L-CARBORANYLALANINE SUBSTITUTED TMV, A HIGHLY BORON LABELED VIRUS AS A MODEL FOR SLOW NEUTRON THERAPY OF TUMORS*

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SUMMARY

The use of the newly synthesized 10-boron atoms containing amino acid L-o-carboranylalanine (Car) as a useful tool in slow neutron therapy of tumors is proposed and its attempted application with a Car-substituted Tobacco mosaic virus (TMV) is described and discussed. Also the synthesis of ^{10}B-enriched decaborane as a precursor of Car is described.

L'utilisation de la nouvelle acide aminée L-o-carboranylalanine (Car) contenant 10 atomes de boron comme agent dans le traitement de tumeurs avec des neutrons thermiques est proposée. L'application sur un TMV substitué avec "Car" est rapportée et discutée. En outre, la synthèse de décaboron enrichie en ^{10}B comme précurseur de Car est décrite.

Die Verwendung der kürzlich synthetisierten 10 Boratome enthaltenden Aminosäure L-o-Carboranylalanin (Car) als leistungfähiges Mittel in der Therapie von Tumoren mit thermischen Neutronen wird vorgeschlagen. Die versuchte Anwendung an einem Car-substituierten TMV wird beschrieben und diskutiert. Im weiteren wird die Synthese von ^{10}B-angereichertem Dekaboran als Vorläufer von Car beschrieben.

KEY WORDS: Boron, Decaborane-^{10}B, TMV, Slow Neutron Therapy.

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Introduction

The boron-isotope ^{10}B is a very strong slow neutron absorber and subsequent α-emitter. This amazing property has led during the past 40 years to many attempts to find an application for this isotope in cancer therapy. In spite of these efforts, no therapeutically satisfying solution has been found until now. The major problems encountered: lack of specificity (boron ratio in tumor tissue vs normal tissue), insufficient boron content, and high toxicity of the boron compounds (1).

The great advantage of a successful application of the slow neutron therapy is that a non-radioactive carrier is introduced which binds specifically to target cells. There it can be triggered by slow neutrons to emit α-radiation, destructing the target cells without damaging other tissue.

The isotope ^{10}B, which occurs up to 18.45% in natural boron, reacts with thermal neutrons in a (n, α)-reaction:

$$^{10}B + {}^{1}n \rightarrow {}^{7}Li + {}^{4}He + 2.79\ MeV \quad 6.3\%$$

$$^{10}B + {}^{1}n \rightarrow {}^{7}Li^{*} + {}^{4}He + 2.31\ MeV \quad 93.7\%$$

$$^{7}Li^{*} \longrightarrow {}^{7}Li + \gamma + (0.48\ MeV)$$

The nuclear cross section σ of ^{10}B for slow neutrons is very high : 3,800 barns (1 barn = 10^{-24} cm^2), whereas common elements range between 32.5 (Cl) and 0.0042 (C) barns (1).

To apply this technique to a simple living system, *Tobacco Mosaic Virus* (2) (TMV, wild type) was modified with Car and the still active virus was exposed to thermal neutrons in order to inactivate the virus.

TMV was selected because of its availability, high chemical stability, simple test methods, simple purification and large quantities (3).

Results

In order to raise the content of ^{10}B in later preparations of Car or similar compounds, decaborane enriched in ^{10}B has been synthesized. Boron trifluoride with 85% ^{10}B content was reduced with lithium aluminium

hydride to diborane which subsequently was pyrolyzed to decaborane (4, 5).

$$BF_3 \xrightarrow{LiAlH_4} B_2H_6 \xrightarrow{\Delta t} B_{10}H_{14}$$

For boron substitution of TMV, a Car containing diazonium reagent was prepared capable of coupling specifically with Tyrosine Histidine and to some extent to Lysine side-chains. Phtalyl protected Car was esterified to give the N-hydroxisuccinimideester (6) (Pht·Car·OSu). This active ester was coupled with p-nitrophenylalanine (4'-NO_2)Phe, yielding the N-protected dipeptide Pht·Car-(4'-NO_2)Phe-OH. Hydrogenation followed by diazotation gave Pht·Car-(4'-N_2^+)Phe-OH which was coupled in aqueous solution to native TMV.

After several purification steps, a soluble, fully active orange TMV was obtained. The substitution was 4.51 Car/subunit or 10'000 Car/TMV, as shown by elementary analysis. Non-covalent incorporation has been ruled out by electrophoretic control. Amino acid analysis was unsuitable because Car does not appear in a normal amino acid spectrum.

