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BIODISTRIBUTION AND TOXICITY OF 2,4-DIVINYL-NIDO-*o*-CARBORANYLDEUTEROPORPHYRIN IX IN MICE

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Abstract—BALB/c mice with transplanted subcutaneous KHJJ mammary carcinomas were given 2,4-divinyl-nido-*o*-carboranyldeuteroporphyrin IX (VCDP), a prospective boron carrier for boron neutron-capture therapy, to determine the dose schedule that results in maximal boron uptake in tumor. A total dose of 270 ± 10 μg/g body weight given in a 4-day multiple intraperitoneal injection schedule (3/day) resulted in 30–50 μg boron/g tumor. After such a dose, thrombocytopenia, granulocytosis and altered liver enzyme levels were measured in the blood. Blood boron clearance was followed for an 18 hr to 6 day post-injection period. Toxic effects of VCDP subsided within 4–6 days after the last injection. In view of the >30 μg/g peak accumulation of boron in tumor from VCDP and the subsequent rapid reversal of VCDP toxicity, further studies of VCDP in small mammals relevant to its distribution, toxicity and potential clinical use for neutron-capture therapy of tumors appear warranted.

Boron neutron-capture therapy (BNCT‖) is based on the $^{10}B(n,\alpha)^{7}Li$ nuclear reaction, whereby densely ionizing charged particles (α and ^{7}Li) are produced from the capture of slow neutrons by ^{10}B [1]. BNCT is a bimodal radiation therapy in that both boron-10 and neutrons must be present for cell-killing to occur. Among the criteria that must be met for BNCT are: (1) the drug must deliver boron to tumor tissue in concentrations greater than 15 μg ^{10}B/g tissue at the time of irradiation [2]; (2) drug toxicity is tolerable and transient; and (3) there is preferential uptake of boron in targeted tissues within the irradiated volume. The presence of boron in unirradiated tissues is not critical. However, because of the ubiquity of blood, the tumor:blood boron concentration ratio is an important factor in BNCT.

Porphyrins accumulate and persist in tumors, a property that enables them to be used for photodynamic therapy [3]. Boronated porphyrins share this property, and are cited [4] as potentially more useful for BNCT than are $Na_2B_{12}H_{11}SH$ monomer [5], dimer [6] and *p*-dihydroxyboronylphenylalanine [7, 8], three boron carriers for BNCT that do not persist in tumor as long as do porphyrins. A water-soluble, boronated natural porphyrin derivative, 2,4-divinyl-nido-*o*-carboranyldeuteroporphyrin IX (2,4-[2′,2′-dinido-*o*-carboranyl]protoporphyrin IX) (VCDP) (Fig. 1A), has been synthesized [9]. The purpose of this study was to determine the bio-distribution and toxicity of VCDP in mice which could then serve as a baseline for VCDP-based BNCT experiments. High doses of the compound were required to elicit substantial toxicity and lethality, which we deemed appropriate for a preliminary *in vivo* study of a new synthetic compound.

In Experiment I, the total dose and the dose rate were varied until maximum boron concentrations in tumor were achieved. The results of Experiment I, which include lethality data and blood boron concentrations from various VCDP dose regimens, were then evaluated so as to establish a convenient "standard experimental dose" (SED) for the succeeding Experiments II and III. The SED is, in effect, the dose that resulted in tumor boron concentrations of ≈40 μg B/g and a tumor:blood ratio of 4:1 at a lethality not exceeding 10% in mice.

Using the SED in Experiment II, the bio-distribution, pharmacokinetics and toxicity of VCDP in tumor-bearing mice were examined. In addition, a reproducible acute toxic dose (LD_{40}) of VCDP was compared to tetraphenylporphyrin tetrasulfonate (TPPS) (Fig. 1B) in tumor-bearing mice. In Experiment III, the toxic effects of VCDP administered to non-tumor-bearing mice at the SED level were evaluated after 2- and 4-day clearance periods and at half the SED level after a 4-day clearance period.

MATERIALS AND METHODS

Drugs. Preparation of the boronated porphyrin VCDP is described elsewhere [9]. The dried red powder was shielded from ambient light and stored

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§ This article is dedicted to Ralph Grandism Fairchild (1935–1990).

‖ Abbreviations: BNCT, boron neutron-capture therapy; PDT, photodynamic therapy; TPPS, tetraphenylporphyrin tetrasulfonate; QNCR, quantitative neutron-capture radiography; SED, standard experimental dose; VCDP, 2,4-divinyl-nido-*o*-carboranyldeuteroporphyrin IX; GLU, glucose, BUN, blood urea nitrogen; BCR, blood creatinine; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; TPR, total protein; ALB, albumin; CPK, creatine phosphokinase; LDH, lactate dehydrogenase; SODH, sorbitol dehydrogenase; and BILE, bile acids.

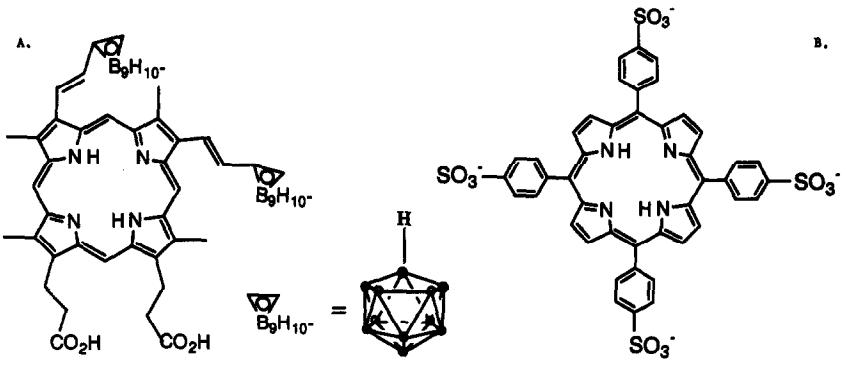

Fig. 1. Structures of (A) VCDP and (B) TPPS.

in dry air at room temperature until used. VCDP and TPPS (Strem Chemicals Inc., Danvers, MA) were dissolved not more than 2 days before injection in isotonic phosphate-buffered saline (PBS) or in 0.5% $NaHCO_3$ solution at pH 7. Injection solutions were shielded from ambient light.

