in vivo *26*: 853-858 (2012)

Obesity Effects on Cyclophosphamide-induced DNA Damage in Hematopoietic Cell Transplant Recipients

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Abstract. *Background: Cyclophosphamide, an alkylating agent, is metabolically activated to phosphoramide mustard, to form toxic DNA–DNA (G-NOR-G) crosslinks. Increased exposure to cyclophosphamide metabolites has been associated with treatment-related toxicity. The effect of obesity on exposure to cyclophosphamide-induced G-NOR-G crosslinks is not known. Therefore we sought to determine whether obesity affects the formation of cyclophosphamide-specific G-NOR-G crosslinks. Patients and Methods: Plasma cyclophosphamide concentrations and blood cell G-NOR-G amounts were measured. Results: Overweight/obese patients received a significantly higher daily cyclophosphamide dose (median 3000 vs. 4450 mg, p<0.01). Despite the higher doses, overweight/obese patients had lower exposure to cyclophosphamide compared to lean patients with an area under the curve ($AUC_{0-\infty}$) =529.24 vs. 867.99 μcg/ml*h respectively, p<0.01. G-NOR-G amounts were similar in overweight/obese and lean subjects, $AUC_{0-\infty}$=142.8 vs. 147.0 adducts/10^6 nucleotides*h, respectively, p=0.59. Conclusion: Overweight/obese patients have altered metabolism and disposition of cyclophosphamide. This altered exposure may be an important determinant of efficacy and may play a role in treatment-related mortality.*

Drug dosing of overweight/obese individuals is a significant clinical problem. Obesity alters the pharmacokinetics of some chemotherapeutic agents; however, its unclear if this is true for all agents. For those agents for which obesity has been shown to be influential, it is not known to what extent these alterations alter clinical outcomes (1). As the number of overweight/obese individuals receiving cancer chemotherapy increase, this has become a highly relevant clinical dilemma.

Cyclophosphamide is a bifunctional alkylating agent that is widely used in combination with hematopoietic cell transplantation (HCT) to treat adults with hematological malignancies. However, there is uncertainty in regard to the effect of obesity on systemic and cellular exposure to cyclophosphamide. Previously, reduced cyclophosphamide clearance has been observed in obese patients with breast cancer as compared to lean individuals (2), while other studies demonstrated that obese adults have higher exposures to the 4-hydroxycyclophosphamide metabolite as compared to lean adults (3). In the HCT setting, administration of high-dose cyclophosphamide is associated with several dose-dependent toxicities, including veno-occlusive disease (VOD), cardiotoxicity, and hemorrhagic cystitis (4). Given that these toxicities are highly dose-dependent, this raises a concern about administering chemotherapy to overweight/obese individuals based on actual body weight.

Cyclophosphamide is metabolically activated primarily by cytochrome P450 2B6 (CYP2B6) monooxygenase to form DNA-reactive metabolites, phosphoramide mustard and nornitrogen mustard. The latter induce cellular death by forming DNA adducts, in particular, *bis*-N-7 guanine interstrand DNA–DNA crosslinks (G-NOR-G). Previous studies have revealed a correlation between the concentration of alkylating agent-induced DNA adducts and therapeutic response (5, 6). Although interstrand DNA–DNA crosslinks comprise only approximately 5-10% of all adducts formed by alkylating agents, these adducts are the most physiologically relevant because of their ability to block DNA replication and are furthermore associated with cytotoxicity (7-10). Previous studies from our group have demonstrated that cyclophosphamide-induced G-NOR-G adducts can be quantified in the blood of patients receiving cyclophosphamide (11). Furthermore, we also demonstrated that the concentrations of G-NOR-G adducts are elevated in patients with Fanconi anemia, who are well-known to have

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Key Words: Transplantation, cyclophosphamide, DNA adducts, obesity, pharmacokinetics, hematopoietic cell transplant recipients.

deficiencies in DNA repair (12). Therefore, quantification of systemic adduct formation may be an important determinant of therapeutic efficacy and toxicity of cyclophosphamide in cancer. The present study sought to explore the effects of body size on cyclophosphamide exposure and formation of cyclophosphamide-specific DNA–adducts in patients with cancer.

