Gene and human tumour cell line specific differences in nitrogen mustard induced DNA alkylation and interstrand crosslinking frequencies

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ABSTRACT

The levels of N-alkyl purine and DNA interstrand crosslink formation, produced by the clinically used nitrogen mustard antitumour drug mechlorethamine (HN2), were quantitated at the level of specific genes in a panel of human tumour cell lines using modified Southern blotting methods. When purified genomic DNA was treated with HN2 in vitro, no significant difference in the extent of N-alkyl purine or interstrand crosslink formation in the N-ras, c-myc or CD3δ genes was observed. When the cell lines LS174T, Colo320HSR, J6 and U937 were treated with HN2, however, there was significant heterogeneity in the levels of N-alkyl purine formation in the three genes. The rank order of the extent of damage in the three genes was also different in the cell lines. The level of alkylation did not correlate with either the transcriptional activity of a gene or drug sensitivity. Crosslinks were not detectable in the N-ras or c-myc genes of LS174T, J6 or U937 cells treated with HN2, and only detectable in the amplified c-myc gene of the Colo320HSR cell line. In the related cell line Colo320DM, which has both native and translocated c-myc alleles which are both amplified and episomal, crosslinks were detected in the amplified native and rearranged c-myc alleles, and also in the N-ras gene which is also amplified in this cell line. For bifunctional alkylating agents such as HN2, therefore, heterogeneity of DNA damage can occur between different genes in human cells and can also vary for different lesions produced by the same agent. In addition, this heterogeneity can differ between human tumour cell lines.

INTRODUCTION

Nitrogen mustards are potent alkylating agents, and are one of the main classes of clinically used anti-cancer drugs (1). The critical cellular target of these drugs is DNA, which is alkylated primarily at the N7 position of guanine with lesser reaction at the N3 position of adenine (2–4). Nitrogen mustards are bifunctional and have the ability to produce crosslinks on DNA (1,4). Although DNA interstrand and DNA–protein crosslinks can be produced, the DNA interstrand crosslink formed between the two complementary strands of DNA is believed to be the major cytotoxic lesion (4–6). A major problem with anti-cancer chemotherapy is the development of drug resistance. In the case of nitrogen mustards this can be due to reduced drug uptake (7,8), detoxification by glutathione (9), or enhanced DNA repair of critical DNA lesions (10).

For DNA lesions such as those produced by UV irradiation, DNA repair is heterogeneous throughout the genome as a result of gene-specific DNA repair and transcription-coupled repair (11,12). Gene-specific repair is characterized by the observation that DNA damage in actively transcribed genes is repaired more rapidly than inactive genes or non-coding regions of the genome, whilst transcription-coupled repair is distinctive in that the transcribed strand of an active gene is repaired at a faster rate than a non-transcribed strand. Gene-specific repair is thought to be mediated largely by the structure of chromatin, whilst transcription-coupled repair is dependent on RNA polymerase II (11,12).

Although less is known about drug-induced DNA damage and its repair there is evidence of heterogeneity in the distribution of DNA damage and repair of some anti-cancer drug lesions. For example, Wassermann et al described a Southern blotting technique that allowed the detection of the alkylation produced by the nitrogen mustard mechlorethamine (HN2) in specific genomic regions (13). In Chinese hamster ovary (CHO) cells there was heterogeneity of HN2-induced DNA damage, with active genes being damaged more than the bulk genomic DNA (13). Repair of HN2 damage was also demonstrated to be faster in the active genes than in the genome as a whole, or in non-coding sequences (13). More recently, repair of HN2 alkylations was shown to proceed more rapidly in the DHFR gene in the CHO cell line in the absence of α-amamin (an RNA polymerase II inhibitor), indicating that RNA polymerase and transcription coupling are involved in the repair (14).

Heterogeneity in the distribution of DNA interstrand crosslinks has also been observed (15). Using a denaturation/renaturation-based Southern blotting technique it was demonstrated that
HN2-induced crosslinks were only detectable in an amplified c-myc gene and not in the single copy N-ras gene of human colon tumour Colo320HSR cells (15). The interstrand crosslinks in the c-myc gene were repaired at a faster rate than those in bulk genomic DNA (15). It has also been shown that interstrand crosslinks produced by chemotherapeutic agents were repaired faster in an active region of the DHFR locus than an inactive 5′ flanking region in CHO cells (16). Both guanine-N7 and adenine-N3 alkylations, as well as the interstrand crosslinks produced by HN2 were repaired faster in the DHFR gene than in the rRNA gene of CHO cells (17).