Animals. Female 10–16-week-old BALB/c mice were purchased from Harlan Sprague–Dawley, Indianapolis, IN, were kept in a temperature-and light-controlled room, and received food and water *ad lib*. Mice were implanted subcutaneously with the KHJJ mammary carcinoma [10]. For these experiments two 1-mm^3 tumor fragments were implanted subcutaneously in the back of each mouse through an 18-gauge trocar. Drug administration was begun 10 days after tumor implantation, at which time tumors weighed 50–100 mg. The mice were shielded from light from the time of the first VCDP administration until they were ether-euthanized.

Right ventricular blood was collected from deeply anesthetized mice in Microtainer® (Becton–Dickinson, Rutherford, NJ) tubes containing lithium heparin for chemical analyses and boron determinations and in tubes containing EDTA for hematological analyses. The mice were then euthanized, and tumor, liver, kidney, spleen, lung and brain tissues were removed for boron analysis and/or histopathological study.

Blood plasma used for the *in vitro* study in Experiment III was obtained from 6–8-month-old CBA mice.

Chemistry and hematology. Glucose (GLU), blood urea nitrogen (BUN), blood creatinine (BCR), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total protein (TPR), albumin (ALB), creatine phosphokinase (CPK), lactate dehydrogenase (LDH), sorbitol dehydrogenase (SODH) and bile acids (BILE) were determined using a Cobas Fara II microanalysis system (Roche Diagnostic Systems, Nutley, NJ). Blood counts were determined with a Serono-Baker System 9000 (Serono-Baker Diagnostics Inc., Allentown, PA) automated hematology micro-analyzer. Differential leukocyte counts were determined by microscopic examination of stained blood smears.

Histopathology. Microscopic sections of liver, spleen and kidney (formalin-fixed, paraffin-embedded, 5 μm-thick, hematoxylin- and eosin-stained) were examined by the pathologist (D.N.S.) in experimental groups that were labeled only by code.

Boron analyses. Boron-10 assays were performed by prompt-gamma spectrometry [11]. All boron concentrations reported here are total boron concentrations which were assumed to be 5.4-fold greater than ^{10}B concentrations due to the use of boron of natural isotope abundance for the synthesis of VCDP. A background correction in the prompt-gamma method was carried out by assay of the tissues of mice that were given buffer solution only. Several extra mice were analyzed by quantitative neutron-capture radiography (QNCR) at each VCDP dose level [12].

Data analyses. Graphs were produced using Kaleidagraph™ (Abelbeck Software, Reading, PA). The Wilcoxon analyses using variable allowable percent difference in data were carried out using a new self-prompting computer program, which is listed in Fig. 2.

RESULTS

Experiment I. This preliminary study determined an experimentally convenient dose schedule (SED), which we define as a dose (total and rate) at which there is ≈40 μg B/g in tumor and minimal boron in blood (≈10 μg B/g) under the constraint that the lethality be less than 10% (see Figs. 3 and 4). The amount of VCDP per injection, the number of injections and the total dose were varied until an SED was found. For the mortality study, tumor-bearing animals received VCDP via multiple i.p. injections (2–3 per day for 2 or 4 days) at a rate of 11–46 μg VCDP/g body wt per injection.

Lethality was minimized (<10% mortality) by keeping the dose rate down to ≤25 μg/g body wt/injection. Total doses of >350 μg/g body wt (at 25 μg/g body wt/injection) given in 4 days resulted in ≈25% lethality, while a smaller total dose, 184 μg/g body wt, administered in larger increments (46 μg/g body wt/injection) given over 2 days, was invariably lethal. Thus, lethality appeared to be more dependent on the dose of porphyrin per injection than on the

