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COMMENTARY

G_2 BLOCK INDUCED BY DNA CROSSLINKING AGENTS AND ITS POSSIBLE CONSEQUENCES

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Numerous clinically used antitumor drugs as well as other antitumor agents have been studied for their effects on the cell cycle of normal as well as tumor cells.

According to the concept of Howard and Pelc [1], the cell cycle is divided into four consecutive phases: G_1, S, G_2 and M. The S and M phases represent the distinct time periods in a cell cycle when DNA synthesis or mitosis occurs, while G_1 and G_2 phases correspond to the "gaps" in time between M and S and S and M respectively. The knowledge of the stage at which the cells stop or slow down their progress through the cell cycle is of primary importance for the understanding of drug antiproliferative action. Though this information can be obtained by a variety of methods, the studies of drug effects on the cell cycle have become increasingly frequent during the last decade after the development of a very efficient technique of flow cytometry [for review see Ref. 2]. Other methods [for review see Ref. 3] for analyzing drug action on the cell cycle have been employed mainly in earlier studies.

Among antitumor agents studied to date, almost all classes of DNA crosslinking compounds are represented. The published data demonstrate that all of these drugs produce qualitatively similar effects on the cell cycle, preferentially inducing accumulation or arrest of cells in G_2 phase of the cell cycle (G_2 block). Nevertheless, even the most recent papers on this subject seem to neglect the question of the relevance of the G_2 block to the antiproliferative action of crosslinking agents. This question is discussed later in this commentary.

In Table 1 the available data on the cell cycle effects of DNA crosslinking agents are summarized. Studies on the preferential toxicity of drugs for cells in the various stages of the cell cycle are not covered by this summary. The cells in G_1, at the G_1/S boundary, and in M phases were found, in general, to be more sensitive to the lethal action of crosslinking agents [for review see, for example, Refs. 4 and 5]. This is, however, a separate issue which remains beyond the scope of this commentary. Table 1 includes the data for adriamycin and daunomycin, which have been shown recently to induce interstrand crosslinks in cellular DNA [6]. Very recently we have found that another drug included in Table 1, mitoxantrone, is also able to form interstrand DNA crosslinks in cultured HeLa S_3 cells [7].

As shown in Table 1, the cell cycle effects of DNA crosslinking agents apparently have a lot of common characteristic features, depending on drug dose and time of treatment. At low drug levels, a slow-down of cell traverse through S-phase along with some accumulation of G_2 phase cells are usually observed [e.g. 11, 14, 22, 29, 35, 40, 55]. Both effects and especially S block seem to be readily reversible upon prolonged incubation of the cells [e.g. 11, 13, 15, 35, 40]. Increased drug doses cause progressive enhancement of cell accumulation in G_2 phase leading to G_2 arrest of the majority of cell population [e.g. 11, 13, 19, 29, 35, 40, 45, 56].

The G_2 block seems to be most obvious and persistent in the case of agents exhibiting the highest antitumor efficacy (in terms of the magnitude of the increase of life span or the decrease of tumor weight in tumor bearing animals). The most efficient antitumor DNA crosslinking drugs, e.g. mitomycin C [29], *cis*-platinum [35], or adriamycin [40], were reported to induce an essentially irreversible G_2 block that persisted for several days. On the other hand, the G_2 arrest caused by melphalan [13, 16], a drug of moderate antitumor efficacy, was partially reversible, whereas Yoshi 864, which is relatively inefficient as an antitumor drug, produced a G_2 block that was reversible upon post-incubation of the cells in drug-free medium [24]. The persistence of the G_2 block depends also on the drug dose and duration of drug treatment, and even in the case of such a very potent drug as adriamycin some cells may escape from G_2 arrest when exposed to the drug given at a relatively low concentration for a short period of time [40].

It should be stressed that, in general, the range of drug concentrations causing "pure" G_2 block seems to be very narrow. This has been particularly well documented in the case of *cis*-platinum [35]. The drug levels exceeding the range which produces G_2 block induce additionally S phase arrest, and even higher drug concentrations produce also G_1 block, freezing thereby completely the cell traverse through the cell cycle [21].