Heterogeneity in DNA damage and repair may be an important determinant of sensitivity of tumour cells to nitrogen mustards. In the present study, heterogeneity of the levels of alkylation and interstrand crosslinking was demonstrated not only between three genes within a single cell line, but also between the same genes in a panel of human tumour cell lines.

MATERIALS AND METHODS

Materials were of analytical grade and purchased from Sigma Chemical Co. (Poole, UK) unless stated otherwise. All tissue culture reagents and restriction enzymes were purchased from Life Technologies (Paisley, UK).

Cell culture

The human colonic adenocarcinoma cell line LS174T was cultured in Iscoves modified MEM containing 10% fetal calf serum (FCS) at 37°C. Cells were passaged following trypsinization. The two human colon carcinoma cell lines Colo320DM and Colo320HSR, the human histiocytic lymphoma cell line U937, and the human leukaemic T cell lymphoblast cell line J6, were cultured in RPMI 1640 medium supplemented with 5 mM glucose and 10% FCS. These cells were passaged by dilution.

Measurement of N-alkyl purine formation in specific gene sequences by Southern blotting

The alkylation of DNA by HN2 in specific gene sequences was measured by a Southern blotting method originally published by Wassermann et al. (13) but modified in this laboratory to include the use of α satellite DNA as an internal standard (18).

Briefly, cells were washed twice in serum-free medium and then treated with drug in serum-free medium. Following drug incubation cells were washed twice with 25 ml of ice-cold PBS, and lysed in 9 ml lysis buffer (400 mM Tris–HCl, 60 mM EDTA, 150 mM NaCl, 1% w/v SDS, pH 8.0). DNase-free RNase A was added to a final concentration of 10 µg/ml, and incubated at 37°C for 1 h. Three millilitres of 5 M sodium perchlorate were added and the mixture was mixed by inversion at room temperature for 20 min and then incubated at 65°C for 30 min. The samples were then cooled in an ambient water bath for 5 min and extracted twice with 15 ml of chloroform. After centrifugation at 2500 g for 20 min, 10 ml of the aqueous supernatant was removed and DNA precipitated by the addition of 25 ml absolute ethanol at −20°C. The genomic DNA was then washed twice in 70% ethanol and dried. DNA was dissolved in water and digested with EcoRI, extracted with an equal volume of chloroform, and ethanol precipitated. The DNA was washed twice in 70% ethanol, dried and resuspended in 2 ml of sterile deionized water. The DNA was then quantified by determining the absorbance at 260 nm using a Pharmacia Gene Quant (Pharmacia, Milton Keynes). Aliquots containing 30 µg DNA were lyophilized overnight. The DNA samples were dissolved in 30 µl of sterile deionized H2O and heated at 70°C for 30 min to depurinate N-alkylated bases. Apurinic sites were converted to single-strand breaks by the addition of 3 µl of freshly prepared 1.1 M NaOH for 30 min at 37°C. Gel loading buffer (4 µl of 10×) was then added to each sample, and the samples were electrophoresed in a 0.6% agarose gel containing 0.05 µg/ml of ethidium bromide in TAE at 2 V/cm for 16 h. The gel was transferred onto Hybond N* (Amersham Life Sciences, Little Chaflont, UK) using the inverted alkaline Southern blotting procedure (19) and hybridized to a specific radiolabelled probe according to manufacturer’s instructions. After autoradiography, the membranes were stripped (according to manufacturer’s instructions), and serially hybridized to other radiolabelled probes. The c-myc gene was detected using the c-myc cDNA insert of the plasmid pSKmyc (a kind gift of Dr Jim Woodgett, University of Toronto) as a probe. The N-ras and CD3δ genes were detected by using BamHI–EcoRI fragments of plasmids pKS8.87 and pt3.4.37, respectively (pt3.4.37 was a kind gift of Dr Brian Flanagan, University of Liverpool). The human α satellite sequence was detected using the oligonucleotide 5′-CAG CAA GAA CCT GCT AAG CAG CAG-3′, 5′-end labelled with [γ-32P]ATP (18). The gene fragment sizes detected following EcoRI digestion of the genomic DNA were: c-myc, 12.5 kb; N-ras, 8.8 kb; CD3δ, 14 kb; α satellite, 342 bp. Autoradiographs were scanned using a Bio-Rad imaging densitometer and band volumes were determined. The number of lesions per fragment (L/F) were calculated using the equation (13):