Program WLCOXNPD [MS DOS 3.21/GW-BASIC 3.20]

```
10  PRINT"Program WLCOXNPD runs Wilcoxon two-sample tests corrected for an
    allowable percent difference between coupled data. Null data are not accepted."
20  PRINT"Any two numbers within two series of data being compared are ranked
    equal if they do not differ by more than an allowable percent difference."
30  DIM N(2),C(3),V(3,100),H(20,100),A(20),W(3,100):PRINT"Up to 20 sets of data, with
    up to 100 data per set, may be entered for possible testing.":FOR G=1 TO 20
40  PRINT "Enter the number of data in set #"G"(If all data sets have been recorded,
    enter 0)":A(G)=100:INPUT A(G):Q$ = "There are no more data to be recorded."
50  IF A(G)=0 THEN PRINT Q$ ELSE PRINT"There are"A(G)"data in set #"G
60  GOSUB 490: IF Z$="N" OR Z$="n" THEN 40
70  IF A(G)=0 THEN 140 ELSE 80
80  FOR I=1 TO A(G):PRINT"Datum"I"of set #"G:INPUT H(G,I):NEXT I
90  PRINT "Data set #"G"may now be reviewed for errors":FOR I=1 TO A(G)
100 PRINT "Datum "I" of set #"G" is " H(G,I):GOSUB 490
110 IF Z$="N" OR Z$="n" THEN 120 ELSE 130
120 PRINT "Datum #" I "of set#" G:INPUT "=" ;H(G,I):GOTO 100
130 IF H(G,I)=0 THEN PRINT"Null datum not accepted":GOTO 120 ELSE NEXT I:NEXT G
140 PRINT "Any pair of data sets may be tested by the modified Wilcoxon two-sample
    test.These are temporarily labeled series 1 and series 2":FOR I=1 TO 2
150 PRINT"Which data set# is temporarily labeled series"I"?":INPUT"Set # ";B(I)
160 PRINT"Series"I" is set #"B(I):GOSUB 490:IF Z$="N"orZ$="n" THEN 150
170 NEXT I:FOR Z=1 TO 2:N(Z)=A(B(Z)):FOR I=1 TO N(Z):W(Z,I)=H(B(Z),I)
180 NEXT I:NEXT Z:P=N(1):Q=N(2):S=P+Q:FOR J=1 to 2
190 FOR I=1 TO N(J):PRINT"Datum #"I"of series"J"is"W(J,I):NEXT I:GOSUB 510:NEXT J
200 FOR L=1 TO 2:FOR K=1 TO N(L)-1:X=W(L,K):FOR J=K+1 TO N(L)
210 IF W(L,J)<=W(L,K) THEN W(L,K)=W(L,J):W(L,J)=X:X=W(L,K)
220 NEXT J:NEXT K:NEXT L:FOR I=1 TO 3:FOR J=1 TO S:V(I,J)=W(I,J):NEXT J:NEXT I
230 PRINT"In the standard Wilcoxon test, the allowable percent difference % = 0"
240 INPUT"Enter allowable percent difference (default %=0), %=?",F:GOSUB 490
250 If Z$="N" OR Z$="n" THEN 230
260 FOR I=1 TO 3:FOR J=1 TO S:W(I,J)=V(I,J):NEXT J:NEXT I:T=0:R=0:D=Q
270 FOR I=1 TO P:FOR J=1 TO D:IF W(1,I)<=W(2,J) THEN 300
280 IF J=D THEN W(2,D+1)=W(1,I):W(3,D+1)=W(1,I)
290 NEXT J:D=J:NEXT I:GOTO 320
300 FOR K=D+1 TO J+1 STEP -1:W(2,K)=W(2,K-1):W(3,K)=W(3,K-1)
310 NEXT K:W(2,J)=W(1,I):W(3,J)=W(1,I):J=D:GOTO 290
320 PRINT"The data in series 1 are now listed in ascending numerical order:"
330 FOR I=1 TO P:PRINT"In series 1,the ordinal rank of"W(1,I)"is"I:NEXT I
340 GOSUB 510:FOR I=1 TO S:IF W(3,I)=0 THEN 360
350 PRINT"In series 1&2 combined, the ordinal rank of"W(2,I)"is"I
360 NEXT I:GOSUB 510:FOR I=1 TO S:IF W(3,I)=0 THEN 380
370 IF W(3,I)=W(2,I) THEN R=I:GOTO 400
380 NEXT I:PRINT"In series 1&2 combined, the corrected rank sum for series 1 is"T
390 PRINT"The allowable percent difference used for this result is"F"%":GOTO 440
400 FOR K=3 TO 2 STEP -1:C(K)=0:FOR J=1 TO S
410 IF ABS(W(3,I)-W(K,J))<=F*ABS(W(3,I)+W(K,J))/200 THEN C(K)=C(K)+1:M=J
420 NEXT J:NEXT K:B$="?":IF C(3)=C(2) THEN T=T+R ELSE R=(2*M-C(2)+1)/2:T=T+R
430 PRINT"In series 1&2 combined, the corrected rank of "W(3,I)" is "R:GOTO 380
440 B$="?":INPUT"Should the rank sum be calculated for a different allowable
    percent difference? (Y/N)",B$:IF B$="Y" OR B$="y" THEN 230
450 C$="?":INPUT"Should another couple of data sets be compared? (Y/N)",C$
460 IF C$="Y" OR C$="y" THEN 500
470 PRINT"Program execution has been halted":INPUT"To end, enter END then RETURN,
    otherwise enter RETURN:",Z$:IF Z$="END" OR Z$="end" THEN 480 ELSE 450
480 PRINT"Program execution has ended.":END
490 Z$="?":INPUT"If wrong,enter N then RETURN.Otherwise,only RETURN:",Z$:RETURN
500 FOR Z=1 TO 3:FOR I=1 TO S:W(Z,I)=0:NEXT I:NEXT Z:GOTO 140
510 PRINT"To continue, enter CONT then RETURN (or key F5) after Ok.":STOP:RETURN
```

Fig. 2. List of a self-prompting MS DOS 3.21/GW-BASIC 3.20 program named WLCOXNPD for handling data sets being compared in pairs by a variant of the Wilcoxon Two-Sample Test. Acceptance regions for rank-sums corresponding to significance limits of 0.10, 0.05, 0.02, 0.01, 0.005, 0.002, and 0.001 ($N_1 \leq 25$; $N_2 \leq 50$) have been calculated by W. Seewald, Swiss Federal Institute of Technology, Zurich (1977), and then tabulated in: *Geigy Scientific Tables* (Ed. Leitner C), 8th Edn, pp. 156–162. Ciba-Geigy Ltd., Basle, Switzerland, 1982. Program WLCOXNPD enables the user to correct the ordinal ranks of the data for virtual ("experimental") as well as for numerical ties when a certain percent uncertainty is attributed to the data, as in Tables 1–4. The corrected rank of a datum is computed by first identifying the uncorrected ordinal rank of each datum in the merged data set. The corrected rank of a datum is then the arithmetic mean of its own uncorrected ordinal rank and the uncorrected ordinal ranks of all other numbers within its "allowable" range in the merged data set. This range is defined by an "allowable percent difference" [F%, $0 \leq F < 200$; lines 240 and 410] which is such that the difference between the datum being rank-corrected and any other datum within the "allowable" range is less than an "allowable" percent of the arithmetic mean of the two data. This program reverts to the uncorrected Wilcoxon Two-Sample Test when zero is assigned to F, either deliberately or by default [line 240].

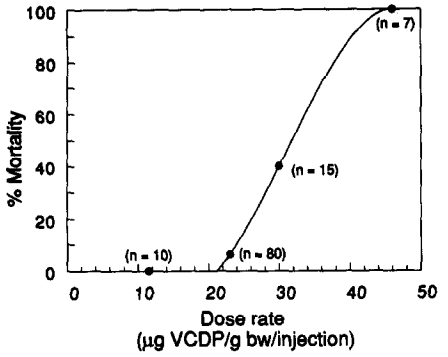

Fig. 3. Lethality of VCDP in tumor-bearing mice as a function of dose per injection. All mice given a lethal dose died during the 4-day period after the last injection. The curve was generated using least squares regression (third-order polynomial curve-fitting).