Patients and Methods

Five overweight/obese [body mass index (BMI) ≥25] and five lean (BMI<25) adult patients underwent HCT for hematological disorders at the University of Minnesota. All patients signed an informed consent form, which was approved by the University of Minnesota IRB Board (IRB#0610M94328). All participants received intravenous cyclophosphamide over 2 h at a constant infusion rate. Patients received one of the following disease-specific regimens: cyclophosphamide at 60 mg/kg/dose *i.v.* daily, given on days −6 and −5 plus 165 cGy total body irradiation (TBI) twice daily on days −4, −3, −2, −1 (n=1); cyclophosphamide at 50 mg/kg/dose *i.v.* on day −6 plus fludarabine at 40 mg/m^2 *i.v.* daily for 5 days, on day −6, −5, −4, −3, −2 and 200 cGY of TBI on day −1 (n=7); cyclophosphamide 50 mg/kg/dose IV on day −7 plus fludarabine 40 mg/m^2 IV daily for 5 days, on day −6, −5, −4, −3, −2 plus Campath at 0.2 mg/kg *i.v.* daily for 5 days on day −10, −9, −8, −7, −6 (n=1); cyclophosphamide at 60 mg/kg daily on days -7 and -6, fludarabine at 25 mg/m^2 daily *i.v.* on days −8, −7, −6 plus 165 cGy TBI twice daily on days −4, −3, −2, −1 (n=1).

Pharmacokinetic sampling was conducted with the first dose of cyclophosphamide. Cyclophosphamide and G-NOR-G adduct concentrations were measured in blood samples obtained at times 0 (prior to the start of infusion), 2, 6, 8 and 22 h after the end of the 2-h cyclophosphamide infusion. At each time point, 5 ml of blood were collected into tubes and placed on wet ice. Plasma was separated from blood cells by centrifugation and stored at −80˚C until analysis. Plasma cyclophosphamide concentrations were measured by high-pressure liquid chromatography (HPLC) using a validated assay with UV detection, as previously described (13). UV absorption for the internal standard (ifosphamide) and cyclophosphamide was recorded at 195 nm and was eluted at 19.2 and 22.2 min, respectively. The cyclophosphamide assay was linear from 0.01-20 μg/ml of cyclophosphamide and the lowest limit of quantification was 0.01 μg/ml. The accuracy and precision of the cyclophosphamide assay was 98.2% and 2.5%, respectively.

Cyclophosphamide-specific DNA adducts (G-NOR-G) were quantified by isotope dilution high-pressure liquid chromatography electrospray ionization (ESI)-tandem mass spectroscopy (HPLC-ESI-MS/MS), as described previously (13). In brief DNA was extracted using the Qiagen DNA extraction kits (Qiagen, Valencia, CA, USA), as per the manufacturer's instructions. DNA amounts were estimated by UV spectrophotometry and determined by HPLC-UV analysis of deoxyguanine (dG) in enzymatic hydrolysates as described by Malayappan *et al.* (13). DNA samples were spiked with [$^{15}N_{10}$]-G-NOR-G (internal standard for mass spectrometry) and subjected to neutral thermal hydrolysis (70˚C for 1 h) to release G-NOR-G as free base adducts. G-NOR-G and its internal standard were purified by solid-phase extraction. Solid-phase extraction recovery averaged between 90 and 95%. Fractions containing G-NOR-G and $^{15}N_{10}$- G-NOR-G were dried under nitrogen and the residues were reconstituted in 20 μl of 15 mM ammonium acetate buffer, pH 6.8 for capillary HPLC-ESI-MS/MS analysis. The lower limit of detection was 50 fmol, with an accuracy and precision of 93% and 7.0%, respectively.

Plasma cyclophosphamide and G-NOR-G adduct concentrations were analyzed by non-compartmental analysis using the standard software (WinNon Lin Professional 5.2; Pharsight Corp, Mountain View, CA, USA). Area under curve ($AUC_{0-\infty}$), and clearance (CL) were determined for cyclophosphamide and DNA adducts. The AUC was estimated by the trapezoidal rule. CL was determined *via* non-compartmental analysis. DNA adduct formation was also normalized to cyclophosphamide exposure by a ratio of G-NOR-G $AUC_{0-\infty}$ to cyclophosphamide $AUC_{0-\infty}$.

Comparisons of cyclophosphamide $AUC_{0-\infty}$, G-NOR-G adduct $AUC_{0-\infty}$, and the ratio of G-NOR-G $AUC_{0-\infty}$ to cyclophosphamide $AUC_{0-\infty}$ were assessed between lean and overweight/obese individuals. Statistical comparisons between lean and overweight/obese were made using two-way ANOVA.

