Chemotherapy-induced Bystander Effect in Response to Several Chloroethylnitrosoureas: An Origin Independent of DNA Damage?

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Abstract. Chloroethylnitrosourea (CENU) chemotherapy is used for the treatment of melanoma tumors. The main mechanism of action of this anticancer agent is via DNA damage. We recently showed in murine experiments using a parental double B16 melanoma tumor model that, after treatment of primary tumors with cystemustine (CENU agent), untreated secondary tumors exhibited growth inhibition and metabolism disorders. The response of secondary untreated tumor was called the chemotherapy-induced bystander effect. To see whether chemotherapy-induced bystander effects were induced with other members of the CENU family, we compared three CENU(s) used in melanoma treatment: cystemustine, carmustine and fotemustine. Our results demonstrate that fotemustine, like cystemustine, but not carmustine induced a protective effect against secondary untreated tumors including alterations in phospholipid derivative and glutathione which are the metabolic signature of the bystander effect. From these data we may conclude that DNA damage to the primary tumor is not sufficient to explain chemotherapy-induced bystander effects.

Melanoma tumor is a medical challenge. Its incidence is increasing in the Caucasian population 50 to 80% per year (1, 2). This disease presents an important metastatic potential and less than 10% of patients have a prolonged survival in metastatic disease (3). There is no standard strategy in metastatic tumor melanoma treatment. However chemotherapy and immunotherapy allow to achieve significant tumor regressions with a median duration of 4 to 5 months. The role of immunotherapy (interferon α and/or interleukin 2 in association with conventional chemotherapy) still needs to be evaluated (4). Recent discovery of a mutation in the BRAF gene (5) coding for a serine threonine kinase (present in more than 70% of melanoma) opens new perspectives for treatment, especially with kinase inhibitors (6).

Dacarbazine, a mono-alkylating agent (7), represents the reference molecule in monotherapy during the metastatic phase with a response rate of 20% and a complete response at 3-4% (8). Other chemotherapeutic agents include nitrosoureas and vinca alkaloids. No chemotherapeutic combinations or immunotherapy have proven their superiority to dacarbazine treatment in monotherapy. Fotemustine, which is the most recently marketed CENU agent, presents the same antitumoral activity and tolerance as dacarbazine, but brain cerebral appear later (4). It was recently shown that cystemustine, a CENU, administred in a syngeneic tumor model (B16 melanoma) induced disturbance of tumor phospholipid metabolism (9, 10) and induced a protective effect against the development of secondary tumors, the so-called bystander chemotherapeutic effect (11). In this murine model, the bystander chemotherapeutic effect induced re-differentiation of secondary tumors untreated by chemotherapy, implanted at distance and four weeks after treatment of primary tumors (9, 11, 12). The mediator of the bystander chemotherapeutic effect has not been identified yet but is of potential therapeutic interest. It was shown that the primary tumors accumulated reactive oxygen species (ROS) and presented metabolic changes observed by 1H-NMR spectroscopy, such as a decrease of glutathione, and an increase of phosphoethanolamine and polyunsaturated fatty acids. Secondary tumors responding...
to the bystander effect presented a re-differentiated aspect, and a metabolic profile similar to that of the primary treated tumors (11).

The mechanism of CENU action is two-fold, with an enzymatic metabolism and a chemical decomposition responsible for the alkylation (by carbocation) (Figure 1) and carbamoylation (by the isocyanate group). The cellular effects of CENUs other than the alkylation of DNA by the carbocation (chloroethyldiazohydroxide group) are still undefined.

In this study, we investigated whether the bystander chemotherapeutic effect was specific to cylumustine (13), or could be found with other CENUs such as fotemustine (14) and carmustine, both used in clinical therapy. Therapy responses to these three CENUs (Figure 1) were evaluated using the tumor mass and the metabolic profile.