\[ \text{L/F} = \log_{10} (\text{fraction of undamaged DNA}) \]

Measurement of DNA interstrand crosslinks

For the detection of DNA interstrand crosslinks, cells were treated as above, except that the pH of the lysis buffer was 10.4. DNA interstrand crosslinks were measured using the Southern blotting method of Futschler et al. (15), but with inverted alkaline bloting and hybridization as above.

Cytotoxicity assays

The cytotoxicity of HN2 against the adherent LS174T cell line was determined by the sulphorhodamine B (SRB) assay as detailed previously (6). The cytotoxicities of Colo320HSR, Colo320DM, J6 and U937 were determined by the MTT assay (20).

RESULTS

N-alkyl purine formation in genomic DNA treated in vitro with HN2

The formation of nitrogen mustard induced N-alkyl purine lesions in genomic DNA was initially evaluated using purified DNA treated in vitro. Aliquots of human LS174T DNA were treated with various concentrations of HN2 for 30 min at 37°C. HN2-induced lesions were then converted to strand breaks by neutral depurination followed by alkaline hydrolysis. The DNA was size-fractionated using a 0.6% agarose gel. Southern blotted and hybridized sequentially with probes specific for the CD3δ, N-ras, c-myc genes and human α satellite. The resulting autoradiographs in Figure 1 show the HN2 dose-dependent loss of signal from the full-length single-stranded band as a result of N-alkyl purine...
HN2-induced DNA damage in the same three genes was determined in the human LS174T, Colo320HSR, J6 and U937 cell lines. All these cell lines express the N-ras and c-myc genes, whilst only the T cell lymphoma cell line J6 expresses the CD3δ gene. This was confirmed using northern blotting and RT–PCR analysis (data not shown). The Colo320HSR cell line has an amplified c-myc gene.

In all cases cells were growing exponentially when they were treated with HN2 for 30 min in serum-free media. Genomic DNA was prepared and N-alkyl purines converted to strand breaks prior to Southern analysis. A representative set of autoradiographs derived from the same Southern blot containing DNA from HN2-treated Colo320HSR cells sequentially hybridized with probes specific for the CD3δ, c-myc and N-ras genes, as well as the α internal standard sequence, is shown in Figure 3. The full-length band for each gene decreases in a dose-dependent manner relative to the untreated control bands. At 100 μM the majority of signal had gone from the gene fragments but was still evident for the cDNA which was only significantly reduced at 200 μM. Scanning densitometry was performed on the autoradiographs where the response of the film was still in the linear range. The data for the N-alkyl purine formation in the CD3δ, c-myc and N-ras genes of the four cell lines is summarized in Figure 4. The data is expressed as lesions/10 kb to allow direct comparisons between the amount of damage in each gene to be made. The extent of DNA damage is summarized in Table 1. Clearly, as expected, the extent of DNA damage was much less in the gene sequences from drug-treated cells (Fig. 4) than treated naked genomic DNA (Fig. 2).

Unexpectedly, however, there was a clear heterogeneity in the extent of monoalkylation damage in the three genes in all of the cell lines, and the ranking order of damage was different in different cell lines (Fig. 4; Table 1). For example, the c-myc gene sustained approximately twice the alkylation of the CD3δ and N-ras genes in the LS174T cell line, whereas both the c-myc and CD3δ genes contained many more alkylations than the N-ras gene in the Colo320HSR cell line. In the J6 cell line, which is the only one to express the CD3δ gene, the alkylation of this gene was significantly lower than the c-myc gene. In contrast, alkylation in the CD3δ gene was greater than in the N-ras and c-myc genes of the U937 cell line.