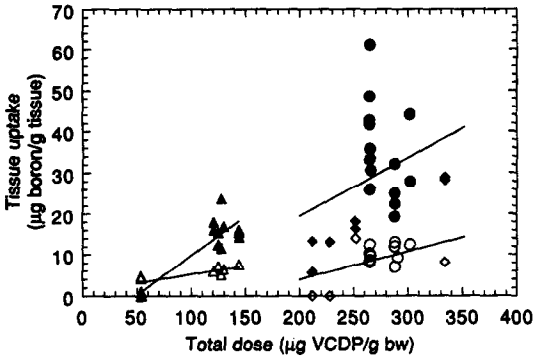

Fig. 4. Boron concentrations in individual tumor and blood samples from BALB/c mice as a function of total dose of VCDP. Key: (▲) tumor (3 injections/day for 2 days); (△) blood (3 injections for 2 days); (◆) tumor (2 injections/day for 4 days); (◇) blood (2 injections/day for 4 days); (●) tumor (3 injections/day for 4 days); (○) blood (3 injections/day for 4 days). The drug was administered via multiple i.p. injections (2–3 per day at 11–32 μg/g body wt/injection) for 2 or 4 days, and mice were euthanized 4 days after the last injection. The 4-day injection data were combined to generate the lines using first-order least squares regression.

total dose given (Fig. 3) at least over the range of doses studied. The LD_{50} dose was estimated to be 28.8 μg (26.6–33.1 μg, 95% fiducial limits) VCDP/g body wt/injection using an SAS Probit Procedure.*

In a preliminary tumor- and blood-uptake study (Fig. 4), tumor-bearing mice received VCDP at a reduced dose rate in the 11–32 μg VCDP/g body wt/injection range to avoid excessive mortality at higher dose rates (Fig. 3). Uptake of boron in tumor 4 days (90 hr) after the last injection of a total dose of 260–280 μg VCDP/g body wt (using multiple i.p. injections) was ≈40 μg B/g tumor tissue. This dose and administration schedule were used in the succeeding biodistribution and toxicity experiments (II and III) (SED: 270 ± 10 μg/g body weight given in a 4-day, 3/day multiple i.p. injection schedule) and it resulted in a lethality of 6/80 before euthanasia.

The boron quantitative neutron-capture radiograph (Fig. 5) shows heterogeneous uptake of boron in the tumor (bright areas correspond to high boron concentrations). There is an outer ring of high uptake (100–200 μg B/g tissue) in the immediate peritumor zone associated with lower uptake (10–30 μg B/g tissue) in the tumor itself. There was no boron detectable by the QNCR method in normal brain parenchyma.

Experiment II. This study determined the biodistribution and toxicity of VCDP injected at the SED and measured boron concentrations in tissues at various time periods (18 hr, 2 days, 4 days, 6 days and 8 days) after the last injection (see Fig. 6). In addition, an acutely toxic dose of VCDP was evaluated (see Table 1). Mice were divided into eight groups (A–H), seven of which bore implanted KHJJ mammary carcinomas. In groups E–H, mice were given the SED, a total dose of 265 μg/g body wt of porphyrin over 4 days (3 i.p. injections/day; 22 μg VCDP/g body wt/injection) and euthanized 18 hr, 2 days, 4 days or 6 days after the final injection. Four of these mice were kept in metabolic cages in which urine and feces were collected during drug administration. The plasma fraction of blood from a few mice in group E was separated by centrifugation. Group D mice were injected with equivalent volumes of PBS and euthanized 6 days after the last injection. Tissues (tumor, liver, kidney, spleen, blood, brain, muscle and skin) and some urine and fecal specimens from groups E–H were analyzed by prompt-gamma spectrometry [11].

Mice in group C received VCDP for 2½ days at the rate of 3 injections/day, 30 μg VCDP/g body wt per injection (total dose 210 μg VCDP/g body wt) which had been determined to be ≈LD_{40} in Experiment I (Fig. 3). Mice in group B received weight-equivalent injections of TPPS, a non-boronated porphyrin, on the same schedule. Another group of mice with no tumors received volume-equivalent injections of PBS on the same schedule. When a mouse died 1 hr after the seventh dose of VCDP (about 48 hr after the first injection), all other mice (in groups A, B and C) were euthanized within 3 hr thereafter.

Visual observations and hematological analyses (Table 1) indicated that VCDP was more toxic than the same weight of similarly injected TPPS. Mice in the VCDP group (C) were visibly more lethargic and showed more piloerection than those in the TPPS group (B). Mice in groups B and C were more lethargic than untreated mice (group A). Weight losses (<5%) and weights of organs were similar in all three groups. The adrenals were darker and the livers appeared slightly yellower in group C mice. Fresh blood seen in a few mesenteric lymph nodes was associated with visible submucosal hemorrhage of the distal small intestine in some VCDP-injected mice. This intestinal hemorrhage might have resulted from, or been associated with, thrombocytopenia (Table 1). There were

* *SAS User's Guide: Statistics*, 6 Edn. SAS Institute Inc., Cavy, NC, 1991.