**Materials and Methods**

**Cell culture.** Transplantable B16 melanoma cells originating from C57BL6/6J Ico mice were obtained from ICIG (Villejuif, France) and adapted to grow in culture. The cells were maintained as monolayers in culture flasks using culture medium consisting of Eagle’s MEM-glutaMAX medium (Life Technologies, USA) supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO₂ atmosphere.

**Chemicals.** Two commercial CENUs, carbomustine [N,N′bis-(2-chloroethyl)-N-nitrosourea] and fotemustine [diethyl-N-[N′-(2-chloroethyl)-N′-nitrosoureido]ethylphosphonate] were obtained from Bristol-Myers Squibb (Suresnes, France) and Servier (Neuilly-sur-Seine, France) company. Another member of the CENU drug family, cylumustine [N′-(2-chloroethyl)-N-(2-methylsulfonyl)ethyl-N′-nitrosourea] (Orphachem, Clermont-Ferrand, France) (13) was used in experiments, and was prepared as 5 mM solution in 0.9% NaCl. All drugs were prepared immediately in 0.9% saline solution with 5% of ethanol before injection into mice (each volume injected was of 150 μl). The doses used are expressed in μg/g of animal weight.

**In vivo models.** Six to 8 weeks old C57BL6/6J male mice were purchased from IFFA CREDO, France. All procedures were approved by the Animal Experiments Ethics Committee. Mice were shaved before s.c. injection of 5x10⁵ B16 cells into their flanks. B16 melanoma tumors became palpable at 8-10 days after cell inoculation and reached 0.26 g (± 0.09g) at day 11 when chemotherapy treatment started. Four groups of mice (20 recipients per group) were used: an untreated group which received a vehicle solution, and CENU-treated groups which received CENU drug family, cylumustine [N,N′bis-(2-chloroethyl)-N-nitrosourea] and fotemustine [diethyl-N-[N′-(2-chloroethyl)-N′-nitrosoureido]ethylphosphonate] were obtained from Bristol-Myers Squibb (Suresnes, France) and Servier (Neuilly-sur-Seine, France) company. Another member of the CENU drug family, cylumustine [N′-(2-chloroethyl)-N-(2-methylsulfonyl)ethyl-N′-nitrosourea] (Orphachem, Clermont-Ferrand, France) (13) was used in experiments, and was prepared as 5 mM solution in 0.9% NaCl. All drugs were prepared immediately in 0.9% saline solution with 5% of ethanol before injection into mice (each volume injected was of 150 μl). The doses used are expressed in μg/g of animal weight.

Tumor surface areas were calculated by measuring the length and perpendicular width, with a calliper scale and the tumor mass was estimated with the Dolan formula (15) (tumor mass (g) = tumor long axis x (tumor small axis)^2 x 0.5). At different times, three mice of each group were sacrificed. All tumors were removed, dissected, weighed and prepared for histology and ex vivo NMR spectroscopy examinations (stored at –80°C).

**Double tumor model.** Mice bearing CENU-treated tumors were used to assess the effect of prime CENU treatment on the fate of secondary untreated parental tumors. On day 30, each group of mice bearing tumors treated by cylumustine, carmustine or fotemustine was challenged on the opposite flank with 5x10⁵ untreated B16 melanoma tumor cells. Secondary tumor development and growth were evaluated twice a week with callipers. Primary and secondary tumors were followed until day 56. Naïve groups of mice were injected with B16 melanoma tumors cells at the same time as the control group (because mice bearing untreated B16 melanoma tumor died after day 35).

**Histological analyses.** Tumor pieces were fixed in a formaldehyde solution. Paraformaldehyde sections were cut into 4 μm sections, and tissue sections were prepared for hematoxilin-eosin staining and routine pathological analysis. The count of mitoses was performed on 10 high power microscopic fields. Two slides per tissue sample were studied. The melanin pigmentation index and anisocytosis index (16) were quantified on 5 microscopic fields with a scale from 1 to 4 (1: non significant, 2: small, 3: moderate, 4: large).