Table 1. Extent of N-alkyl purine formation produced by 50 μM HN2 in four human tumour cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lesions/10 kb&lt;sup&gt;a&lt;/sup&gt;</th>
<th>c-myc</th>
<th>CD3δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS174T</td>
<td>0.69 ± 0.01</td>
<td>2.5 ± 0.42</td>
<td>1.14 ± 0.05</td>
</tr>
<tr>
<td>Colo320HSR</td>
<td>0.23 ± 0.11</td>
<td>1.18 ± 0.12</td>
<td>1.63 ± 0.16</td>
</tr>
<tr>
<td>J6</td>
<td>0.8 ± 0.08</td>
<td>2.08 ± 0.42</td>
<td>0.65 ± 0.08</td>
</tr>
<tr>
<td>U937</td>
<td>0.76 ± 0.07</td>
<td>1.5 ± 0.3</td>
<td>2.38 ± 0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are the means ± SEM of three independent experiments.
HN2-induced DNA interstrand crosslinking in genomic DNA treated in vitro

The extent of DNA interstrand crosslinking in the N-ras and c-myc genes was measured in naked genomic DNA treated with HN2, using a modification of the Southern blotting method of Futscher et al. (15). In this case, alkylations are not converted to strand breaks and DNA is denatured prior to gel electrophoresis and Southern blotting. Under the conditions employed, gene fragments containing DNA interstrand crosslinks re-anneal in the gel and migrate as double-stranded DNA. Uncrosslinked material remains denatured and migrates as single-stranded DNA. Figure 5 shows the formation of DNA interstrand crosslinks in the N-ras gene with increasing dose of HN2 following treatment of genomic DNA for 30 min at 37°C. Crosslinks are evident at 1 µM, and by 10 µM the majority of fragments contain crosslinks. For comparison, the extent of N-alkyl purine formation in the N-ras gene at the same doses is shown. Clearly, many more monoalkylation events are evident than interstrand crosslinks at each dose. The extent of both monoalkylation and interstrand crosslinking in the N-ras and c-myc genes are shown in Figure 6. When the data are normalized for the size of the restriction fragment there is no significant difference in the extent of alkylation or interstrand crosslinking between the two genes. In each case the yield of interstrand crosslinks in vitro is ~15% of total N-alkyl purines.

DNA interstrand crosslink formation in the N-ras and C-myc genes in HN2-treated cells

No evidence of double-stranded (crosslinked) DNA was evident in the N-ras or c-myc genes from LS174T, U937 or J6 cells treated with HN2 with doses up to 100 µM. Crosslinking was detected, however, in the amplified c-myc gene of Colo320HSR cells, but not in the single copy N-ras gene of these cells (Fig. 7a). Since crosslinking was only detected in the amplified gene in Colo320HSR cells it was also assessed in the related Colo320DM cell line. This latter cell line has both native and translocated c-myc alleles which are both amplified and episomal (21). Crosslinks were detected in the amplified native and re-arranged
Figure 5. DNA interstrand crosslinking and N-alkyl purine formation in the N-ras gene from DNA of Colo320HSR cells treated with HN2 in vitro.

Figure 6. HN2-induced N-alkyl purine (a) and interstrand crosslink formation (b) in the N-ras (▲) and c-myc (■) genes of purified Colo320HSR DNA treated in vitro. Results are from a single representative experiment.

c-myc alleles of this cell line and also in the N-ras gene which is also amplified in this cell line (Fig. 7b). The extent of crosslinking in the two Colo320 cell lines is summarized in Figure 8.

Cytotoxicity of HN2 in the cell lines

The cytotoxicity of HN2 in each of the cell lines was determined using microtitre plate assays. In all cases the optimum number of cells plated was determined experimentally such that the control cells that were sham treated were still in exponential growth at the endpoint of the assay (6 days). The IC50 values (dose of drug required to give 50% loss of cell survival) are shown in Table 2. The IC50 values varied over a 15-fold range with the order of sensitivity: LS174T > J6 > colo320HSR > Colo320DM > U937.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (µM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS174T</td>
<td>0.27 ± 0.21</td>
</tr>
<tr>
<td>Colo320HSR</td>
<td>0.79 ± 0.3</td>
</tr>
<tr>
<td>Colo320DM</td>
<td>1.0 ± 0.14</td>
</tr>
<tr>
<td>J6</td>
<td>0.55 ± 0.22</td>
</tr>
<tr>
<td>U937</td>
<td>4.1 ± 0.43</td>
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</table>

aData are the means ± standard deviations of three independent experiments.