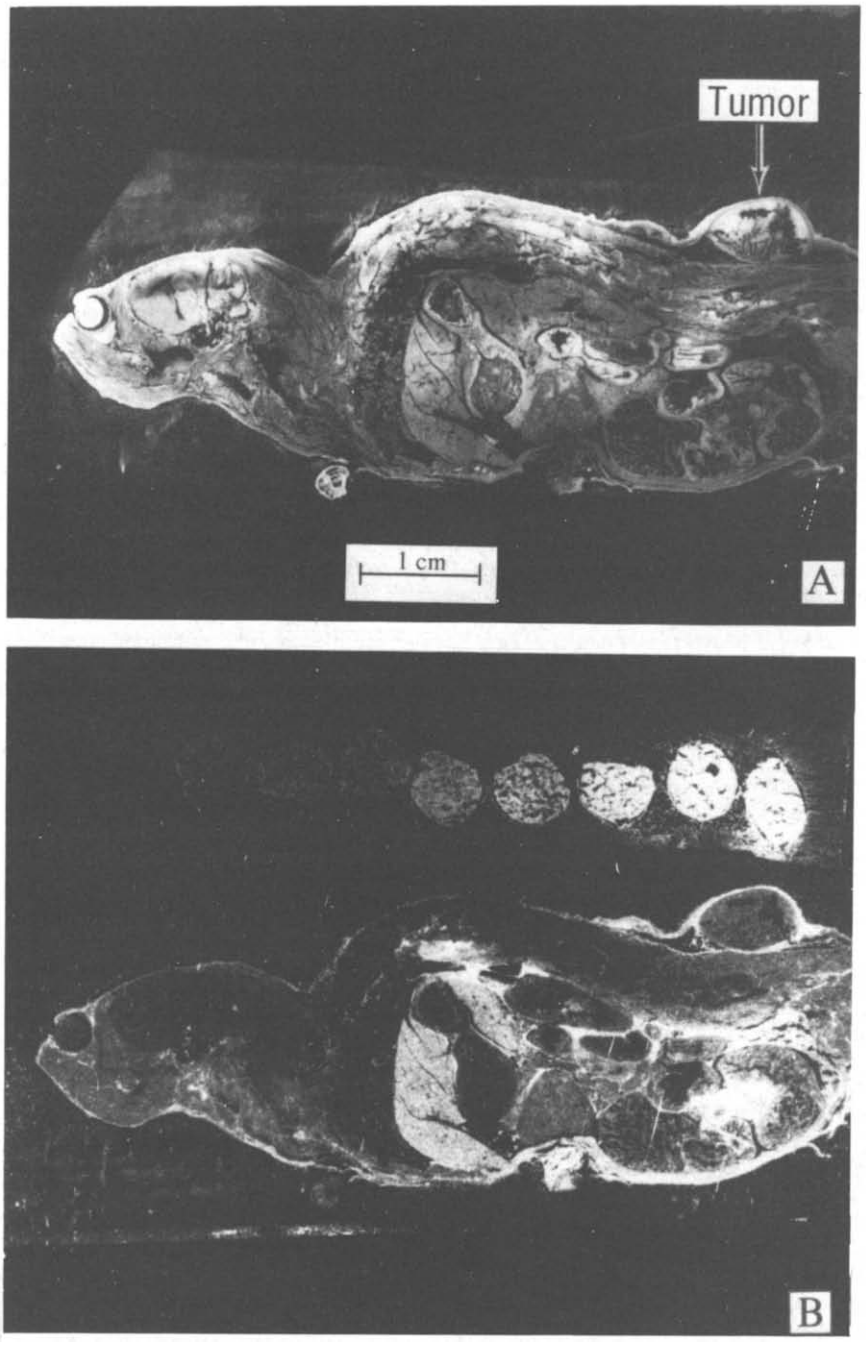

Fig. 5. Quantitative neutron capture radiographs (QNCR) of 50 μm-thick whole body sections of mice. (A) A photograph of a section of a BALB/c mouse given a total dose of 264 μg/g body wt of VCDP via multiple i.p. injections and allowed a 4-day post-injection clearance period. (B) A QNCR of the same section where light areas correspond to high boron concentration.

no formed feces in the mice so affected, an observation that we could not explain. In all mice of group C and in several mice of group B, parts of the small intestines were focally and weakly adherent to the liver, presumably from transient sterile chemical peritonitis occasioned by the i.p. injections. The brain, lungs, heart, spleen and kidney appeared normal. The mice in group C evidently contained porphyrin in the bile, as clear bile aspirated from the gallbladder at necropsy fluoresced intensely red under low-intensity long-wave UV illumination. The internal organs as well as the skin and eyes of porphyrin-bearing mice were easily visible as red-fluorescent under illumination by the same long-wave UV light source.

Table 1. Experiments II (A–H) and III (K,L) hematologic parameters

Group	Clear. time	Number of mice	Dose (μg/g body wt)	Tumor (yes/no)	Leukocytes (10^3/mm^3)	Platelets (10^3/mm^3)	Blood urea nitrogen (mg/dL)
A	3 hr	11	(PBS only)	n	3.5 (1.5–4.4)	1005 (547–1217)	28 (25–37)
B	3 hr	11	210 (TPPS)	y	4.2 (2.1–9.7)	652 (216–906)[a]	27 (20–32)
C	3 hr	16	210 (VCDP)	y	6.6 (2.6–13)[a]	528 (103–796)[a]	24 (14–60)
D	6 days	6	(PBS only)	y	12.7 (8.1–14.2)[a]	979 (840–1182)	23 (20–25)[b]
E	18 hr	6	265 (VCDP)	y	8.7 (4.7–10)[a]	137 (78–398)[a]	60 (22–140)[c]
F	2 days	5	265 (VCDP)	y	12.4 (6.7–19.5)[a]	762 (635–798)[c]	18 (16–19)[a]
G	4 days	5	265 (VCDP)	y	10.8 (7.1–15.1)[a]	819 (735–952)	17 (14–33)
H	6 days	3	265 (VCDP)	y	25.4 (18.8–34.2)[a]	905 (796–965)	22 (19–30)
K	4 days	6	($NaHCO_3$)	n	4.4 (3.2–4.8)	1141 (1040–1396)	27 (22–37)
L	4 days	4	275 (VCDP)	n	6.0 (3.5–9.7)	828 (788–1050)	20 (17–24)
M	4 days	6	($NaHCO_3$)	n	3.8 (2.8–4.6)	1154 (992–1265)	26 (22–30)
N	4 days	6	130 (VCDP)	n	3.7 (2.3–3.8)	1158 (557–1247)	26 (18–32)

Tabulated values are median (or the arithmetic mean of the two median numbers in even-numbered groups) and range (in parentheses). Mice in groups B–H had transplanted KHJJ tumors. Group A was comprised of normal mice given the same volume of PBS as were groups B and C. Group B mice were given TPPS (210 μg/g body wt) at a rate of 30 μg/g body wt per injection. Group C mice were given VCDP at the same total dose and dose rate. Group D mice were given the same volume of PBS as were groups E–H, and euthanized 6 days post-injection. Groups E–H were given VCDP (265 μg/g body wt) at a rate of 22 μg/g body wt per injection, and euthanized after the clearance periods indicated. Groups K–N were comprised of normal mice. Groups L and N were given VCDP (275 and 130 μg/g body wt, respectively), and groups K and M were given equivalent volumes of solvent. All four groups were euthanized 4 days post-injection. Erythrocyte tests (hemoglobin, hematocrit, cell count, mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration) showed no differences among groups A–H.

[a–c] The non-parametric Wilcoxon Two-Sample Test, using a 10% allowable percent difference (Fig. 2), shows that these values differed from normal mice (group A) with the following uncertainties: [a]$P < 0.001$, [b]$P < 0.01$, and [c]$P < 0.05$.

Noteworthy hematological differences between groups are the white blood cell (WBC) and platelet (PLT) counts shown in Table 1. Although the TPPS WBCs were not statistically different from those of normal mice (group A), the VCDP mice had elevated WBCs ($P < 0.001$), primarily by granulocytosis. The PLTs in groups B and C were lower than those of group A ($P < 0.001$ for both). The PLTs of mice in groups B and C, however, were not statistically different from each other. Other hematological tests (red blood cell counts, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration) showed no inter-group differences.

