In vitro studies on the tumorigenic potential of halonitromethanes

Alicia Marsà[1], Constanza Cortés[1], Elisabet Teixidó[1], Alba Hernández[1,2], Ricard Marcos[1,2,*]

[1]Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain.

[2]CIBER Epidemiología y Salud Pública, ISCIII, Spain.

[*]Corresponding author at: Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, Campus de Bellaterra, 08193 Cerdanyola del Vallès (Barcelona), Spain. Phone: +34 93 581 20 52; Fax: +34 93 581 23 87; E-mail address: ricard.marcos@uab.es

Running Title: Carcinogenic potential of water disinfection by-products

ABSTRACT

Epidemiological data indicate that chronic exposure to water disinfection by-products (DBPs) results in an increased risk of cancer in humans. However, the real carcinogenic potential of the compounds within the DBPs is not known. In this study, we assessed the *in vitro* carcinogenic potential of trichloronitromethane (TCNM) and bromonitromethane (BNM), two unregulated halonitromethanes commonly found in DBP mixtures at comparably high concentrations. Human lung BEAS-2B cells were exposed for 8 weeks to environmentally relevant doses of TCNM and BNM. The acquisition of different *in vitro* cancer-like features was evaluated throughout the exposure. Matrix metalloproteinase (MMP) activities were measured by zymography while colony formation and promotion were assessed by soft-agar assays. Alterations in cellular morphology and proliferation were also analysed. The results indicate that long-term exposure to low doses of TCNM and BNM did not cause carcinogenic transformation of BEAS-2B cells as indicated by the absence of morphological changes, no effects on cell growth, no changes in the level of MMP secretion, and no increased anchorage-independent cell growth capacity. Furthermore, TCNM- and BNM-exposed BEAS-2B cells were unable to enhance tumour growth directly or by indirect influence on the lung stroma. Our results indicate that the demonstrated carcinogenic effects in human populations exposed to DBP mixtures cannot be attributed to the evaluated halonitromethanes. To our knowledge, this is the first study evaluating TCNM and BNM under a long-term exposure scenario, and using suitable hallmarks of the cancer process.

1. Introduction

Disinfection by-products (DBPs) are produced by reactions between the organic matter present in raw water and the chemicals used to disinfect it (Ngwenya et al., 2013; Banach et al., 2015). DBPs in drinking water are a complex mixture containing many different chemical groups with a number of compounds having mutagenic and carcinogenic potential, as extensively reviewed (Richardson et al., 2007). Therefore, DBP exposure can have important long-term human health implications (Grellier et al., 2015), with bladder cancer as one of the main documented effects (Villanueva et al., 2007; Costet et al., 2011; Hrudey et al., 2015).

Due to their potential risk to public health, some DBPs are regulated in many countries, although there are also some chemical species that belong to non-regulated classes which could present health risks (Jeong et al, 2015; Kim et al., 2015; Li et al., 2015). Halonitromethanes (HNMs) constitute a highly abundant emerging class of non-regulated DBPs (Weinberg et al., 2002; Krasner et al., 2006; Serrano et al., 2015). These soluble, low molecular weight compounds are produced when chlorine and/or ozone are used for water treatment. They are structurally similar to the halomethanes, but have a nitro-group in place of hydrogen bonded to the central carbon atom. The presence of nitrogenous DBPs in drinking water is of great concern due to their higher genotoxicity and cytotoxicity as compared to regulated DBPs (Bond et al. 2011). Trichloronitromethane (TCNM) and bromonitromethane (BNM) are two well-known HNMs. Although TCNM is the most common HNM, special attention must be focused on BNM because brominated DBPs are more reactive than their chlorinated forms (Woo et al., 2002; Kim et al., 2015).

