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Causative agent of vascular pain among photodegradation products of dacarbazine

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Abstract

The photodegradation products of the anticancer drug, dacarbazine, cause adverse reactions including local venous pain when injected intravenously. In this study, we attempted to identify which of these products is responsible. We synthesized or purchased five photodegradation products of dacarbazine (dimethylamine, 5-diazoimidazole-4-carboxamide (Diazo-IC), 4-carbamoylimidazolium-5-olate, 5-carbamoyl-2-(4-carbamoylimidazol-5-ylazo)imidazolium-5-olate and 2-azahypoxanthine) and examined the pain reaction induced by their intraperitoneal administration in mice using an abdominal stretching or constriction assay. Only Diazo-IC clearly induced pain reaction in mice in a dose-dependent manner, the other products caused no pain reaction. The threshold concentration for pain reaction in mice was estimated to be about 0.1 mg mL^{-1}. While diclofenac sodium significantly reduced acetic-acid-induced pain reaction in mice, it did not influence those induced by Diazo-IC. This result suggests that the mechanism of Diazo-IC-induced pain is different from that of acetic-acid-induced inflammatory pain. Dacarbazine itself produced marked relaxation of rat thoracic aorta strips in a concentration-dependent manner, but there was no difference between the activity of dacarbazine and its photo-exposed solution, so constriction or relaxation of blood vessels is unlikely to be a factor in the pain reaction. In conclusion, Diazo-IC generated by photodegradation of dacarbazine solution causes the side-effect of venous pain. Dacarbazine solution that has turned pink should not be used, because Diazo-IC is an intermediate in the formation of the reddish product, 5-carbamoyl-2-(4-carbamoylimidazol-5-ylazo)imidazolium-5-olate. Drip infusion preparations of dacarbazine should be shielded from light.

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Introduction

Dacarbazine, 5-(3,3-dimethyl-1-triazenyl)-1 *H*-imidazole-4-carboxamide, is an anticancer drug used in the treatment of several malignant disorders, including metastatic malignant melanoma, sarcoma and Hodgkin's disease, in combination with other anticancer drugs (Sutow & Maurer 1981). Dacarbazine is supplied as a sterile, lyophilized powder for injection, which is reconstituted with water before use. Shealy et al (1962, 1968) reported that the reconstituted drug rapidly decomposed in sunlight, producing dimethylamine and 5-diazoimidazole-4-carboxamide (Diazo-IC), which subsequently cyclized to 2-azahypoxanthine, and the solution turned pink. Horton & Stevens (1981a) re-investigated the drug degradation to establish the influence of pH, and summarized the mechanisms of dacarbazine photodegradation as shown in Figure 1. The pharmacological significance of the presence of the degradation by-products in terms of the overall efficacy of dacarbazine therapy is uncertain. Some authors have reported side-effects of photo-exposed dacarbazine, including local venous pain at the injection site.

In 1994, a patient (male, 28 years old) at Kanazawa University Hospital with soft-tissue sarcoma in the retroperitoneal space complained of severe pain along the vein throughout neoadjuvant therapy with CYVADIC (cyclosphosphamide, vincristine, adriamycin and dacarbazine). We considered that the pain might be related to

photodegradation of dacarbazine (Baird & Willoughby 1978; Kirk 1987), and therefore shielded the infusion solution from light. Thereafter, the vascular pain did not recur. Nevertheless, the precise relationship between the photodegradation products and the venous pain is still unclear. In this study, therefore, we attempted to identify the photodegradation product of dacarbazine that is responsible for the side-effect.

Materials and Methods

Compounds

Dacarbazine was purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan. Dacarbazine Injection Kyowa was purchased from Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan. Among the known photodegradation products of dacarbazine, diazo-IC (**3**) and 2-azahypoxanthine hydrate (**6**) were prepared by the method of Shealy et al (1961); 4-carbamoyl-imidazolium-5-olate (**4**) and 5-carbamoylimidazol-5-ylazo-imidazolium-5-olate (**5**) were prepared by the method of Horton & Stevens (1981b). Compound **5** was synthesized by adding Diazo-IC (**3**) containing citric acid to a solution of 4-carbamoylimidazolium-5-olate (**4**) in water–dimethyl sulfoxide (98 : 2). The solution was illuminated with a 100-W mercury-vapour lamp via a Pyrex filter (Riko Kagaku Sangyo KK, Chiba, Japan) for 10 h. The synthesized compounds were used after appropriate purification and their structures were confirmed by elemental analysis, and by IR, mass and NMR spectra. Other chemicals were of reagent grade.