**Proton HRMAS NMR spectroscopy.** Proton high resolution magic angle spinning (HRMAS) NMR spectroscopy was performed at 500 MHz (Bruker DRX 500, Germany). Samples, consisting of pieces of intact tumor of tissue between 20 and 50 mg, were inserted into 4 mm diameter zirconia rotors and rotated at 4 kHz. ¹H-NMR spectra were obtained from one-dimensional 1H saturation recovery sequences (repetition time: 10 s, spectral width: 10 ppm, complex data points: 16 K, number of samples: 64, water signal presaturation at low power). Fourier transformation and spectrum analysis were performed on an O₂ workstation (Silicongraphics, USA). The attribution of melanoma tumor NMR spectrum signals was previously reported (17, 18).

**Statistical analysis.** Data are presented as mean±standard deviation. Comparisons between CENU-treated and untreated groups were performed using the Mann-Whitney test. Time series were analysed using ANOVA, followed by a post-hoc test (SEM software, France) (19) with paired comparisons between groups.

**Results**

**Primary tumor response to CENU(s).** Previous data have shown that cylumustine treatment induced two phases: a growth inhibition phase (GI) for day 11 to 32 then a growth recovery phase (GR) with a lower proliferation rate than in untreated tumors (9). At a dose equimolar to that of cylumustine (15 μg/g), carmustine (12.5 μg/g) provoked tumor growth inhibition much less marked by cylumustine: at day 28, tumor mass reduction was respectively 5- and 17-fold lower for carmustine and...
cystemustine respectively versus the untreated group (p<0.0001) although fotemustine (18.4 µg/g) provoked tumor growth inhibition similar to that of cystemustine (Figure 2).

Body weight curves of all recipients bearing CENU-treated tumors showed a similar loss of 10% given a recipient weight of 22 ± 1g before chemotherapy (data not shown). However this secondary effect was reversible after chemotherapy arrest.

**Histological analysis.** CENU treatment between day 12 and day 28, the period of tumor growth inhibition of treated primary B16 melanoma tumors, provoked: a reduction in the mitotic index (-50% of control), a 2-fold increase of anisocytosis, and a 4-fold increase in melanin pigmentation. These effects were similar to those induced by cystemustine, fotemustine or carmustine treatment (Figure 3).

**Double tumor model.** Untreated secondary tumors in recipients bearing carmustine-treated primary tumors grew as untreated control tumors (a naïve group of mice was injected with B16 melanoma tumors cells at the same time as a second control). At day 21, the untreated secondary tumor mass reached 0.5 g ± 0.3g in the fotemustine group versus 0.3 g ± 0.1g in the cystemustine group. This absence of any protective effect by carmustine treatment was found whatever the dose used whether equimolar (12.5 µg/g) or clinical (16.5 µg/g) (p=NS). We conclude that carmustine treatment did not induce a protective effect untreated secondary B16 melanoma tumor (Figure 4 B,C). Primary fotemustine-treated tumors modified the fate of untreated secondary tumors. Growth of secondary tumors was restrained whatever the management of treatment (equimolar or clinical dose) (p<0.0001). (Figure 4 D,E). At day 21 untreated secondary tumor mass reached 0.5 g ± 0.3g in the fotemustine group versus 0.3 g ± 0.1g in the cystemustine group. Taken together these data show a hierarchy in bystander response: carmustine << cystemustine, fotemustine.