The same sequence of blocks appearing in response to increasing drug concentrations was also observed when the cells were incubated with DNA crosslinking agents for various times. Higher drug levels given for shorter periods were found to be equivalent in their effects to lower drug con-

Table 1. Influence of interstrand DNA crosslinking agents on cell cycle progression

Compounds	Cells*	Method†	Effects/Remarks	Reference(s)
Nitrogen mustard	Ehrlich ascites tumor (EAT), *in vivo*	MI, A	G_2 block after a single dose (25 μg/mouse); inhibition of DNA synthesis as a secondary effect	8
	EAT, *in vivo*	MI, FLM, CM	G_2 block after single dose (0.3, 0.6 or 1.2 mg/kg)	9
	H.Ep. No. 2	FLM	G_2 block, dose-dependent	10
	Normal human fibroblast	FCM	S delay followed by G_2 block (at 0.1 to 1 μg/ml)	11
	Fanconi anemia human fibroblast		G_2 block (at 0.01 to 0.1 μg/ml)	
	Human lymphoblastoid Raji and TK 6 cell lines	FCM	Dose-dependent delay in S phase traverse followed by an accumulation of cells in G_2 phase	12
Melphalan	Ehrlich ascites tumor/ 3 lines/*in vivo*	CM, MI	G_2 block (at 1–5 μg/ml); reversible after prolonged post-incubation	13
	T_1 human lymphoma	FCM	Concentration- and exposure time-dependent S delay and G_2 block (at 0.5 to 5 μg/ml)	14, 15
	RPMI 6410	FCM	G_2 block (at 1 μg/ml); reversible after short exposure time	16
Peptichemio	T_1 human lymphoma	FCM	Concentration- and exposure time-dependent S delay and G_2 block (at 0.5 to 10 μg/ml, 1–48 hr); S block at higher concentration	15, 17
Cyclophosphamide	M 5076 ovarian reticuluar sarcoma, *in vivo*	FCM	G_2 block in sensitive tumor, transient S and G_2 blocks in resistant tumor subline (at 200 mg/kg single dose)	18
Nitrosoureas				
BCNU, CCNU, Me-CCNU	CHO (Chinese hamster ovary)	FCM	Dose-dependent late S and G_2 blocks	19
BCNU, CCNK, Me-CCNU	CHO	CC	Terminal point at late G_2	20
CCNU	CHO	PCC	G_2 block (at 50 μg/ml, 1 hr)	21
BCNU	9L Rat brain tumor	FCM, PCM	S delay followed by G_2 block (1 or 5 μg/ml)	22
CCNU	KHT sarcoma, *in vivo*	FCM	G_2/M block, accumulation of 47% of cells at 48 hr after a single dose of CCNU (7.5 mg/kg)	23
Yoshi 864	T_1 human lymphoma	FCM	Concentration- and exposure time-dependent S delay and G_2 block; reversible	15, 24
Mitozolomide	Lewis lung carcinoma, *in vivo*	FCM	Accumulation of cells at G_2/M 24 hr after drug treatment, reversed 94 hr after inactive dose (10 mg/kg) but not after effective dose (40 mg/kg)	25
Spirohydantoin mustard	9L Rat brain tumor	FCM, PCM	S delay followed by G_2 block (at 5–20 μg/ml, 1 hr)	26, 27
	9L—Spheroids	FCM	S delay followed by G_2 block for cycling (50%) cells (at 1.5 to 6 μg/ml)	28