For mice given the SED, toxic effects appeared to be reversible, as blood urea nitrogen and blood platelets returned to normal 6 days post-injection. Leukocytes, mainly granulocytes, were still elevated at 6 days. However, tumor-bearing control mice euthanized 6 days after the last injection also had elevated WBC.

Tumors contained a substantial amount of boron (≈60 μg/g) at 18 hr (Fig. 6). The liver, spleen and kidney contained even greater amounts.Tumor boron concentrations remained in the BNCT range [2] 4 days after the last injection (≈40 μg B/g), when boron in blood had decreased significantly (≈10 μg B/g). At this time, these tissues (Fig. 6) contained ≈23% of the injected dose. All tissues containing boron in concentrations >20 μg B/g appeared to be losing boron within an 8-day post-injection time frame. Figure 7 suggests that the rate of decrease in average tumor boron concentration may be caused in large part by dilution of tissue-bound boron into tumor cells at division. The concentration of boron in formed feces was about twice that in urine. Most of the boron in blood was in its plasma fraction.

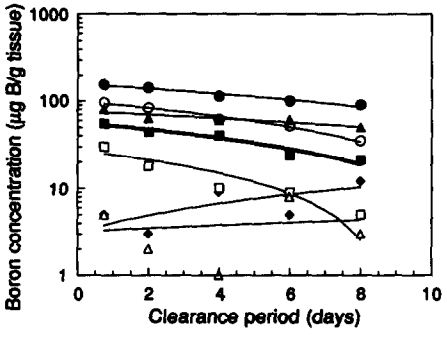

Fig. 6. Boron concentrations in various tissues of BALB/c mice given 265 μg VCDP/g body wt as a function of time after the last injection. Key: (●) liver, (▲) kidney, (○) spleen, (■) tumor, (◆) muscle, (□) blood, and (△) brain. The lines were generated using first-order least squares regression.

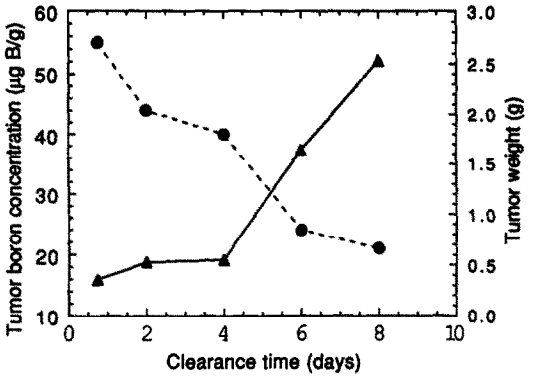

Fig. 7. Tumor weight (▲) and tumor boron concentration (●) of BALB/c mice given 275 μg VCDP/g body wt as functions of time after the last injection.

Table 2. Experiment III plasma chemistry of VCDP *in vitro*

	Control (plasma only) (N = 6)	VCDP + plasma (N = 6)
GLU (mg/dL)	184 (181–187)	182 (179–189)
BUN (mg/dL)	29 (29–30)	29 (29–31)
BCR (mg/dL)	0.4 (0.3–0.4)	0.5 (0.5–0.6)[a]
ALT (U/L)	30 (29–35)	23 (21–24)[a]
AST (U/L)	64 (63–64)	8 (7–9)[a]
ALP (U/L)	100 (96–105)	63 (57–69)[a]
TPR (g/dL)	5.3 (5.2–5.4)	5.3 (5.2–5.4)
ALB (g/dL)	2.8 (2.8–3.0)	2.8 (2.8–2.9)
CPK (U/L)	97 (85–107)	12 (10–15)[a]*
LDH (U/L)	292 (278–297)	240 (230–247)
SODH (U/L)	13.4 (13.1–13.5)	3.8 (3.1–3.9)[a]
BILE (mmol/L)	12.0 (9.3–15.1)	7.0 (5.0–7.7)[b]†

Tabulated values are median and range. VCDP was dissolved in CBA mouse plasma so that the solution contained 36 μg B/g (equivalent to ≈18 μg B/g of whole blood). Controls were untreated mouse plasma samples.

[a–b] The non-parametric Wilcoxon Two-Sample Test, using a 10% allowable percent difference in the data (Fig. 2), shows that these values differed from the controls with the following uncertainties: [a]$P < 0.005$, and [b]$P < 0.05$.

* A 40% allowable percent difference was used in the Wilcoxon analysis.

† A 20% allowable percent difference was used in the Wilcoxon analysis.

Experiment III. A study was carried out to compare normal plasma to which VCDP was added *in vitro* (Table 2) with plasma from VCDP-injected mice (Tables 3 and 4). These data provide a basis for assessing possible VCDP artifacts in plasma chemistry determinations.

VCDP was added to normal mouse plasma *in vitro* to a concentration of 36 μg B/g solution, a concentration that corresponds to ≈18 μg B/g whole blood. These concentrations are equivalent to the *in vivo* value at the 2-day clearance period. Blood chemistries were analyzed for all groups.

Non-tumor-bearing BALB/c mice were divided into two groups. One group of mice was given VCDP at the SED level in 12 i.p. injections at 3 injections/day. The other group of mice was given a bicarbonate solution (0.5%, w/w) in an identical manner. Mice were euthanized 2 or 4 days after the last injection.

Hematologic data for mice given the SED of VCDP at 4-days post-injection are shown in Table 1 (groups K and L). The chemical analyses of blood plasma from the 2- and 4-day groups are shown in Table 3. At 2 days (group J) there were significant decreases in GLU, BUN, TPR and ALB, as well as an increase in ALT. By 4 days (group L), four of these five indices had returned to normal. Apparent depressions in AST, ALP, BILE, SODH and CPK are attributable to *in vitro* chemical effects of VCDP on the microanalysis procedures. The entries in Table 3 that are most likely affected by these analytical artifacts are indicated in the footnotes to Table 3.

Hematologic data for mice given half the SED (130 μg VCDP/g body wt over 4 days at a rate of 3 injections/day and 11 μg VCDP/injection/g body wt) are shown as group N and are compared with control group M in Table 1. There were no differences in these values between groups M and N. The chemical analyses of the plasma of these groups are shown in Table 4. Wilcoxon analyses show that these plasma chemistries for mice given VCDP at the half-SED dose were not different from those for mice given buffer only.