Photolysis experiments

A solution of dacarbazine (1.0 mg mL^{-1}, pH 6.8) was exposed to ultraviolet (UV) irradiation (4 J m^{-2}). A 0.1-mL sample was diluted 100-fold at designated times, and the UV–visible spectral changes were monitored for 360 min on a UV-260 spectrometer (Shimadzu, Kyoto, Japan). For pharmacological studies, 10 mg mL^{-1} dacarbazine solution was exposed to UV irradiation (4 J m^{-2}) for 120 min.

Abdominal stretching or constriction assay

All animal experiments were performed in strict compliance with the guidelines of the Institutional Animal Care and Use Committee of the University of Kanazawa. DDY male mice, about 30 g (Japan SLC, Shizuoka, Japan), were intraperitoneally injected with saline, a test compound solution or 0.6% acetic acid (0.1 mL/10 g body weight). Immediately after the injection, mice were placed in a plastic animal cage measuring 25 × 50 cm, and the pain reaction, defined as constriction of the abdomen with stretching of the hind limbs, was measured by the method of Collier et al (1968). The number of reactions in each 5-min interval after the intraperitoneal injection was counted. For each treatment, values are expressed as the mean (±s.d.), using groups of at least five mice. Each mouse was subjected to only one treatment.

Preparation of thoracic aortic strips and assessment of relaxant effect of dacarbazine

Male Wistar rats (Japan SLC, Shizuoka, Japan), 200–300 g, were stunned and exsanguinated. The thoracic aorta was removed and placed in Krebs–Henseleit solution (composition in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 11 glucose). The aorta was cut into helical strips (3 mm wide, 20 mm long). Each strip was mounted in a 10-mL organ bath, with one end anchored to the bottom of the bath and the other connected to a force-displacement transducer (Oriectec Japan, Tokyo, Japan). The strips were allowed to equilibrate for 1 h at a resting tension of 1 g. The bathing solution was continuously bubbled with 95% O_2–5% CO_2, and the temperature was maintained at 37°C. The relaxant effects of dacarbazine and its UV-exposed solution were examined as follows. The helical strips were contracted by exposure to 10 μM phenylephrine or high-K^+ Krebs–Henseleit solution in which 118 mM NaCl was replaced with equimolar KCl. When the contractions reached a plateau and became constant, portions of test solution were cumulatively applied. The magnitude of relaxation induced in each case was expressed as a percentage of the amplitude of the contraction.

Data analysis

The data were analysed using Student's *t*-test to compare the unpaired mean values of two sets of data. A value of $P < 0.05$ was taken to indicate a significant difference between sets of data.

Results

Photodegradation of dacarbazine

During exposure to UV light, dacarbazine solution became pink to dark red in colour. Figure 2 shows the UV spectra of dacarbazine solution (1.0 mg mL^{-1}) after UV exposure. The absorption in the range 240–330 nm decreased with time in proportion to the increase of absorption at about 210 nm. Given that the absorbance maxima (λ_{max}) of dacarbazine are 230 and 330 nm, it appears that dacarbazine was degraded and compounds **3** (λ_{max} 206 nm) **4** (λ_{max} 210 nm) or **6** (λ_{max} 210 nm) were produced. Compound **5** (λ_{max} 518 nm) was ruled out as a major product because there was little increase in absorbance near 520 nm.