Results

Five lean and five overweight/obese Caucasians were assessed for cyclophosphamide exposure and G-NOR-G adduct formation. The median (range) BMI of the lean subjects was 20 (19-24) kg/m^2, and the median (range) BMI in the overweight group was 29 (27-36) kg/m^2. There was no difference in age between the lean and overweight/obese groups with a median (range) of 51 (25-54) and 55 (42-56) years, respectively (p=0.24). The lean individuals underwent HCT for aplastic anemia (n=1), acute myelogenous leukemia (n=2), multiple myeloma (n=1) and non-Hodgkin's lymphoma (n=1). The overweight/obese individuals underwent HCT for acute lymphocytic leukemia (n=1), acute myelogenous leukemia (n=3), and chronic myelogenous leukemia (n=1). Time-to-donor stem cell engraftment, defined as absolute neutrophil count >500 cells/μl for three consecutive days, was not different in the two groups. Lean patients engrafted at a median (range) of 9 (6-25) days post- transplant, whereas the overweight/obese individuals engrafted at 6 (6-15) days. Treatment-related mortality of the lean patients was 0% at day 100 and 20% [95% confidence interval (CI)= 0-50%] at day 180 post-transplant. There was no treatment related mortality at day 100 or 180 in the overweight/obese group.

Pharmacokinetic parameters for cyclophosphamide in plasma following *i.v.* drug administration are given in Table I. Overweight/obese patients received approximately ~45% higher total daily cyclophosphamide dose than lean patients (p<0.01). Despite this, the median (range) cyclophosphamide $AUC_{0-\infty}$ was 60% lower in the overweight/obese *vs.* lean (529.2 *vs.* 867.9 μcg/ml*h) (p<0.001) (Table I). Median (range) cyclophosphamide plasma concentrations in the lean patients at 2, 6, 8 and 22 h after the end of the infusion were 66.62 (29.3-72.3), 46.6 (22.1-50.7), 37.2 (14.4-42.9), and 8.3 (0.65-11.4) μcg/mL, respectively. Median (range) cyclophosphamide plasma

Table I. *Cyclophosphamide and G-NOR-G exposure in lean and overweight/obese subjects.*

Patient	Weight (kg)	BMI kg/m^2	Total daily cyclophosphamide dose (mg)	Gender M/F	Cyclophosphamide AUC (μg/ml*h)	DNA adduct AUC (G-NOR-G/10^6 dG nucleotides*h)
Lean n=5						
1	74	24	3700	M	908.70	276.07
2	54.6	19	2750	F	867.99	19.03
3	60.78	23	3000	M	647.43	33.54
4	57	20	3400	F	961.05	147.04
5	60.4	20	3000	F	300.62	177.49
Median (range)			3000 (2750-3700)		867.99 (300.62-961.05)	147.04 (19.03-276.07)
Overweight/obese n=5						
1	94.1	27	5640	M	515.33	96.69
2	79.7	29	4000	M	529.24	142.83
3	91.7	28	4225	F	590.92	1176.76
4	88.4	29	4600	M	659.56	425.27
5	103.2	36	4450	M	463.00	50.81
Median (range)			4450 (4000-5640)		529.24 (463.00-659.56)	142.83 (50.81-1176.76)
p-Value*			<0.01		<0.01	0.59

**p*-Values are comparison of lean and overweight/obese pharmacokinetic measures.

concentrations in the overweight/obese patients at 2, 6, 8, and 22 h after the start of infusion were 60.93 (42.5-64.0), 34.4 (21.2-41.2), 26.45 (16.3-31.8), and 2.63 (1.85-4.63) μg/ml, respectively. Cyclophosphamide $AUC_{0-\infty}$ was inversely correlated with BMI with (R^2=–0.72, Figure 1A) and cyclophosphamide clearance was proportionally correlated with BMI (R^2=0.74, Figure 1B)

The post-dose $AUC_{0-\infty}$ G-NOR-G adducts found in the lean and overweight/obese patient groups were similar (147.04 *vs*. 142.83 G-NOR-G/10^6 dG nucleotides*h respectively, p=0.59). The median (range) concentrations at 2, 6, 8 and 22 h after the end of cyclophosphamide infusion of the lean patients were 1.23 (0.1-58.59), 0.99 (0.44-3.61), 1.39 (0.42-21.99), and 0.25 (0.11-0.816) G-NOR-G adducts/10^6 nucleotides, respectively. The concentrations of G-NOR-G in DNA of overweight/obese patients at 2, 6, 8, and 22 hours, concentrations were 8.01 (3.44-16.27), 12.96 (4.65-268.93), 4.86 (2.97-15.93), and 1.82 (0.03-13.56) G-NOR-G adducts/10^6 nucleotides, respectively. As expected, no adducts were detected before cyclophosphamide administration (time 0) in either group. After normalization of G-NOR-G adducts for cyclophosphamide plasma concentrations, twice the number of G-NOR-G adducts, were formed in overweight/obese patients as in the lean group (0.26 *vs*. 0.15 G-NOR-G $AUC_{0-\infty}$ to cyclophosphamide $AUC_{0-\infty}$ ratio, p=0.26, Figure 1C) than lean. Maximum observed G-NOR-G concentrations occurred between 2 and 6 hours after the completion of infusion. There was high (15-23-fold) interpatient variability in G-NOR-G AUC in both treatment groups. Cyclophosphamide plasma exposure was poorly correlated with G-NOR-G adduct formation (R^2=0.031, Figure 1D).