Cross section calculations show that substituted TMV in solution requires high slow neutron doses which readily inactivate native TMV. Using lyophilizate instead of solutions, the cross section increases drastically because the nuclear fission products can now hit viruses up to 10μ apart. The volume of the capture cell is at a radius of 10μ: $4.2 \cdot 10^3$ μ^3 and contains $4 \cdot 10^7$TMV. The cross section σ thus is: σ_{TMV} $.4 \cdot 10^7$ barn = $2.8 \cdot 10^{-9}$ cm^2. To inactivate a virus, the RNA has to be cut or destroyed, so only RNA hits are to be considered. The RNA content is 5.7% in TMV, there are $4 \cdot 10^7$ virus per cell and one ^{10}B-fission is producing $7.5 \cdot 10^7$ ionizations, resulting in a required flux of $3.4 \cdot 10^{12}$ (n/cm^2).

Native TMV was rather stable against thermal neutrons up to 10^{15} n/cm^2 from a reactor when contaminating γ and fast neutrons were filtered off. Irradiation of Car substituted TMV with $1.6 \cdot 10^{15}$ n/cm^2 (500 times the calculated dose) gave products which had the same virulence as non-irradiated substituted or irradiated non-substituted samples. A tendency to lesser aggregation in irradiated samples was observed (Table).

ACTIVITY TEST

TMV	Dilution	1:10	1:50	1:250
Native, non-irradiated		16	14	5
Native, irradiated		44	12	1,5
Substituted, non-irradiated		51	24	6
Substituted, irradiated		118	35	5

The activity is expressed as necrosis spots per tobacco leaf and an average of two TMV probes is taken. Each point consists of twice three plants of about three leaves (see experimental).

Discussion

This failure may be due to the decreasing sensitivity of TMV towards increasing ionizing power of the radiation, whereas tumor cells behave inversely (7). TMV RNA fragments seem to reaggregate back to active viruses. Since the α- and ^{7}Li particles have a range = 10μ (8) in tissue and since the whole energy is deposed in about 75,000 ionization per decay, it is reasonable to assume that the energy of a single ^{10}B-decay of 2.3 MeV provokes death of a tagged or an adjacent cell. Assuming that on the average each cell of the tumor has to suffer one decay, one can calculate the required boron content per gm of tissue at a given neutron flux. Since thermal neutrons have serious radiobiological effects, the flux is limited by the maximum tolerated whole body irradiation dose. Depending on the seriousness of the tumor, irradiation may be up to 25 rem. The action of thermal neutrons comes mainly from the $^{14}N(n, p)^{14}C$-reaction (9), which gives at a flux of 10^{10} n/cm^2 25 rem whole body dose.

Taking the above considerations into account, one can estimate a boron requirement of 10^{10} ^{10}B/cell or 20-100 mg/kg tumor. This is in good agreement with *Javid et al* (10) who have calculated that in tissue containing 50 mg ^{10}B/kg, 86% of the total radiation dose results from the (n, α) reaction. Carboranylalanine (Car) (6) is particularly attractive for this purpose since it contains 10 boron atoms per molecule. Accordingly, 5 x 10^9 amino acids are required per cell for natural boron or 10^9 Car enriched in boron 10. However, one cell may contain only up to 10^4 receptor sites for hormones or

antibodies (11). A high boron charge of 5 x 10^5 per single carrier molecule would be needed. Carriers which have been considered include tumor specific antibodies (12), and to a lesser extent, peptide hormones i.e. for melanoma. However, until now all efforts to label an antibody with the requested amount of boron have failed due to insolubility of the modified antibody (13) or too low boron substitutions (14).

Also toxicological problems have to be considered; boron compounds in general are very toxic (1). In contrast, Car seems to have a low toxicity (15). The use of Car by the method of *Fuchs and Sela* (16), and *Mallinger et al* (14) could yield such a soluble antibody. Studies to this extent are presently in progress in our laboratory. For further biological experiments, we intend to use cell cultures or animals, i.e. transplantable rodent tumors and their specific antibodies, similar to the work of *Mishima et al* (17).