DISCUSSION

No significant difference in the level of N-alkyl purine formation was observed between three gene sequences when genomic DNA was treated with HN2 in vitro, indicating that the primary base sequence composition of the gene fragments analysed does not significantly effect the overall distribution of HN2 lesions. The heterogeneity in DNA damage observed between the same genes in HN2-treated cells was therefore a cellular effect. There was no clear relationship between the levels of DNA damage observed in the genes studied in the different cell lines and IC50 values, suggesting that the initial levels of alkylation damage induced by HN2 at the gene level are alone not predictive of cytotoxicity, and that differences in drug sensitivity are determined by other factors, including DNA repair capacity, the coupling of DNA damage to cell death and the number of cytotoxic lesions in the whole genome. Indeed, the heterogeneity of damage in the various genes was such that there was more damage in the CD3δ gene in the least sensitive cell line (U937) than the most sensitive (LS174T) following the same HN2 treatment.

In the LS174T and J6 cell lines the highly expressed c-myc gene is damaged more than the weakly expressed N-ras gene, or the CD3δ gene, which is only expressed at lower levels in J6 cells, and not at all in LS174T cells. In the Colo320HSR cell line, however, the levels of N-alkyl purine formation in the amplified c-myc and single copy CD3δ genes were very similar. In Colo320HSR the expression of c-myc is high, as measured by northern blotting (data not shown), whilst CD3δ is not expressed. Both the CD3δ and c-myc genes were damaged more extensively than the N-ras gene, which is expressed at a low level. In U937 cells the damage induced in the silent CD3δ gene was greatest. No obvious correlation, therefore, existed between the extent of initial damage and the gene transcriptional activity. A previous study in human lymphoma cell lines has shown that both transcriptionally active translocated and silent native copies of c-myc produced HN2-induced N-alkyl purines to a similar extent (22). It has also been demonstrated that the extent of gene-specific repair of cyclobutanepyrimidine dimers in the inactive and active c-myc genes of Colo320DM is not significantly different (21).

It has been suggested that active genes are damaged and repaired more extensively by some agents due to the structure of the chromatin being more open to allow the transcriptional machinery access to the coding sequences (13,22–24). It is a reasonable assumption that the more open conformation of...
Figure 7. HN2-induced interstrand crosslinking in the c-myc and N-ras genes of (a) Colo320HSR and (b) Colo320DM cells. In the autoradiograph from the c-myc genes derived from the Colo320DM cell line, the upper band corresponds to the translocated allele, whilst the lower band corresponds to the native allele. Some loss of signal is observed consistently at the highest doses used, presumably due to degradation of DNA.

Figure 8. Quantitation of HN2-induced DNA interstrand crosslink formation in the N-ras (A), native c-myc (B), and rearranged c-myc (C) genes of (a) Colo320HSR and (b) Colo320DM cells. Results in (a) are from a single representative experiment and in (b) are the means of three independent experiments with error bars corresponding to the SEM.