Genotoxicity assessment is usually accepted as a surrogate biomarker of potential cancer risk. Due to the complexity and ethical issues posed by long-term carcinogenesis studies using mammalian models, *in vitro* genotoxicity assays are used to evaluate potential for carcinogenic risk. This approach has been exploited to determine the

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genotoxic potential of various DBPs (Richardson et al., 2007; Liviac et al., 2011; Manasfi et al., 2015; Teixidó et al., 2015). Nevertheless, a more direct way to measure the potential carcinogenic risk of individual DBPs remains to be developed. *In vitro* cell transformation assays have been proposed as suitable alternatives to long-term animal studies to measure carcinogenic effects (Vasseur and Lasne, 2012). In fact, there is increasing evidence that the cellular and molecular processes involved in *in vitro* cell transformation are similar to those that occur during *in vivo* carcinogenesis (Creton et al. 2012). It is generally accepted that an exposed cell line becomes tumorigenic when a battery of different cancer hallmarks are evident (Hanahan and Weinberg, 2011).

It is also interesting to note that the detection of the harmful effects induced by chemical exposures, including those induced by DBPs, are usually measured in unrealistic scenarios of acute treatments at high concentrations. Nevertheless, when the mechanisms of induced carcinogenicity are studied *in vitro*, it is particularly important to design studies involving more relevant types of exposure in terms of human risk; thus, *in vitro* long-term or chronic exposures with low or non-toxic doses would seem to be a good alternative system to assess realistic exposure conditions (Bach et al., 2014).

Using an experimental approach with long-term (8 weeks) exposure and subtoxic concentrations, we evaluated the potential transforming capacity of two HNMs (TCNM and BNM) in a human bronchial epithelial cell line (BEAS-2B), considering inhalation as the main route of exposure to DBPs (Villanueva et al., 2011). Different hallmarks of cell transformation such as morphological cell changes, anchorage-independent cell growth, and secretion of matrix metalloproteinases (MMPs) were analysed.

2.   Materials and methods

2.1. Cell culture conditions and in vitro DBP exposure.

Bromonitromethane (BNM, $BrCH_2NO_2$; CAS 563-70-2, 90% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and trichloronitromethane (TCNM, CCl_3NO_2; CAS 76-06-2, 97.5% purity) from Riedel-de-Haën (Seelze, Germany). Human bronchial epithelial cells (BEAS-2B), human colorectal carcinoma cell line (HCT116) and human lung fibroblast cell line (MRC5) were maintained in DMEM high glucose medium (Life Technologies, NY, USA) supplemented with 10% foetal bovine serum (FBS; PAA®, Pasching, Austria), 1% of non-essential amino acids (NEAA; PAA®) and 2.5 µg/mL plasmocin (InvivoGen, CA, USA) at 37 ºC in a humidified 5% CO_2 incubator. BEAS-2B cells were long-term exposed to low concentrations of the HNMs (1 and 5 µM BNM; 5 and 25 µM TCNM) for 8 weeks. The DBP-containing medium was changed every 48 h, and subconfluent cells were passaged weekly. Three separate 75-cm^2 flasks were maintained for each treatment, and DBP-exposed cells were in all cases compared with unexposed passage-matched controls.

2.2. Cell viability.

BEAS-2B cells were plated in 6-well plates in triplicates at a density of 50,000 cells per well and incubated overnight in complete medium. Cells were then placed in fresh medium with increasing concentrations of 1 to 50 µM of each DBP. After 24 h of treatment, the cells were washed with PBS, and the cell number was assessed after trypsinization by the Beckman counter method with a ZTM Series coulter-counter (Beckman Coulter, CA, USA). Cell toxicity curves were derived from averaging three independent experiments, and the IC_{50} values were calculated using GraphPad prism version 5.03.