Experimental

Activity tests of TMV were done at the "*Landwirtschaftliche Forschungsanstalt Reckenholz*" Zürich, Switzerland. TMV samples were dissolved in 3 ml water and smeared on corund powder pretreated leaves of 6 weeks old Tobacco seedlings ("*nicotinea glutinosa*"). After five days, necrosis spots were counted and the virus activity as spots per leaf expressed. TMV has been prepared by the method of *Van Wechmar* and *Van Regenmortel* (3) and was stored as standard solution containing 25.2 mg TMV/ml in 0.125 mMolar phosphate buffer pH 6.8 at 4.0°C. TMV was verified after each step by electron microscopy and only intact fractions were used. Electron microscopic work was done at the Institute of Cellular Biology, ETH Zürich. The microanalytical laboratory "*Alfred Bernhardt*" D-5251 Elbach über Engelskirchen, G.F.R. carried out the boron elementary analysis. Mass-spectroscopic measurements were conducted on a HS-30 double beam instrument (AEI Co., England) from the Nuclear Medical Department of the University of Sherbrooke. All reagents used were reagent grade from Fluka AG Buchs, Switzerland, if not otherwise noted.

Pht·Car-(4'-NO_2)Phe·OH: 79 mg Pht·Car·OSu (MW 459.54; 0.172 mMol) (6) were dissolved in 3 ml dry dimethylformamide (DMF), solid (4'-NO_2)Phe (MW

210.2; 0.2 mMol) and 23 mg N-methylmorpholine was added. The reaction was left in the thawing ice-bath and stirred overnight. DMF was evaporated, the residue redissolved in ethylacetate and washed with potassium sulfate-bisulfate pH_2-buffer. The organic phase was dried over anhydrous sodium sulfate and evaporated. Recrystallization from isopropanol/water gave 80 mg (MW 553.60; 0.145 mMol) product in 84% yield. Thin layer chromatography (TLC): three migrations in $CHCl_3$/MeOH/AcOH = 100·5 3, Merck Silicagel precoated plates, detection by UV and I_2. Rf starting material 0.31, product 0.35, ninhydrine negative. NMR 60 mHz: (Varian T60, recorded in $CDCl_3$, TMS standard, Chemical shift is δ in ppm, b = broad, s = singlet, d = dublet, m = multiplet); δ = 9.1 (bs, IH,-COOH); 7.9 (bs, 4H, Pht); 7.2 - 8.1 (m, AA'-BB' System, 4'-NO_2-aromate); 6.8 (bd, 1H, Amid-H). Melting point: 146.3°C.

Pht·Car-(4'-NH_2)Phe·OH: 68 mg Pht·Car-(4'-NO_2)Phe·OH (MW 553.6; 0.123 mMol) were hydrogenated in 5 ml methanol at room temperature and normal pressure with a spatule tip of 10% palladium on charcoal during one hour. The product was filtered off from the catalyst, evaporated and dissolved in 1 ml glacial acetic acid. TLC gave one spot, Rf 0.08, ninhydrin coloration pink. The substance was not further characterized and directly used for the next step.

Substitution of TMV: 9.0 mg sodium nitrite (0.013 mMol) were dissolved in 10 ml 2N hydrochloric acid and chilled to 0°C. The solution of the previous step was added under vigorous stirring, a precipitation was redissolved by adding a further 3 ml of acetic acid. After one hour, the yellowish solution was filtered off, excessive nitrite was destroyed with sulfamic acid and the mixture was neutralized to pH8 with sodium bicarbonate solution. To this diazonium solution was slowly added 51.14 mg TMV in 2 ml standard solution. The mixture was rotated at room temperature for six hours, followed by twenty hours dialysis against 20 l water with one change. The turbid solution was centrifugated at 20,000 rpm, the clear orange upper layer diluted to 100 ml and the pellet discarded. Addition of 4 g Carbowax and sodium chloride followed by 10 min centrifugation at 10,000 rpm gave a

TMV pellet. This TMV was redissolved in 10 ml water and dialyzed for three days against 10 l water at 4°C with three changes to yield the saltfree standard solution of modified TMV.

Protein content (Lowry): substituted TMV: 1.3 mg ^{TMV}/ml. An equally treated native TMV solution gave 3.85 ^{mg}/ml. Boron content in lyophilized substituted TMV: 2.82% = 4.57 ^{Car}/subunit or about 10,000 ^{Car}/TMV.