Chromatin in active genes would also allow greater access to DNA binding drugs as well as to the proteins associated with transcription and repair. Indeed, this is the basis for the DNase hypersensitivity assay where the nuclease sensitivity of a gene is associated with transcription, or the readiness to undergo transcription (25,26). DNase I hypersensitive sites in the intergenic region between the CD3γ and CD3δ genes are only found in cell types where these genes are actively transcribed, namely those derived from T cells (26). If transcriptional activity alone predicted the extent of gene-specific HN2 damage, the CD3δ would be predicted to be damaged most extensively in the J6 cell line, the only one tested that is actively transcribed. However, a DNase I hypersensitive site downstream of the CD3δ gene which was found in all the cells assayed (including HeLa cells) in the study of Flanagan et al. (26), which may account for the accessibility of drug in non-T-cell lines. The Southern blotting-based assay used to detect HN2-induced DNA interstrand crosslinking showed that, similar to the case for N-alkyl purines, there was no heterogeneity in the distribution of DNA interstrand crosslinks when the DNA was treated in vitro. The ratio of interstrand crosslinks to monoadducts was calculated to be 15% in each gene. This is higher than the 5–10% value proposed by Kohn et al. (27). However, the latter value was calculated using the ratio of crosslinked DNA to total adducts where the total adducts were quantitated from the total amount of radiolabelled drug binding to DNA (27). In the results shown here, the adducts that were measured were only N-alkyl purines, and do not include other adducts such as alkylated phosphates. In the present study, DNA interstrand crosslinks could only be detected in genes that were amplified. No interstrand crosslinking was detected at the doses used in any single copy gene in the five cell lines tested. The ratio of interstrand crosslinks to monoadducts in the amplified c-myc genes in Colo320HSR and DM cells appeared to be higher than when the same DNA was treated in vitro. There was, however, no difference in the amount of either N-alkyl purine alkylation or interstrand crosslink formation between the N-ras or c-myc genes of Colo320HSR DNA treated in vitro, again indicating that the heterogeneity observed was a cellular phenomenon.

Those reports of gene-specific interstrand crosslink formation, as detected by denaturation/renaturation Southern blotting methodologies, have all demonstrated crosslink formation in actively transcribed amplified genes. In the study of Futscher et al. (15), Colo320HSR cells were used to demonstrate HN2-induced crosslinking only occurring in the transcriptionally active amplified c-myc gene. This was confirmed in the present study and extended to the amplified c-myc and N-ras genes in Colo320DM cells. In CHO cells, HN2-induced crosslinks were detected both in the coding and non-coding regions of the DHFR gene, but again, this
gene is highly amplified in this cell line (16). In the same CHO cell model, crosslinks are repaired more slowly in the highly reiterated rRNA genes than the amplified DHFR gene (17). It therefore appears that the formation of interstrand crosslinks is more favourable in amplified genes in intact cells. This phenomenon may not be universal for all DNA interstrand crosslinking agents, however, since interstrand crosslinks produced by cisplatin are detectable in both the N-ras and c-myc genes of LS174T and U937 cells (data not shown).

The most likely explanation for the observed heterogeneity of DNA interstrand crosslink formation is that the amplified c-myc gene in Colo320HSR, and N-ras and c-myc genes in Colo320DM cells have a chromatin structure that is different in some way to that of single copy alleles. Crosslinking by HN2 has been shown to occur preferentially at 5′-GNC sequences, with the crosslink between two guanine-N7 sites spanning three base pairs when isolated DNA is treated (28–30). This results in considerable distortion of the DNA structure. It is not clear, however, if this is the preferred sequence for crosslink formation in intact cells. Certain structural features of DNA in amplified sequences, such as torsional stress or supercoiling, could conceivably allow the formation of a crosslink from a monofunctional alkylation to be more favourable, even in the absence of increased initial quanine-N7 alkylation. Indeed, Tang et al. suggest that gene amplification affects the formation of the monofunctional lesions produced by CC-1065 in the minor groove of DNA (24).

Heterogeneity in the extent of DNA damage produced by chemical and physical agents at the level of individual genes has been shown previously. The data presented here, however, indicate that for bifunctional alkylating agents such as HN2, heterogeneity of DNA damage can occur between different genes in human cells, and this can vary for different lesions produced by the same agent. In addition, this heterogeneity can differ between human tumour cell lines. The heterogeneity observed does not appear to correlate with either the level of transcription of the genes, or with the sensitivity of individual cell lines to the drug. This suggests that other factors, in particular the extent and efficiency to which cells can repair relevant DNA lesions and couple critical DNA damage to the cell death response will determine the drug sensitivity of a particular cell line. The critical lesion produced by HN2 is generally assumed to be the interstrand crosslink. Previous studies have indicated that for nitrogen mustard based drugs, cytotoxicity is related to the total number of interstrand crosslinks when measured at the level of the genome (5,6). The present study indicates that their formation in cells appears to be most efficient in amplified DNA sequences. These results have important consequences for the future design of more efficient alkylating and crosslinking based agents for clinical use.

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