Discussion

Cyclophosphamide is widely used as an anticancer and immunosuppressive agent, and the dosage given to patients varies considerably depending on the indication. To our knowledge, this is the first study to report increased clearance of cyclophosphamide in overweight/obese individuals undergoing HCT and then further examine the effect of obesity on the formation of clinically relevant DNA–DNA adducts (G-NOR-G). The overall impact of obesity on the pharmacokinetics of chemotherapeutic agents is not well-understood, making exposure predictions difficult. In the case of cyclophosphamide prior to HCT, some protocols use actual body weight to determine dosing whereas others use ideal weight. Therefore, there is inconsistency in exposure of overweight/obese individuals and this may account for differences in clinical efficacy.

Dosing overweight/obese adults with cancer is an important and a clinically relevant problem. For example, studies evaluating the efficacy of combination chemotherapy (cyclophosphamide plus 5-fluorouracil or cyclophosphamide plus doxorubicin) in adult women with advanced breast cancer found that obesity was associated with decreased drug

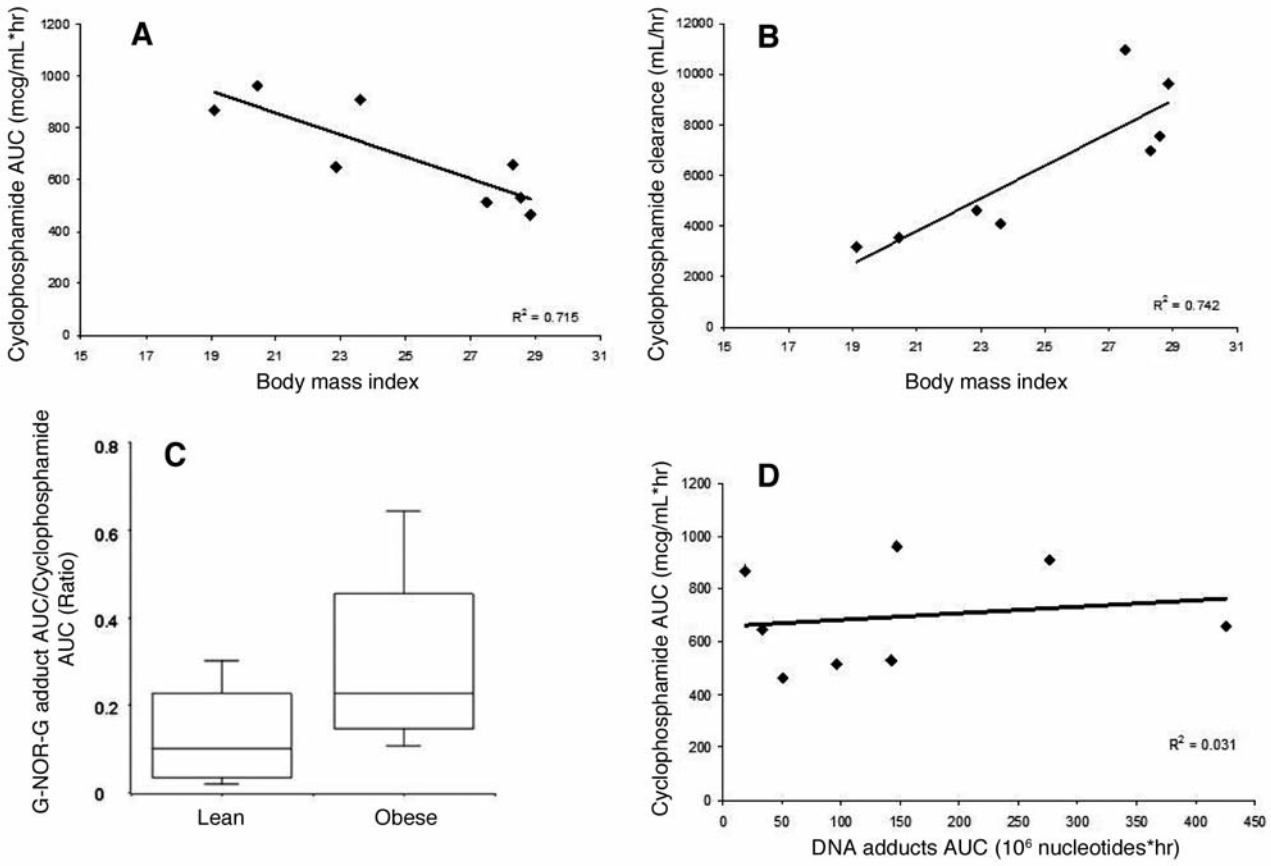

Figure 1. *A. The correlation between BMI and cyclophosphamide exposure. B. The correlation between cyclophosphamide clearance and BMI. C. The G-NOR-G/cyclophosphamide area under the curve (AUC) ratio in overweight/obese and lean subjects. Data are mean±S.D. D. The correlation between cyclophosphamide exposure and G-NOR-G adduct formation in all study subjects.*

clearance (2). Our data show that cyclophosphamide clearance is increased in overweight/obese patients. This is consistent with observations in an obese patient with metastatic breast cancer with high cyclophosphamide clearance, as evident by the elevated levels of 4-hydroxycyclophosphamide metabolite, a precursor to phosphoramide mustard, when compared to lean patients (3). Other chemotherapeutic agents that exhibit altered clearance in obese *vs*. healthy-weight cancer patients include cisplatin, paclitaxel, troxacitabine and doxorubicin (1). Cyclophosphamide induces cellular death by forming DNA adducts, in particular, the interstrand DNA–DNA crosslink (G-NOR-G), through the formation of phosphoramide mustard. Despite increased clearance of cyclophosphamide in the overweight/obese group we did not observe a significant difference in G-NOR-G cross-link formation. However, the trend suggests that in overweight/obese individuals, after normalization for cyclophosphamide plasma exposure, more G-NOR-G is formed (Figure 1C). We hypothesize that the increased clearance of parent cyclophosphamide leads to greater formation of the active metabolites and ultimately of G-NOR-G. The correlation between cyclophosphamide exposure and G-NOR-G was weak, suggesting that cyclophosphamide levels may not be clinically