Pain reaction in response to dacarbazine and its photodegradation products

The time-course of pain reaction in mice after intraperitoneal injection of UV-exposed dacarbazine solution (10 mg mL^{-1}) was compared with that induced by 0.6% acetic acid solution, and the results are shown in Table 1. The pain reaction to dacarbazine solution increased with increasing exposure time of the solution to UV light. Pain

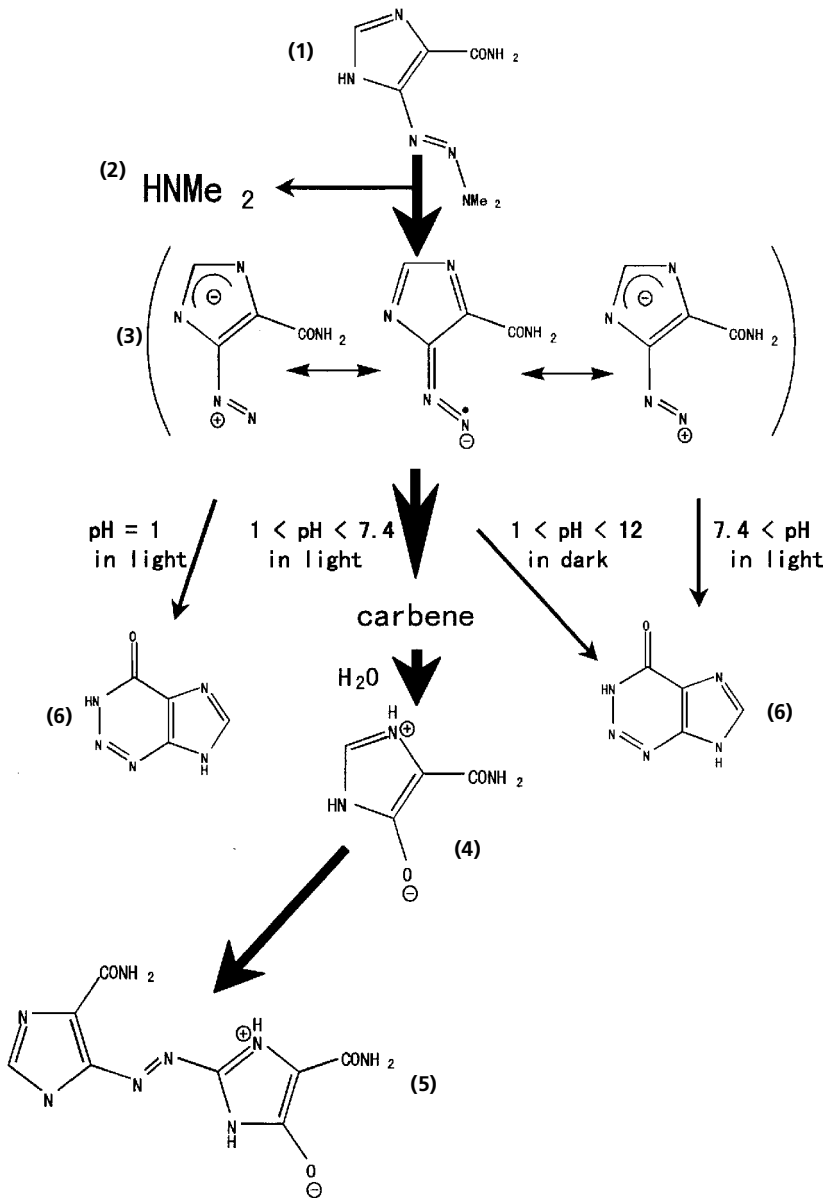

Figure 1 Suggested scheme of dacarbazine photodegradation. Horton & Stevens (1981a) showed that dacarbazine (**1**) is photolysed to afford dimethylamine (**2**) and 5-diazoimidazole-4-carboxamide (Diazo-IC) (**3**), and some of the Diazo-IC (**3**) is photohydrolysed to the 4-carbamoylimidazolium-5-olate (imidazolium-olate) (**4**) at $1 < pH < 7.4$. The activated imidazole ring of (**4**) is susceptible to electrophilic substitution and couples with unphotolysed Diazo-IC (**3**) to afford 4-carbamoyl-2-(4-carbamoylimidazole-5-ylazo)imidazolium-5-olate (azo dye) (**5**), which has a deep red colour. Diazo-IC (**3**) cyclizes exclusively to 2-azahypoxanthine (**6**) in the dark or at pH 1 or pH > 7.4.