2.3. Cell proliferation.

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123   BEAS-2B long-term exposed cells and unexposed passage-matched controls were

124   plated in 6-well plates at a density of 50,000 cells per well in regular conditions. At 24 h

125   intervals, the cells were collected by trypsinization and counted by the Beckman counter

126   method. Proliferation was defined as the time necessary for doubling the cell population,

127   and it was calculated according to the equations referred in *http://www.doubling-time.com*

128   */compute.php*

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130   *2.4. Anchorage-independent cell growth.*

131   Colony formation in soft-agar was assessed in long-term exposed BEAS-2B cells and

132   passage-matched controls to determine the capacity for anchorage-independent growth

133   (Bach et al., 2014). For this study, BEAS-2B cells were collected and individualized using

134   a 30-µm filter. Triplicates of a total of 10,000 cells were suspended in a 1:1:1 mixture

135   containing 1x DMEM (supplemented with 10% FBS, 1% NEAA, 1% L-Glu 200 mM and 2.5

136   µg/mL Plasmocin), 2x DMEM (with 2x supplements) and 1.2% Bacto Agar (DIFCO, MD,

137   USA). The mixture was then plated in 6-well plates over a 0.6% base agar (in

138   supplemented 2x DMEM). Plates were incubated at 37 ºC for 21 days. After this time, cell

139   colonies were stained overnight with 1 mg/mL of (2-p-iodophenyl)-3-3(p-nitrophenyl)-5-

140   phenyl tetrazolium chloride (Sigma, MO). Plates were scanned and colony counting was

141   performed using OpenCFU, open-source Software. A modified version of the assay was

142   performed using 48-h conditioned medium (CM) from long-term exposed BEAS-2B cells

143   and passage-matched controls to assess their capacity to promote the malignant growth of

144   tumour cells. Triplicates of a total of 10,000 individualized HCT116 cells were resuspended

145   in CM and mixed in a 1:1:1 ratio with supplemented 2x DMEM and 1.2% bacto agar. The

146   remainder of the protocol was performed as described above. Plates were incubated for

147   15 days. In normal conditions, transformed cells stimulate the secretion of promoting

148   factors by cells from the stroma. Thus, to further analyse the cancer-like phenotype in

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149 long-term exposed BEAS-2B, their 48-h CM was used to grow the lung fibroblast MRC5

150 cells. Then, the 48-h CM of stimulated MRC5 stroma cells was collected and used for the

151 soft agar assay with HCT116 cells as indicated above.

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153 *2.5. Secretion of MMP-2 and MMP-9.*

154   The activity of secreted matrix metalloproteinases 2 and 9 was examined in long-term-

155 exposed BEAS-2B and passage-matched controls. Cells were cultured in basal DMEM

156 medium (without serum or supplements) for 72 h to obtain a CM that was analysed by

157 standard zymography, following the manufacturer's instructions (Bio-Rad, Hercules, CA).

158 The area of protease activity was measured densitometrically using the ImageJ analysis

159 program. Since MMPs are mainly secreted by stroma cells, MRC5 fibroblasts were grown

160 in 48-h CM of long-term exposed BEAS-2B and passage-matched controls. Subsequently,

161 the 48-h CM of stimulated MRC5 stroma cells was collected and used for zymography as

162 indicated above.

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164 *2.6. Statistical analysis.*

165   One-way analysis of variance followed by Dunnett's multiple comparison test was

166 performed to compare treatments with untreated time-matched controls. In all cases, a

167 two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were

168 performed using GraphPad Prism 5 version 5.03.

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3. Results

3.1. BNM and TCNM are toxic for BEAS-2B cells at low-micromolar levels.

An initial toxicity experiment was carried out using a wide range of compound concentrations to determine appropriate concentrations to study the effects of long-term exposures to non-toxic concentrations of TCNM and BNM. The obtained results showed differences in sensitivity to the two compounds (Fig. 1). Thus, BNM was more toxic than TCNM given their IC_{50} values of 32 ± 10 μM and 111 ± 17 μM, respectively. Both compounds were clearly cytotoxic at 50 μM, with a mean viability of 32.4 ± 7.2% and 64.2 ± 7.4% for BNM and TCNM, respectively, when compared to control cells. Given these results, two different concentrations of each HNM inducing less than a 20% decrease in viability were chosen to carry out the subsequent experiments. The selected doses were 1 and 5 μM for BNM and 5 and 25 μM for TCNM.