Table 1—*continued*

Compounds	Cells*	Method†	Effects/Remarks	Reference(s)
Mitomycin C	LoVo human colon adenocarcinoma	FCM	Concentration- and exposure time-dependent S delay followed by G_2 block. At higher concentrations also block in S and G_1 (at 0.1 to 5 μg/ml, 1–24 hr)	29
	Fanconi anemia, 8 lines	FCM	G_2 block (at 0.05 to 0.1 μg/ml, 24 hr)	30
	CHO	MCS	Terminal (transition) point at G_2, dose-dependent	31
Porfiromycin (*N*-methyl mitomycin)	HEP-2, human epidermoid carcinoma	MI, PCM	G_2 block (at 0.1 μg/ml, 4 hr)	32
cis-Dichlorodiammineplatinum (II)	Ehrlich ascites tumor, *in vivo*	CM	G_2 block still persisting on day 6 after a single dose (4.5 mg/kg)	33
	Chick embryo fibroblast, freshly prepared	CM	G_2 block (at 0.5 to 5 μg/ml, 24 hr)	34
	LoVo human colon adenocarcinoma	FCM	Concentration- and exposure time-dependent S delay, followed by irreversible G_2 block; at higher concentration also blocks in S and G_1 (at 0.5 to 10 μg/ml, 1–48 hr)	35
	Ehrlich ascites	FCM	G_2 block, reversible at low concentration; at higher concentration also blocks in S (at 5×10^{-7} to 5×10^{-5} M, 4–48 hr)	36
	Zea Mays L root meristematic	CM	G_2 block (at 5 μg/ml, 24–48 hr)	37
Adriamycin	HeLa S3	MI	Inhibition of G_2 progression (at 0.1 μg/ml)	38
	CHO	LF, MI	G_2 block (at 0.4 to 2 μg/ml)	39
	T_1 human lymphoma	FCM	Concentration- and exposure time-dependent delays in G_1, S and G_2, followed by G_2 block, reversible at low concentration and irreversible at higher concentration (at 0.05 to 0.5 μg/ml, 1–48 hr)	40
	CHO	FCM, CC	G_2 block, terminal point located 72 min prior to prophase (at 1–10 μg/ml, 2 hr)	41
	CHO	MCS	Dose-dependent terminal (transition) point at S/G_2 boundary for 0.1 μg/ml, and late G_2 for higher concentration	31, 42
	CHO synchronized	FCM	G_2 block (at 1 μg/ml, 1 hr)	43
	CCRF-CEM human lymphoblast	FCM	Cell accumulation at late S and G_2 (at 0.1 to 0.5 μg/ml), inhibition of cell cycle traverse at higher concentration	44, 45
	CHP-100 human neuroblastoma	FCM, MI	Concentration- and exposure time-dependent G_2 block (at 10–100 μg/ml, 1–24 hr)	46
	Sarcoma 180	FCM	G_2 block (at 0.2 and 2 μg/ml, 1 hr)	47
	V 79 hamster	FCM	G_2 block (at 0.2 to 4 μg/ml, 1 hr)	48
	P 388 mouse leukemia, *in vivo*	FCM	Cell accumulation in S and G_2 after 12 hr of drug treatment (4 mg/kg)	49

Continued

Table 1—*continued*

Compounds	Cells*	Method†	Effects/Remarks	Reference(s)
Daunomycin	Ehrlich ascites	FCM	G_2 block (at 10–100 μg/ml)	50
	HeLa S3	MI	Inhibition of G_2 progression	38
	CHO	LF, MI	G_2 block (at 0.5 to 4 μg/ml)	39
	Chick and rat embryo fibroblast	CM, MI	G_2 block (at 0.5 μg/ml)	51
	L-929 mouse fibroblast	FCM	G_2 block (at 0.05 μg/ml, continuous exposure or at 1 μg/ml, 4 hr) in synchronized cultures	52, 53
	Ehrlich ascites tumor, *in vivo*	CM	G_2 block after a single dose (25 μg/mouse), reversible after a few days	54
	CHO	FCM	Concentration- and exposure time-dependent delay in S, followed by G_2 block (at 2×10^{-8} to 2×10^{-5} M, 2–24 hr)	55
Dihydroxyanthraquinone, mitoxantrone	FL 745 Friend leukemia, L1210 mouse leukemia, CHO	FCM	Concentration-dependent G_2 arrest at dose range of 1–10 μg/ml. Terminal point for FL cells at late G_2	56, 57
	CHO	MCS	Dose-dependent terminal (transition) point at S/G_2 boundary and at G_2/M boundary for 10^{-4} μg/ml and 100 μg/ml respectively	58
Psolarens				
3,5′-8-Trimethylpsolaren and UV	S-91 Cloudman murine melanoma	FCM	G_2 block (at 2×10^{-7} M, UV 0.3 J/cm^2)	59
	S-91 Cloudman, CRL-1295 diploid human skin fibroblast	FCM	G_2 block (at 2×10^{-7} M, UV 1 J/cm^2)	60
8-Methoxypsolaren and UV	FM3A mouse mammary carcinoma	FCM	G_2 block (at 0.1 μg/ml, UV 5 kJ/m^2 or at 1 μg/ml, UV 1 to 2.5 kJ/m^2)	61
4′-Hydroxymethyltrioxalen and UV	S-91 Cloudman	FCM	Concentration-dependent G_2 block (at 1–3 μM, UV 0.3 J/cm^2), S and G_1 block at higher concentration	62

