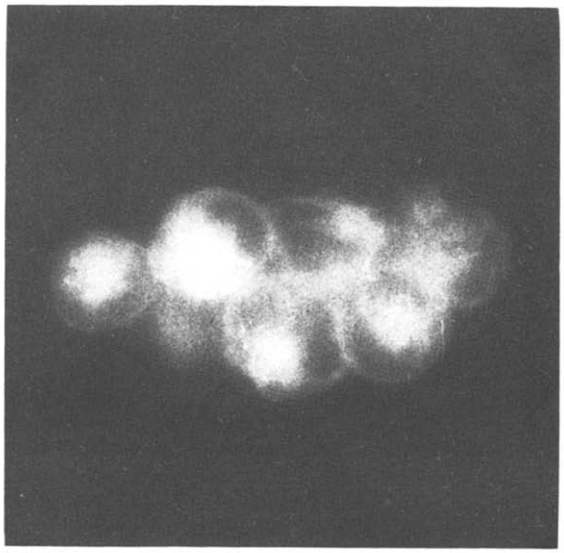
L 1210 cells (1 × 10^6/ml) were incubated in the presence of 3 μg free or bound daunorubicin/ml. Cells were collected by centrifugation and washed 3 times with phosphate-buffered saline. The pellet was extrated with chloroform/ethanol (2:1, v/v). The organosoluble compounds were analyzed by thin-layer chromatography using solvent A. Results are mean values from 3 individual expt.

by spectrofluorimetry, decreased continuously. The concentration of Glc–S-Et–Arg–Leu–D decreased also, but much more slowly. The supernatant concentration of daunorubicin in the case of SWGA–A–D was mainly due to protein-bound daunorubicin during the first hours; the concentration of organosoluble daunorubicin increased and became the only form present at 4–8 h incubation.

Upon incorporation of SWGA–A–D into the cells, daunorubicin was released within the cells as free daunorubicin (R_f 0.25, solvent C), as well as an organosoluble derivative having R_f 0.65 similar to that of Glc–S-Et–Arg–Leu–D, and an organosoluble derivative having R_f 0.75 similar to that of leucyldaunorubicin. It is noticeable that cells incubated in the presence of Glc–S-Et–Arg–Leu–D did absorb the drug but that no trace of free daunorubicin or of leucyl daunorubicin was detected within the first 4 h incubation.

Finally, the cytotoxic effect of SWGA–A–D was assessed by measuring the trypan blue exclusion from cells incubated in the presence of daunorubicin, the lectin and their derivatives (table 3). The minimal concentration of SWGA–A–D expressed as bound daunorubicin required to give a similar cytotoxic effect is found to be lower than the concentration of free drug, especially in the case of short time incubation. When used at 3 μg bound daunorubicin/ml the Glc–S-Et–Arg–Leu–D and SWGA–A–D in the presence of 0.1 M di-*N*-acetylchitobiose were not cytotoxic.

A



B

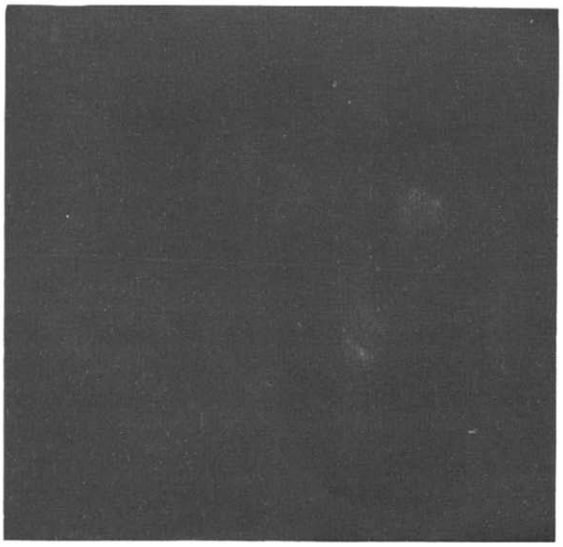


Fig.2. Fluorescence photomicrographs of L 1210 cells incubated in the presence of: (A) SWGA–A–D (100 μg/ml) for 1 h at 37°C; (B) SWGA–A–D (100 μg/ml) and 0.1 M di-*N*-acetylchitobiose for 1 h at 37°C.