3.2. Long-term exposure to HNMs induced no changes in BEAS-2B cellular morphology or proliferation.

Cells undergoing tumoural transformation are known to progressively decrease their doubling times and to start a process in which cells lose their intrinsic morphological characteristics, known as anaplasia. To determine whether the long-term exposure to TCNM or BNM induces tumoural transformation, cellular proliferation and shape were monitored as markers of cancer-like phenotypic changes throughout 8 weeks of long-term treatment. No noticeable changes in cell morphology were observed throughout the whole exposure time (Fig. 2A), and results analysed at the end of the exposure period indicated that neither BNM nor TCNM treatment increased the proliferation rates significantly when compared to time-matched controls (Figs. 2B and 2C).

3.3. Long-term exposure to HNMs did not alter BEAS-2B cell capacity to grow in soft agar.

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196   One of the main characteristics of cancer cells is their anchorage-independent growth

197   capacity (Hanahan and Weinberg 2011). To assess this feature in the HNM-long-term

198   exposed cells, BEAS-2B cells were subjected to a soft-agar colony formation assay.

199   BEAS-2B cells formed a considerable number of small-sized colonies spontaneously,

200   with an average size of 143.49 ± 0.20 µm. Nevertheless, important variations were not

201   observed after the HNM treatments (Fig. 3). Representative pictures of the colony plates

202   are indicated in Figure 3A where no significant changes are apparent. As shown, control

203   samples formed a mean of 273 ± 21 colonies, while cells treated with BNM long-term-

204   exposed cells displayed 332 ± 16 and 246 ± 40 colonies for its lowest and highest

205   concentrations, respectively. On the other hand, TCNM long-term-exposed cells formed an

206   average of 288 ± 16 colonies at 5 µM concentration and 157 ± 25 colonies at 25 µM. When

207   colony sizes were taken into account (

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209   Figure 3~~Fig. 3~~C), the mean size was similar in all cases (142.65 ± 1.51 µm for controls *vs.*

210   145.68 ± 1.58 and 145.06 ± 0.77 µm for BNM 1 and 5 µM treatments, respectively, and

211   141.84 ± 0.74 and 142.24 ± 1.81 µm for TCNM 5 and 25 µM treatments, respectively).

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213   *3.4. Long-term exposure to HNMs did not alter the BEAS-2B cell secretome.*

214   It is known that the interplay between the different cell populations and their

215   environment determine the formation and malignancy of a tumour. Cells undergoing

216   malignant transformation secrete paracrine signals that enhance the tumorigenic potential

217   of nearby cells (del Pozo Martin et al. 2015). Thus, the capability of long-term exposed

218   BEAS-2B cells in promoting the growth of the tumoural cell line HCT116 in soft-agar was

219   evaluated. As observed in Figure 4, no differences in HCT116 colony number or size were

220   observed after the exposure. Cells grown with untreated BEAS-2B CM displayed an

221   average of 1,338 ± 44 colonies. Similar mean values were displayed by HCT116 cells

222   grown in CM of long-term exposed BEAS-2B, showing an average of 1,382 ± 91 and 1,329

223   ± 62 for the CM of 1 and 5 µM BNM, and 1,309 ± 68 and 1,309 ± 61 colonies for the CM of

224   5 and 25 µM TCNM.

225   As CM contains cell-secreted factors known to enhance and not initiate tumour

226   effects, the comparisons of big colonies or colony sizes are of significance when assessing

227   the promotion of anchorage-independent cell growthThe average size of HCT116 cells

228   exposed to control CM was 216.00 ± 1.17 µm, whereas HCT116 colonies grown in HNM-

229   exposed BEAS-2B CM showed mean values of 214.70 ± 2.84 and 213.90 ± 4.34 µm for 1

230   and 5 µM BNM, and 210 ± 2.74 and 206.53 ± 5.70 µm for 5 and 25 µM TCNM,

231   respectively. There were no significant differences in the percentage of big colonies

232   among the treatments (Fig. 4C).