* The cited studies were performed under *in vitro* conditions unless indicated otherwise.
† MI, mitotic index; A, autoradiography; CM, cytometry (static); FLM, fraction of labeled mitosis; FCM, flow cytometry; CC, cell counting; PCC, premature chromosome condensation; PCM, pulse chase method; MCS, mitotic cell selection; and LF, labelled fraction.

centrations present during prolonged incubation [e.g. 35, 40].

It is striking, however, that all the DNA crosslinking drugs tested to date for their cell cycle effects were found to induce G_2 block. Thus, it seems justified to suggest that induction of G_2 block represents a typical cellular effect of DNA crosslinking compounds.

The 9-amino-1-nitroacridines, a new class of DNA crosslinking agents [63, 64], seem to be an exception since they preferentially induce accumulation of HeLa S_3 cells in S phase [65]. However, lysis of the treated cells, observed at higher concentrations of 9-amino-1-nitroacridines, may preferentially affect the cells in one particular phase, thereby concealing a block in other than S phases of the cell cycle.

The mechanism of G_2 block induced by various antitumor agents remains largely obscure. By using a premature chromosome condensation technique, Rao [66] found that G_2 arrest caused by a number of antitumor drugs, including several DNA crosslinking compounds, is associated with extensive chromosome damage, such as gaps and breaks. This author also found a direct correlation between the chromosome damage and cell accumulation in G_2 [66]. Further, Rao has suggested that the cells arrested in G_2 by drug action are depleted of mitotic factors, specific proteins required for mitotic transition [for review see Ref. 67].

Crosslinking agents usually induce many more single-strand DNA lesions than interstrand crosslinks [for review see Refs. 68 and 69]. The latter lesion, however, is considered more lethal and only a few crosslinks per cell are sufficient for inhibition for cell proliferation or even for cell death [e.g. 63, 68]. Such a small number of DNA crosslinks is not sufficient to cause a measurable inhibition of DNA replication but, conceivably, it could suffice for G_2 arrest induction, e.g. by preventing the strand segregation (the separation of the two copies of replicated DNA) and thus disturbing the formation of specific higher order chromatin structure which is a prerequisite for the cell to enter the mitotic phase. The recent studies by Sognier and Hittelman [70] have demonstrated that the cells damaged by the crosslinking agent mitomycin C undergo chromosome condensation even though their DNA synthesis is not fully completed, which in turn leads to the deletions in the unreplicated sites of genome. Although single-strand lesions and some secondary mechanisms, e.g. DNA repair, may contribute to the generation of G_2 block, their role is likely to be limited.

The hypothesis that interstrand crosslinks, as opposed to other lesions, play a crucial role in the mechanism of G_2 block induced by DNA crosslinking agents seems to be worth investigating in detail in specially designed studies. If this hypothesis is true, one may expect that persistence of G_2 block will be parallel to the stability of the crosslinks in cellular DNA. Yoshi 864, an agent which induces easily reversible G_2 block [24], may be useful in this kind of studies.

The numerous papers on G_2 block induction by DNA crosslinking agents reviewed in this article allow one to draw some general conclusions concerning the mechanism of cytotoxic and antineoplastic action of this class of antitumor drugs. It has been commonly observed that interstrand DNA crosslinks give rise to the impairment of DNA synthesis. The latter effect is widely believed to be responsible for inhibition of cell proliferation and eventually for cell killing by crosslinking agents. On the other hand, the observation that DNA crosslinking compounds induce G_2 block at lower concentrations than S phase block implies that under certain conditions these agents are able to inhibit cell proliferation without considerably affecting DNA synthesis. Such a straightforward interpretation seems to have been overlooked in the literature, though it stems from the obvious fact that the cells entering G_2 phase, to become arrested at this stage, have already completed or almost completed their DNA synthesis. Hence, G_2 arrest which precedes S phase block can be considered as a major factor in antiproliferative action of DNA crosslinking drugs even it its role is limited only to a certain narrow range of treatment conditions. This also means that inhibition of DNA synthesis leading to S phase block could become a major cause of growth inhibition only at higher drug concentrations or after a prolonged treatment. G_1 block induced by crosslinking agents is of negligible importance to growth inhibition since it occurs only at very high drug concentrations.