Irradiation experiments - Neutron irradiations of TMV have been carried out in a D_2O moderated reactor *"Diorit"* of the *Eidgenössisches Institut für Reaktorforschung (EIR) in Würenlingen, Switzerland.* The stability of unmodified TMV has been estimated with various γ and fast neutron contaminations from *"Diorit"* and the *EIR* swimming pool reactor *"Saphir"*. TMV was stable only against rather pure thermal neutrons filtered through 10 cm lead up to a flux of 10^{15} (^{n}/cm^2) and rapidly inactivated above 10^{16} (^{n}/cm^2). 500 µg TMV samples were lyophilized into 1 ml polyethylene tubes. Modified and unmodified TMV samples, two of each, were exposed during two days to a dose of $1.6 \cdot 10^{15}$ ^{n}/cm^2 and afterwards their viral activity was measured.

Synthesis of ^{10}B enriched decaborane - NOTE: - This work is extremely dangerous, if air contaminates the substance, vigorous explosions are inevitable. All work has to be carried out behind explosion shields and greatest care has to be taken (4, 5, 18).

Synthesis of ^{10}B-diborane: - 2 l boron trifluoride (80 mMol) 85% ^{10}B (from EIR Würenlingen, Switzerland) at 700 torr in 1,000 ml glass tubes were condensated in liquid nitrogen, opened under nitrogen and 20 ml of dry diglyme were added. The tubes were evacuated, closed, warmed up to room temperature and the pressure was adjusted with nitrogen. The brown diglyme-borontrifluoride solutions were combined and the tubes washed with 2 ml of diglyme. This solution was added, over a period of one hour, to 3 g lithium aluminium hydride (80 mMol) in a nitrogen flushed no-air apparatus equipped

with a 250 ml flask with coolfinger (diglyme condensation), magnetic stirrer and three subsequent cooltraps. The last cooltrap was filled with glass wool to avoid solid diborane to fly into the vacuum pump. The coolfinger was kept to -25°C and the traps in liquid nitrogen. After an additional hour at plus 20°C, the reaction vessel was heated up to 60°C for 30 min. The cooltrap chain was separated from the flask and evacuated to 1 torr for purification. To the residue in the flask still under nitrogen, 50 ml of ethanol was added over a period of 30 min to destroy residual hydride. Afterwards, the content was rejected. The first cooltrap was heated up to -75°C and diborane distilled in the second trap, then the first trap was disconnected. To the closed second cooltrap, containing ^{10}B-diborane, a 100 ml steel autoclave with manometer was connected, flushed several times with nitrogen, evacuated and cooled down to -200°C. The diborane was distilled into the autoclave during 30 min, evacuated to 1 torr, and the valve closed. Warming-up to room temperature gave, after three equal syntheses, 22 atm = 93 mMol ^{10}B-diborane or 80% yield.

Synthesis of ^{10}B-decaborane from ^{10}B-diborane: the method of *de Acetis et al* (5) was tested and found unsuitable. A new pyrolysis apparatus had to be developed consisting of a 500 ml cylindric flask with a greaseless 34/45 female joint with Teflon-Viton sealing on top and a similar 28/15 spherical joint on the upper side. Through the first, a water jacketed coolfinger was placed in the reaction vessel and, on the side arm, a vacuum line with six stopcocks. The apparatus was flushed with nitrogen and evacuated to 1 torr. The following filling has led to acceptable results: 50 torr dimethylether (catalyst), 200 torr diborane, 250 torr nitrogen. A 100°C hot oil bath was carefully lifted to the flask until totally immersed and slowly heated to 160°C during one hour, keeping the pressure at abqut 700 torr. The reaction flask was cooled to 20°C and the content evacuated into three subsequent cooltraps in liquid nitrogen to a residual pressure of 5 torr. The flask was refilled with the mentioned mixture and this operation was repeated five times. The coolfinger was carefully extracted and the white layer

washed off with ethanol into a sublimation apparatus. The ethanol was evaporated and the residual borane sublimated in a 50 torr nitrogen atmosphere overnight.

In order to work up the content of the cooltraps, diborane was distilled back into the reaction vessel by heating the cooltrap to -75°C. The recovered diborane was processed as described above. The residue in the cooltraps contained dimethylether and lower boranes. The latter are extremely explosive and were flushed with nitrogen into the vent. Overall yield of decaborane $B_{10}H_{14}$ was between 16 and 28%. MS: M^+ peak at 118 - 110, boronic acid $B(OH)_3$ (decomposition in the ion source) at 62/61. 62/61 ^{11}B: ^{10}B = 15:85. Normal decaborane gave boronic acid; 62/61 = 85:15 .

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