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234   *3.5. Long-term exposure to HMNs did not influence the lung stroma secretome.*

235   Another method by which transformed cells induce malignant transformation is to

236   prompt stroma cells to secrete growth factors that potentiate tumour effects (Cammarota

237   and Laukkanen, 2016). To assess whether BNM or TCNM could enhance tumour growth

238   through this indirect mechanism, MRC5 lung stroma fibroblasts grown in HNM long-term-

239   exposed BEAS-2B CM were evaluated by the soft agar assay with HCT116 cells (Fig. 5).

240   As shown, HCT116 cells grown in CM of control MRC5 presented an average number of

241   colonies of 1,822 ± 119, non-significantly different than the number of colonies formed by

242   HCT116 cells grown in CM of exposed MRC5 cells, which was 1,848 ± 127, 2,306 ± 149,

243   1,889 ± 178 and 2,171 ± 204 for 1 µM BNM, 5 µM BNM, 5 µM TCNM and 25 µM TCNM,

244   respectively. When mean colony sizes were analysed, the results consistently showed no

245   HNM-associated effect. Thus, HCT116 cells grown in CM of control MRC5 cells showed a

246   mean colony size of 198.61 ± 2.15 µm, whereas colony sizes of HCT116 cells grown in

247   CM of exposed MRC5 cells were 199.25 ± 1.66, 201.06 ± 4.74, 202.19 ± 2.83 and 200.56

248   ± 2.24 µm for 1 µM BNM, 5 µM BNM, 5 µM TCNM and 25 µM TCNM, respectively. The

249   CM of MRC5-exposed cells was also unable to induce changes in the percentage of big

250   colonies (Fig. 5C).

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252   *3.6. Long-term exposure to HNMs induced no changes in the secretion of MMP-2 and*

253   *MMP-9.*

254   Extracellular proteinases, such as matrix metalloproteinases (MMPs), are known to

255   influence cancer cell invasion at a local and distant level (Kessenbrock et al., 2010). For

256   this reason, increased MMP activity levels are generally used as indicators of cell

257   malignant transformation, especially in the case of MMP2 and MMP9. No significant

258   changes were observed when the secreted MMP 2+9 were analysed in HNM long-term-

259   exposed BEAS-2B cells compared to control cells (Fig. 6A). As MMPs are mainly secreted

260   by stroma cells after stimulation by tumoral cells, we found it interesting and necessary to

261   confirm these negative results by analysing the secretion of MMP 2+9 in MRC5 cells

262   grown in CM of long-term-exposed BEAS-2B cells. Consistent with previous results, no

263   changes in MMP secretion were observed (Fig. 6B).

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4. Discussion

DBPs constitute a very complex mixture of chemicals. The amount and variety of their chemical components depend on both the disinfection procedure used and the characteristics of the raw water source. So far, more than 600 DBPs have been discovered, with many of them showing different levels of toxicity, genotoxicity and carcinogenic properties (Richardson et al., 2007).

Although there is ample epidemiological evidence linking cancer risk to DBP exposure (Hrudey et al., 2015; Villanueva et al., 2007, 2015), none of the individual species tested to date in the limited assays employed has shown sufficient carcinogenic potency to account for the cancer risks projected from the epidemiological studies (Bull et al., 2011). This implies that a more systematic approach to determine the carcinogenic potential of DBPs is required. In this sense, our study analysing the carcinogenic potential of two HNMs is an attempt to provide new information to fill in this gap. Our data suggests that long-term exposures to low doses of BNM and TCNM are not able to trigger any of the *in vitro* cancer phenotypic hallmarks analysed in lung epithelial cells. This would support the view that exposure to these particular DBPs does not increase the carcinogenic risk in humans.

There are very few *in vitro* studies together with several *in vivo* studies dealing with the carcinogenic risk of DBPs. Among the *in vivo* studies, McDorman et al. (2003) evaluated four DBPs by using rats phenotypically prone to the effects of renal carcinogens. Potassium bromate, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), chloroform, and bromodichloromethane were tested. No significant increases in renal adenomas and carcinomas were detected. Nevertheless, elevated hyperplasia and tumours were observed when rats were exposed to the highest dose of chloroform, as well as in the mixture containing the highest doses of the four DBPs tested. In another study from the same group using the same DBP compounds, transitional epithelial hyperplasia and cell proliferation in the urinary bladder were observed in rats exposed to the highest

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293　dose of MX. Furthermore, treatment with the four individual DBPs and the mixture induced

294　the development of aberrant crypt foci, considered as putative preneoplastic colon cancer

295　lesions (McDorman et al., 2003b). A third study evaluating the effects of dichloroacetic

296　acid (DCA) in early life observed that DCA is carcinogenic in mice, inducing increases in

297　the incidence of hepatocellular tumours comparable to those of life-long exposures (Wood

298　et al., 2015).