The higherto reviewed observations on cell cycle effects of DNA crosslinking drugs come almost exclusively from the studies performed on cultured cells *in vitro*. The relevance of the mentioned findings to the situation *in vivo* remains to be established. However, the fact that the G_2 block is the first (in terms of drug level) cause of growth inhibition *in vitro* tempts one to hypothesize that the maximal antitumor effects observed at optimal curative drug doses are mainly due to the induction of G_2 block rather than to the inhibition of DNA synthesis. The studies *in vivo* are scarce, and most of them have been carried out on ascites tumors (chiefly Ehrlich ascites tumor) where the drugs were given intraperitoneally [8, 9, 13, 15, 49, 54]. It should be pointed out that the conclusions from such studies are of limited value since the model where tumor cells grow in peritoneal fluid and the drug is administrated directly to tumor cell suspension provides conditions resembling closely those in tissue culture *in vitro*. In this light, it seems especially interesting to note that a few studies performed *in vivo* on solid tumors have provided support for the mentioned hypothesis. In the case of cyclophosphamide, D'Incalci *et al.* [18] have found that this drug clearly induces G_2 block in ovarian reticular sarcoma (M 5076) cells when given to tumor-bearing mice at doses required to produce the maximal increase of animal life span. This incidental finding remained beyond the main focus of these studies and has not been commented on by authors [18]. In another study performed *in vivo* on KHT in mice, a nitrosourea derivative, CCNU, has been shown to induce G_2 block and to cause maximal increase of life span at the same dose [23]. Very recently Broggini *et al.* [25] have showed that mitozolomide, a new DNA crosslinking agent, induces G_2 block in solid Lewis lung carcinoma cells in mice at therapeutic doses.

These three publications suggest that G_2 block may contribute to the *in vivo* antitumor effects of DNA crosslinking agents. However, the additional verification of this hypothesis would be provided by specially designed and more detailed studies on the influence of other DNA crosslinking compounds, especially those with high antitumor activity, on the cell cycle in solid tumors *in vivo*. The above hypothesis is supported also by the previously mentioned *in vitro* observation that the drugs of very high antitumor efficacy produce persistent G_2 block, whereas less effective agents seem to induce only transient G_2 arrest. In the latter case, it seems plausible that repeated or relatively higher drug doses (which may be required for maintenance of growth inhibition) could give rise to enhanced toxic side effects, thereby decreasing overall antitumor efficacy.

It should be stressed that the suggested mechanism of *in vivo* tumor growth inhibition by G_2 arrest may well be limited only to a narrow range of doses (presumably close to, or lower than, optimal dosage). Beyond this range and, perhaps, even within it, some other mechanisms, e.g. inhibition of DNA synthesis, are likely to contribute to tumor growth inhibition. This seems particularly likely in the case of DNA crosslinking agents that are less efficient as antitumor drugs.

The implications of G_2 block for the mechanism of antitumor action have been apparently overlooked in the literature not only for DNA crosslinking agents but also for other classes of antitumor DNA-damaging agents that are known to block cell cycle traverse in G_2 phase. The question why the G_2 phase is such a common blockage point remains unresolved at present. The G_2 phase involves dynamic, extremely complex changes in chromatin structure at several levels of its organization. Segregation of daughter DNA molecules and subsequent condensation of chromatin before mitosis comprise the entire genome (as opposed to replication which occurs locally at a given time). It is conceivable that these processes can be dramatically disturbed even by a relatively low number of lesions. This could be the reason why other DNA-damaging agents (e.g. strand-scission drugs such as bleomycin) also induce G_2 block.

Though further extensive studies are required to provide the information lacking about the mechanism and biological role of G_2 block induced by DNA crosslinking agents, the confirmation of the hypothesis presented may have considerable significance for tumor chemotherapy in general, and especially for the use of these agents in combination with other drugs.

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