**Proton HRMAS NMR spectroscopy.** Primary tumor treatment with carmustine or fotemustine modified the tumor metabolic profile similarly to cystemustine as reported by Morvan et al. (10) involving a transient increase in choline, glycerophosphocholine and glycerophosphoethanolamine during the growth inhibition phase, and a sustained increase in phosphocholine and phosphoethanolamine during the growth inhibition phase and re-growth. Moreover, polyunsaturated fatty acids increased after day 25 and remained strongly elevated during the regrowth phase. NMR spectroscopy analysis of untreated secondary tumors was performed between days 46 to 56 of the double tumor experiment. In cystemustine-treated recipients, metabolic biomarkers of growth-inhibited secondary tumors included an increase in phosphoethanolamine and in polyunsaturated fatty acids and a decrease in glutathione (11). This phenotypic change was not found in secondary tumors in carmustine-treated recipients. In particular, the expression of polyunsaturated fatty acids was identical to that of the control group. This metabolic profile is in agreement with the fact that carmustine treatment did not induce bystander chemotherapeutic effect on secondary tumors. In contrast, in fotemustine-treated recipients the metabolic profile of secondary tumors was similar to that observed under
fotemustine and/or cystemustine treatment, consistent with a bystander chemotherapeutic effect involving an increase in phosphoethanolamine and polyunsaturated fatty acids and a decrease of glutathione content (Figure 5).

**Discussion**

Cystemustine treatment inhibits primary B16 melanoma tumor growth and confers a protective effect against untreated secondary B16 melanoma tumors, called the bystander chemotherapeutic effect. This effect is associated with histological modifications (increase melanin pigmentation, anisocytosis and mitotic index reduction) and a metabolic reprogramming of tumors cells which has been characterized by $^1$H-NMR spectroscopy (17), both testifying to the acquisition of a re-differentiated phenotype (9, 10). The comparative study of CENU treatment in double tumor models shows that a response of secondary tumors testifying to bystander chemotherapeutic effects is only found with fotemustine and cystemustine treatment. Proton HRMAS NMR spectroscopy analysis of carmustine-treated tumors showed a phenotypic change of treated primary tumors (10, 17), but the absence of metabolic changes in untreated secondary tumors where growth was not restrained. In contrast, the metabolic profile of secondary tumors in recipient bearing primary fotemustine-treated tumors clearly showed an accumulation of polyunsaturated fatty acids, an increase of phosphoethanolamine and a decrease of glutathione, in agreement with the acquisition of a histological phenotype of re-differentiation (11). The differential response of secondary tumors observed with cystemustine and fotemustine is in favour of a mechanism at the origin of the bystander chemotherapy effect not dominantly explained by DNA damage to the primary tumor. The observed protective effect was not found with carmustine which contains two carbocations group as shown Figure 1. The function of the isocyanate group has not yet been determined.

New generation CENUs could present specific properties related to the non carbocation fraction. As reported by
Slater (20), a chemotherapy drug can have double properties: (i) a cytotoxic effect against a population of tumor cells with the induction of apoptosis, a well-established pharmacological concept (21), (ii) the induction on the tumor population of resistant cells of cytotoxic effects of re-differentiation. This latter cell fraction could be at the origin of the chemotherapy-induced bystander effect. As a whole our results are in favour that the carbocation fraction of CENUs is not sufficient to induce the bystander chemotherapeutic effect. The non carbocation fraction could be implicated at the origin of the bystander effect. Further studies are required to investigate the pharmacological mechanism at the origin of the chemotherapy-induced bystander effect by using for example pharmacometabolomics (22).

In conclusion, we found that new generation CENUs are able to induce a bystander chemotherapeutic effect. The expression of this effect appears to have an origin not principally related to DNA damage to the primary tumor. The treatment of cancerous disease is becoming more and more multimodal and associates different molecular therapeutics by their mechanism of action, the final objective being to control every tumoral clone. In this respect, the elucidation of the mechanism at the origin of the bystander chemotherapeutic effect could open a new path for cancer treatment.

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Figure 5. Proton HRMAS NMR spectra of B16 secondary tumors, in recipients with primary tumor treated with carmustine, cystemustine or fotemustine. From top to bottom: untreated primary tumor (day 18 from B16 cell inoculation); untreated secondary tumor in a recipient with a primary tumor treated by carmustine; untreated secondary tumor in a recipient with a primary tumor treated by cystemustine; untreated secondary tumor in a recipient with a primary tumor treated by fotemustine; PE: phosphoethanolamine, PUFA: polyunsaturated fatty acids, GSx glutathione.

References

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