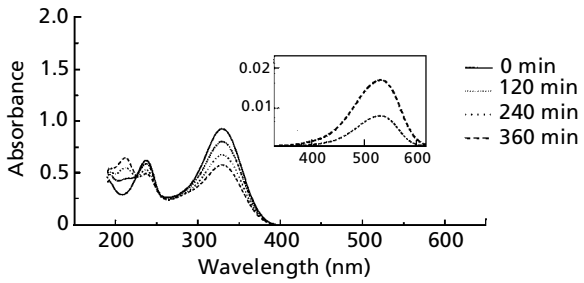

Figure 2 UV spectra of dacarbazine solution (1.0 mg mL^{-1}) after UV exposure at 120-min intervals.

reaction was observed in all mice within 5 min of injection of a dacarbazine solution exposed to UV light for 120 min. Both the number of pain reactions per affected mouse and the number of affected mice gradually decreased with the time after injection. After injection of 0.6% acetic acid as a positive control, the pain response was maximal at 10–15 min. Therefore, the pain caused by photodegradation products of dacarbazine seems to of a different nature to that induced by acetic acid. Table 2 shows the changes in pain reaction in mice after an intraperitoneal injection of each photodegradation product (1.0 mg g^{-1} body weight). Among these compounds, only **3** caused pain reaction in

Table 1 Time-course of pain reaction (number of abdominal constrictions) in mice intraperitoneally injected with dacarbazine solution exposed to UV light.

Drug	Time of exposure to UV light (min)	Number of constrictions				
		0–5 min	5–10 min	10–15 min	15–20 min	20–25 min
Saline	—	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Dacarbazine solution (10 mg mL^{-1})	0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	5	0.8±1.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	30	2.3±2.9	0.0±0.0	0.0±0.0	1.7±2.4	1.3±2.0
	60	3.0±4.2	0.8±1.8	0.0±0.0	0.0±0.0	0.2±0.5
	90	4.3±3.3	0.8±1.8	0.8±1.8	0.7±1.5	0.2±0.5
	120	6.8±8.8	3.6±3.4	3.0±5.6	2.6±3.8	1.6±3.6
Acetic acid (0.6%)	—	1.8±3.1	6.2±9.0	8.8±9.2	7.4±4.8	7.8±7.5

Each value represents the mean±s.d. (n = 5 or 6).

Table 2 Time course of pain reaction in mice intraperitoneally injected with a solution of dacarbazine photodegradation products (10 mg mL^{-1}).

Compound	Number of constrictions				
	0–5 min	5–10 min	10–15 min	15–20 min	20–25 min
1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
2	0.0±0.0	0.8±1.6	0.0±0.0	0.0±0.0	0.2±0.9
3	14.4±10.3	8.4±8.9	5.4±5.7	3.4±5.0	2.6±4.6
4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
5	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
6	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Chemical structures of the compounds are given in Figure 1. Each value represents the mean±s.d. (n = 5).

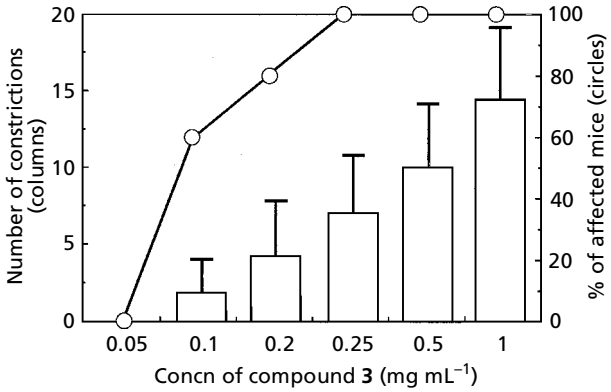

Figure 3 Induction of pain reaction in mice by compound **3**. The indicated concentrations of compound **3** were intraperitoneally injected into mice at a volume of 0.1 mL g^{-1} body weight and the number of abdominal constrictions per mouse and the number of affected mice were recorded for 5 min immediately after injection. The number of pain reactions represents the mean±s.d. (bar) of five mice.

all mice and the greatest number of reactions was observed within 5 min of injection, as with UV-exposed dacarbazine solution.