Table 3
Cytotoxicity of daunorubicin and derivatives for L 1210 cells after 4 h and 24 h incubation

Form	Drug (μg/ml)[a]	Viability (%)	
		4 h	24 h
–	0	100	100
D	0.09	100	100
	0.4	100	46
	3	96	45
A–D	3	100	100
	10	95	86
SWGA + A–D	3	100	100
SWGA–A–D	0.09	89	73
	0.4	63	42
	3	44	12
SWGA–A–D + CB	0.09	100	100
	0.4	100	100
	3	100	86
SWGA	[b]	100	80

[a] Expressed as daunorubicin concentration; [b] concentration of SWGA (100 μg/ml) corresponding to that of 3 μg daunorubicin in SWGA–A–D
Results are mean values from 3 expt.

4. Discussion

The activity of a cytotoxic drug in vitro is mainly based on the capability of the drug to enter the cell and to act specifically inside the cell. The penetration of a drug into a cell depends upon several parameters such as hydrophilicity, hydrophobicity, solubility and presence of charges. To increase the activity of a drug one or several properties may be changed by chemical modification. However, such chemical modification may induce a decrease of the activity at the level of the target reaction inside the cell [12,13]. The penetration of a drug may be greatly facilitated if the drug is bound to a carrier which is easily internalized [2–8]. In order to keep the activity of the drug as high as possible, the use of a peptide as a spacer arm between the drug and the carrier seemed to be convenient. Because of the known activities of lysosomal proteases [13], a peptide containing an arginine residue was chosen. Because leucyl–daunorubicin is known to be as active as free daunorubicin, the arginyl–leucine dipeptide seemed to be convenient. Finally, compound, VI was selected to test the hypothesis of the usefulness of a peptide spacer arm. WGA could not be used as protein carrier, because this lectin is cytotoxic by itself. However, SWGA, which has many properties in common with the unsubstituted lectin, is not cytotoxic at all and so was a suitable carrier. The number of daunorubicin molecules bound to each protein molecule could not exceed 1.5 mol/mol because highly substituted WGA is insoluble. Glc–S-Et–Arg–Leu–D is incorporated into cells very slowly; furthermore its degradation is still slower, no trace of splitting products are found in cells within 4 h. These results together with the lack of cytotoxicity at $\leqslant 3$ μg/ml suggest that the Glc–S-Et–Arg–Leu–D does not meet the specificity requirements of the hydrolytic enzymes.

SWGA–A–D binds readily to the lectin receptors of the cell surface at 4°C as well as at 37°C. This binding is reversed by the presence of di-*N*-acetylchitobiose, a specific inhibitor. When cells are incubated in the presence of SWGA–A–D and di-*N*-acetylchitobiose, no cytotoxic effect was observed. At 37°C, SWGA–A–D is rapidly internalized, and free daunorubicin as well as a small M_r derivative are found inside the cells within 30 min. After several hours, a part of the free daunorubicin is found also in the supernatant, and after 8 h, the supernatant does not contain any more daunorubicin bound to the carrier. It is interesting to notice that the cytotoxic activity of SWGA–A–D is higher than that of free daunorubicin, when assayed after 4 h incubation, and that the cytotoxic activity of both free daunorubicin and SWGA–A–D is similar after 24 h incubation. These results suggest:

(i) That the carrier increases the uptake of the drug by inducing an endocytosis process; and
(ii) That the internalized carrier is promptly hydrolyzed, releasing free daunorubicin and small molecular weight derivatives.

So, the covalent binding of daunorubicin to a protein carrier via a simple peptide spacer arm allows one to introduce the fully active drug inside the cells. In vivo experiments based on this drug–arm–carrier system require carriers specific for cancerous cells, such as monoclonal antibodies [14,15]. The preparation and study of monoclonal antibodies substituted with daunorubicin and other drugs via the above described peptide (DAC conjugate) may provide a new immunochemotherapeutic agent with very specific and high antitumor activity.

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