informative (Figure 1D). In patients with solid tumors receiving oxaliplatin, platinum- specific DNA adduct formation was poorly correlated with platinum blood concentrations (5). Previous studies have shown no association between cyclophosphamide pharmacokinetics and clinical outcomes after HCT (14). Because cyclophosphamide is a prodrug, it must be converted to active metabolites; therefore a better correlation may be observed between phosphoramide mustard concentrations and G-NOR-G formation. In this study, we were unable to measure the phosphoramide mustard metabolite due to its limited stability. Further studies are needed to examine the relationships between cyclophosphamide, its active metabolites, G-NOR-G formation , and clinical outcomes. We acknowledge that there are genetic variants which may affect cyclophosphamide disposition. For example genetic variants of cytochrome

(CYP) P450 monooxygenases involved in cyclophosphamide metabolism may contribute to the pharmacokinetic variability and may be linked to altered DNA adduct formation. Studies by Xie *et al*. demonstrated that genetic variants in the CYP2B6 enzyme resulted in an increased rate in 4-OH cyclophosphamide formation (15). However, a study by Ekhart *et al*., of select few variants in the cyclophosphamide metabolic pathway found that such variation did not explain the pharmacokinetic variability in patients (16) . One limitation of our study is that we were unable to determine the effect of variants on cyclophosphamide metabolism due to our limited sample size. Future studies should evaluate variants in drug metabolizing enzymes and DNA repair.

Our results suggest that cyclophosphamide clearance is significantly increased with increasing BMI. Interestingly, there was no difference in absolute G-NOR-G exposure between the overweight/obese and lean group, despite the higher doses given to the overweight/obese patients. However, once G-NOR-G concentrations were normalized for the plasma concentrations of cyclophosphamide, there was a trend for increased G-NOR-G concentrations in overweight/obese patients. Future studies are now necessary to determine the relationship between G-NOR-G formation and engraftment, treatment-related mortality, toxicity and disease response in a larger number of subjects. Identification and quantification of other drug-induced adducts (*e.g*. G-NOR-OH monoadducts and acrolein-DNA lesions) may also be an important determinant of cyclophosphamide toxicity.

Acknowledgements

We gratefully acknowledge the dedication and hard work of our coordinators Jill Nagorski and Pat Fidler. This work was supported by Children's Cancer Research Fund Minneapolis, MN, a Grant in Aid (L.J.) from the University of Minnesota Graduate School, and NCI grant RO1-CA-100670 (N.T.).

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Received April 13, 2012
Revised July 2, 2012
Accepted July 3, 2012