DISCUSSION

We have found an experimental dose regimen for VCDP, a potential BNCT drug, that permitted us to analyze the most severe toxic effects of VCDP to mice *in vivo*. The greatest tumor boron uptake (≈60 μg B/g) was observed 18 hr after the last injection following a total dose of ≈270 μg VCDP/g body wt via i.p. injections at a rate of 3 injections/day for 4 days. The tumor:blood ratio was optimal 4 days post-injection at 4:1 when tumor still retained ≈40 μg B/g. Unlike the loss of boron from other tissues after the injections of VCDP, the loss of boron from tumor (to ≈20 μg B/g at 8 days) was substantially attributable to the high growth rate of the tumor. Total tumor boron increased from ≈18 μg at 18 hr to ≈50 μg at 8 days post-injection.

The tissue distribution of VCDP is similar to that of the non-boronated porphyrin Photofrin II, the commercial porphyrin used in photodynamic therapy (PDT), at least with respect to the increased uptake of VCDP in tissues with increased vascular permeability to serum proteins (porphyrins are generally tightly bound to albumin): Liver > spleen ≈ kidney > tumor > brain [13]. That VCDP was primarily excreted as feces and that it was found mainly in the plasma fraction of blood were not surprising, as similar distributions have been observed for Photofrin II [14].

The range of measured boron concentration in a tissue is partly due to the limited accuracy of prompt-gamma boron determination in tissues containing

Table 3. Experiment III plasma chemistry of VCDP after SED *in vivo*

Group	I Control (2 days) (N = 6)	J VCDP (2 days) (N = 8)	K Control (4 days) (N = 14)	L VCDP (4 days) (N = 13)
% Wt loss	3.5 (0–7.8)	16.4 (11.3–17.8)[a]	−3.3 (−16.3–8.8)	8.4 (−7.3–23.5)[d]
GLU (mg/dL)	177 (165–241)	130 (92–153)[b]	190 (164–269)	152 (110–206)[b]
BUN (mg/dL)	30 (26–31)	18 (12–20)[b]	29 (21–40)	25 (11–29)
BCR (mg/dL)	0.6 (0.4–0.6)	0.5 (0.4–0.6)	0.5 (0.4–0.7)	0.7 (0.4–0.8)
ALT (U/L)	32 (14–46)	58 (28–110)[c]	25 (3–41)	33 (14–68)
AST (U/L)	93 (80–105)	58* (10–125)	95 (50–107)	48† (12–111)[d]
ALP (U/L)	92 (75–100)	33† (17–48)[c]	79 (63–97)	52† (40–62)[a]
TPR (g/dL)	5.7 (5.6–5.9)	5.0 (4.8–5.5)[c]	5.6 (5.3–5.8)	5.3 (5.0–5.5)
ALB (g/dL)	3.2 (3.1–3.3)	2.2 (2.1–2.4)[c]	3.1 (2.9–3.1)	2.8 (2.3–3.1)
CPK (U/L)	240 (138–307)	96† (66–198)[c]	127 (55–284)	105* (42–280)
LDH (U/L)	376 (297–513)	296 (200–461)	168 (116–269)	200 (130–349)
SODH (U/L)	11.8 (6.3–16.7)	11.0* (7.6–29.1)	15.5 (10.8–17.4)	12.1* (8.7–20.8)
BILE (mmol/L)	—	—	30.7 (17.8–41.9)	34.2* (17.8–47.9)

Tabulated values are median and range. Groups J and L were comprised of normal mice given VCDP at 275 μg/g body wt in 12 i.p. injections at a rate of 22 μg/g body wt/injection. Groups I and K were identical mice given a buffer solution in an identical manner. Groups I and J were allowed a 2-day clearance period and groups K and L were allowed a 4-day clearance period before euthanasia.

[a-c] The non-parametric Wilcoxon Two-Sample Test shows these values to differ from controls with the following uncertainties: [a]$P < 0.001$, [b]$P < 0.002$, [c]$P < 0.005$, [d]$P < 0.01$, and [e]$P < 0.05$. All tests used a 10% allowable percent difference except CPK, 40% and BILE, 20%.

* Probable artifact; likely to be elevated by VCDP *in vivo* (cf. Table 2).

† Probable artifact; unlikely to be greatly altered by VCDP *in vivo* (cf. Table 2).

Table 4. Experiment III plasma chemistry of VCDP at one-half the SED *in vivo*

Group	M Control (N = 6)	N VCDP (N = 6)
GLU (mg/dL)	195 (154–219)	192 (127–219)
BUN (mg/dL)	26 (22–30)	26 (18–32)
BCR (mg/dL)	0.6 (0.5–0.8)	0.6 (0.5–0.7)
ALT (U/L)	11 (10–21)	14 (8–47)
AST (U/L)	58 (53–75)	44 (12–91)
ALP (U/L)	58 (51–82)	52 (33–79)
TPR (g/dL)	5.4 (5.0–5.6)	5.1 (4.8–5.3)
ALB (g/dL)	3.2 (2.7–3.2)	2.6 (2.5–2.6)
CPK (U/L)	152 (110–212)	85* (58–268)[a]
LDH (U/L)	164 (143–224)	188 (131–334)
SODH (U/L)	16.2 (9.2–17.8)	15.6 (10.6–18.0)
BILE (mmol/L)	19.5 (17.1–27.9)	24.4 (18.5–26.5)

Tabulated values are median and range. Group N was comprised of normal mice given VCDP at 130 μg/g body wt in 6 i.p. injections at a rate of 11 μg/g body wt/injection. Group M was comprised of normal mice given a buffer solution in an identical manner to group N. Groups M and N were allowed a 4-day clearance period before euthanasia.

[a] The non-parametric Wilcoxon Two-Sample Test shows that this value differed from normal mice with the uncertainty $P < 0.10$. A 10% allowable percent difference was used for all tests except CPK, 40% and BILE, 20%.

* Probable artifact; unlikely to be greatly altered by VCDP *in vivo* (cf. Table 2).

low concentrations of boron such as brain and blood. However, this range in tumors may also be due to the heterogeneous uptake of boron as shown by QNCR. Prompt-gamma spectrometry confirmed that the inner, necrotic areas of a tumor contained only half the amount of boron as the outer, viable regions. The size of the tumor, therefore, plays a significant role in the boron uptake since malignant tumors generally display more necrosis as they enlarge. Thus, the normalized tumor boron concentration appears to be inversely related to tumor weight (Fig. 8) as expected.