To examine the dose dependency of compound **3** for induction of pain reaction, the number of pain reactions in affected mice and the number of affected mice were observed after an intraperitoneal injection of various concentrations of compound **3**. As expected, compound **3** showed a concentration-dependent effect. Figure 3 shows the results for the 5-min period just after injection of the solution. Compound **3**, 0.25–1 mg mL^{-1}, caused pain in all mice; the mean number of pain reactions in response to 1 mg mL^{-1} was similar to that with 10 mg mL^{-1}, whereas 0.05 mg mL^{-1} was ineffective.

Effect of diclofenac sodium on the pain reaction

Figure 4 shows the effect of diclofenac sodium, which has strong anti-inflammatory and analgesic actions, on the mouse pain reactions after intraperitoneal injection of 0.6% acetic acid or compound **3** (0.5 mg mL^{-1}). The number of pain reactions induced by 0.6% acetic acid was significantly decreased by pretreatment with diclofenac, but the effect of compound **3** was not influenced by diclofenac. Therefore, the mechanism of the pain reaction to

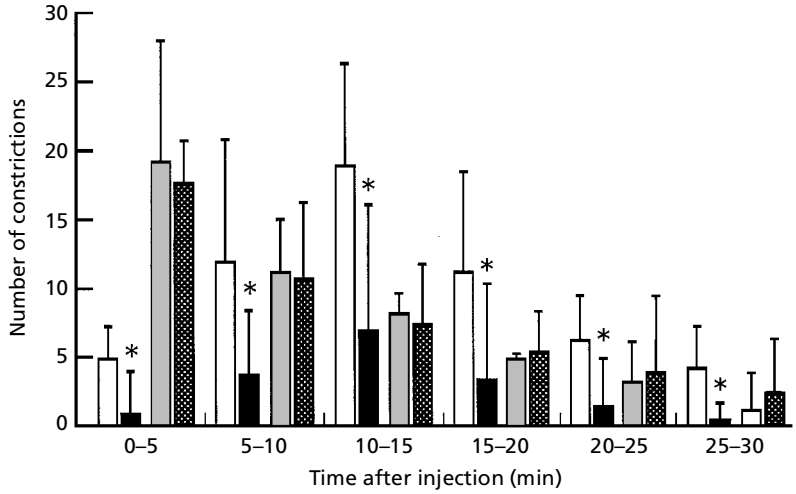

Figure 4 Effect of diclofenac sodium on pain reaction induced by 0.6% acetic acid (□, ■) or 0.5 mg mL^{-1} compound **3** (▨, ▩). Mice were subcutaneously injected with saline (□, ▨) or diclofenac sodium (50 mg kg^{-1}) (■, ▩) 15 min before intraperitoneal injection of 0.6% acetic acid or 0.5 mg mL^{-1} compound **3**. Values represent the mean number of abdominal constrictions (±s.d.) of 5 mice. *$P < 0.05$, vs saline control of 0.6%-acetic-acid group.

compound **3** may be distinct from the inflammatory process that is suppressed by diclofenac sodium.

Relaxant effects of dacarbazine on aortic strips

Dacarbazine itself did not cause constriction of non-contracted aortic strips, although at a concentration of 0.1 mg mL^{-1} it decreased the resting tension. We examined the relaxant activity of dacarbazine and its UV-exposed solution on strips contracted by 10 μM phenylephrine (Figure 5). In phenylephrine-contracted strips, dacarbazine produced marked relaxation in a concentration-dependent manner and fully relaxed the strips at 0.3 mg mL^{-1}. The photolysed dacarbazine similarly, though slightly less potently, relaxed the strips. Dacarbazine and its photolysed solution also relaxed high-K^+-contracted strips (data not shown).

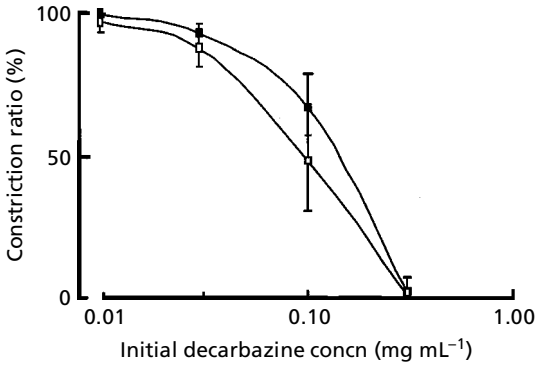

Figure 5 The relaxant effect of dacarbazine and its photodegradation products on aortic strips contracted by phenylephrine. Aortic strips contracted by 10 μM phenylephrine were cumulatively treated with dacarbazine (□) or its solution exposed to light for 120 min (■). Values represent the mean±s.d. (bar) of at least three experiments.