There were no obvious differences in the histological appearances of hematoxylin- and eosin-stained sections of liver and kidney among mice of groups A, B and C (Experiment II, Table 1). In Experiment II, mice in groups E and G showed scattered necrobiotic cells among the hepatocytes and the renal tubules. Isolated mitotic figures were also seen in these cells. These changes were more marked in group G than in group E and were not seen in TPPS- and VCDP-treated mice that were euthanized within several hours after the last of seven i.p. injections of the porphyrin. We attribute these observations to hepatic and renal damage caused by the 4-day regimen of VCDP injections and to delays in initiation of tissue regeneration following VCDP-mediated damage.

These experiments show that VCDP toxicity abates spontaneously after cessation of its administration. This is supported by our histopathological observations and by the return toward normal of blood platelet counts after cessation of VCDP injections. The continued leukocytosis appears to be attributable largely to the KHJJ tumor itself, as shown by WBC differences between group A or K (normal mice) and group D (6-day tumor control) ($P < 0.001$) and by the lack of difference in WBC between group A or K (normal mice) and group L

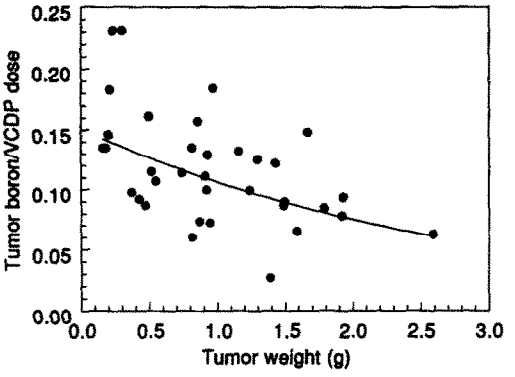

Fig. 8. Normalized tumor boron concentration (tumor boron concentration divided by the total dose of VCDP) as a function of tumor weight. Data from the preliminary tumor boron uptake study (Fig. 4) in Experiment I were used in which all mice were euthanized 4 days after the last injection. The lines were generated using least squares regression (exponential curve-fitting).

(normal mice given VCDP). VCDP-induced leukocytosis tends to persist after clearance of VCDP, since the WBC counts of group H (6-day clearance) are greater than those of group D (6-day tumor control, $P < 0.001$). In contrast, thrombocytopenia is not caused by the KHJJ tumor, as seen from the comparison of PLT counts in groups A, D and H. Thrombocytopenia was also observed in mice following administration of TPPS (group B) and has been observed in mice given a boron-containing derivative of tetraphenylporphyrin [15].

Although $\approx$15–50 μg ^{10}B/g is calculated as the therapeutic dose needed for BNCT [2], factors other than the average ^{10}B concentration in tumor, such as the microscopic distribution of ^{10}B in the tumor, are critical to the efficacy of BNCT. Perhaps the primary targets of both BNCT and PDT should include vascular regions surrounding tumors. *In vivo/in vitro* clonogenic survival assays with two different boron compounds (*p*-dihydroxyboronylphenylalanine and $[Na_2B_{12}H_{11}S]_2$ dimer) suggest that at least part of the efficacy of dimer-based BNCT might be due to damage to the tumor vasculature.* The prompt-gamma boron analyses performed in our experiments give an average concentration of boron in a tissue sample. The QNCR in Fig. 5, however, shows highest boron concentrations around the perimeter of the tumor in a zone (pseudocapsule) that most likely contains neovasculature at the periphery of the tumor stroma where centrifugal tumor growth occurs. It has been reported that the pseudocapsule of a fast-growing murine mammary carcinoma accumulates hematoporphyrin derivative preferentially [13]. In our study, assay by QNCR showed 100–200 μg B/g in this outer presumed pseudocapsular region in contrast to 10–30 μg B/g in the inner regions of the KHJJ murine mammary carcinoma.

Recent studies of PDT mechanisms indicate that PDT effectiveness is determined by damage to tumor blood vessels rather than by direct damage to tumor cells [13, 16]. Hemorrhage, thrombosis and vascular ablation are events associated with tumor control by PDT. These findings, together with the results of our study, lend credence to the concept that irradiation of tumor vasculature may be also desirable in BNCT.

Autoradiograms of Harding-Passey and B-16 melanoma-bearing mice given similar doses of VCDP or [^{35}S]TPPS show similar distribution patterns [17, 18]. Such patterns have not been reported, to our knowledge, with any other boronated compound so tested in these tumor models [19]. If this distribution pattern will later prove to be similar to those associated with human grade IV malignant gliomas where the main tumor mass is (1) largely necrotic, (2) treatable by gamma radiation therapy, and (3) accessible to surgical debulking, VCDP-mediated BNCT could be an adjunct to other therapies used in achieving long-term tumor control.

Another interesting attribute to VCDP is that, unlike some other prospective drugs for BNCT, it persists longer ($\approx$weeks), obviating a need to irradiate immediately or within hours after administration of the drug. If a multiple irradiation regimen proves to be as advantageous for VCDP-mediated BNCT as it is for photon radiotherapy, a full injection regimen of VCDP would not be necessary for each subsequent irradiation, even if these were delayed for days after VCDP administration. On the contrary, if increasing the tumor:blood ratio plays a role in improving BNCT efficacy, then a delay after the last VCDP injection would be desirable since there would be a substantial increase in the ratio by that time.

Although a total dose of $\approx$270 μg VCDP/g body wt exhibited toxic effects (primarily hematologic and hepatic), most toxic effects appeared to be quickly reversible. These experiments were carried out to establish a baseline for VCDP toxicity to which the toxicities of other porphyrins in mice can be compared. At doses of VCDP used in these experiments, high concentrations of boron are delivered to tumors in distribution patterns that should be effective for tumor control. The blood–brain barrier, compromised wholly or in part by human malignant gliomas, allows selective entry to brain tumors by compounds that are not able to penetrate the surrounding non-edematous brain parenchyma. The peritumor edema zone, which harbors occult malignant glioma cells [20], is also expected to be accessible to VCDP. We speculate that VCDP might be applicable to human brain tumor BNCT if, as in mice, it proves to similarly respect the human blood–brain barrier and to similarly accumulate and persist in human tumors. Prior to BNCT applications, the toxic effects of VCDP would have to be understood and controlled to within clinically tolerable limits for humans.

* Coderre JA, Brookhaven National Laboratory, unpublished data, cited with permission.

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