Discussion

Various researchers have reported adverse effects of dacarbazine photodegradation products (Baird & Willoughby 1978; Horton & Stevens 1981a; Islam & Asker 1994, 1995), and Kirk (1987) reported the usefulness of a new intravenous system to avoid adverse reaction to injection of dacarbazine. However, the specific photodegradation product responsible, and the conditions that resulted in the appearance of venous pain remained elusive. In this study we found that, among the dacarbazine photodegradation products examined, only compound **3** (Diazo-IC) caused pain reaction in mice. This is consistent with the observation of Horton & Stevens (1981a) that the process of dacarbazine photodegradation hardly advanced to compound **6** with exposure to light when the pH was less than 7.4 (Figure 1), which may reflect the clinical situation.

Dacarbazine solution at lower concentrations appears to undergo photodegradation more rapidly, the degradation rate at 0.1 mg mL^{-1} being over ten times that at 1 mg mL^{-1}. Therefore, dilution is unlikely to be useful to prevent adverse reaction caused by photodegradation of dacarbazine. Based on the report of Shetty et al (1992), we could estimate the concentration of compound **3** as about 0.01 mg mL^{-1} in a pharmaceutical preparation of dacarbazine, 100 mg in 250 mL saline (0.4 mg mL^{-1}, pH 3.6–4.0), under room light (470 lux) for 30 min. From our in-vivo study, we can estimate the threshold of pain reaction as being approximately 0.1 mg mL^{-1} in mice (Figure 3). Taking into account the species differences, it is reasonable

to assume that compound **3** is predominantly responsible for the side-effect of severe venous pain under clinical conditions. The colour of dacarbazine solution is changed subtly to reddish pink by UV irradiation. A solution of one photodegradation product, compound **5**, which is generated from compound **3** and has λ_{max} of 518 nm (Figure 1), is pink to red in colour. Therefore, we suggest that reddish dacarbazine preparations should not be injected into patients, to avoid venous pain.

Possible mechanisms of venous pain upon intravascular injection include leakage of injection solution, constriction or relaxation of the blood vessel, inflammation at the injection site or change of conduction rate of the nerve impulse of pain. With intravenous injection of dacarbazine, it seems very improbable that the leakage of injection solution causes the venous pain because we did not observe necrosis at the injection site in patients who complained of venous pain in our hospital. To elucidate whether constriction or relaxation of the blood vessel is a factor, we used rat thoracic aortic strips with endothelium, since veins could not be used because of experimental limitations. Dacarbazine itself and its UV-exposed solution did not cause constriction, but tended to relax the aortic strips. To examine the relaxation by dacarbazine or its photolysed compounds, their effect on strips contracted by phenylephrine or K^+ was examined. Although intact dacarbazine produced marked relaxation in a concentration-dependent manner, there was no significant difference between dacarbazine and its photolysed solution. Therefore, constriction or relaxation of the blood vessel is unlikely to be the cause of venous pain. Inflammation at the injection site was also not involved, because diclofenac sodium, a cyclooxygenase inhibitor, did not prevent the pain reaction produced by compound **3**. Further, there was no lag time in the pain reaction caused by compound **3**, while pain reaction induced by acetic acid appeared late after the injection and was significantly inhibited by diclofenac sodium (Figure 4). Therefore, change of synaptic response of mice after injection of the photodegradation product might be the mechanism of the pain reaction, but detailed electrophysiological studies will be needed to confirm this.

In summary, our results indicate that dacarbazine is rapidly decomposed by UV irradiation or sunlight, and compound **3**, which is the first product of photodegradation, is the cause of the side-effect of vascular pain that is seen on intravenous injection. Therefore, to avoid adverse reaction and to obtain the desired antitumour effect, it will be essential to shield dacarbazine injection solution from light immediately after its preparation and thenceforward until it has been injected.

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