299　　　　Although the *in vivo* studies are an accurate approximation of the actual tumour-

300　inducing scenario, the high costs and complexity of these type of studies make them non-

301　applicable to the vast number of potential genotoxic/carcinogenic DBP compounds. This is

302　the basis of our proposal to use *in vitro* cell transformation assays. Indeed, the

303　performance of such assays in predicting carcinogenic potential has been established on

304　several hundreds of chemicals. In fact, the Organisation for Economic Co-operation and

305　Development (OECD) has specific guidelines on "Cell transformation assays for the

306　detection of chemical carcinogens" (Vasseur and Lasne, 2012), with accumulated

307　evidence that the cellular and molecular processes involved in *in vitro* cell transformation

308　are similar to those occurring during *in vivo* carcinogenesis (Creton et al., 2012). In spite of

309　the advantages of these *in vitro* approaches, the limited number of studies carried out with

310　DBPs is surprising, mainly taking into account the environmental risks posed by these

311　chemicals. In one of the few available studies, the ability of MX to promote cell

312　transformation was determined in mouse embryonic fibroblasts. MX was used in both the

313　initiation and the promotion phase, after a previous exposure to 3-methylcholanthrene

314　(MC). When MX was added during the promotion phase in the MC-initiated cells, it

315　promoted the development of the transformation foci in a dose-dependent manner.

316　Exposure to MX as the initiator slightly enhanced the development of foci, suggesting that

317　MX may rather act via promoting tumour development (Laaksonen et al., 2001). In a

318　posterior study, different chlorohydroxyfuranones such as MX, MCA [3,4-dichloro-5-

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319   hydroxy-2(5*H*)-furanone],   CMCF   [3-chloro-4-(chloromethyl)-5-hydroxy-2(5*H*)-furanone],

320   and MCF [3-chloro-4-methyl-5-hydroxy-2(5*H*)-furanone] were tested to demonstrate their

321   ability to promote foci formation in the two-stage cell transformation assay in BALB/c3T3

322   cells. The results indicate that MX acted as a potent inhibitor of gap-junctional intercellular

323   communication. This action was associated with its ability to promote malignant foci

324   formation (Hakulinen et al., 2004). In addition, four DBPs namely tribromomethane,

325   bromochloroacetic acid, dibromonitromethane and tribromonitromethane were tested for

326   their potential to transform normal human colonocytes into malignant cells. The results

327   indicated that all DBP-exposed colon cells acquired the ability to grow in soft agar to some

328   extent; however, only cells exposed to tribromomethane were able to grow in media

329   lacking serum and enriching growth factors (DeAngelo et al. 2007). Finally, the cell-

330   transforming potential of the two unregulated DBPs iodoacetic acid (IAA) and iodoform (IF)

331   were tested in mouse NIH3T3 cells. Exposure to IAA increased the frequencies of cells

332   with anchorage-independent growth abilities. In addition, IAA-transformed cells were found

333   to form aggressive fibrosarcomas after inoculation into Balb/c nude mice (Wei et al., 2013).

334   The above-mentioned studies show the usefulness of cell transforming assays to

335   demonstrate the tumorigenic potential of DBPs. Nevertheless, due to the complexity of the

336   cell transformation process, it is preferable to investigate the acquisition of a wide range of

337   cancer-like phenotypic features to decipher carcinogenic risk potential. This is the basis of

338   the strategy used in our study. Morphological changes in the exposed cells are considered

339   to be typical cancer-like phenotypic indicators (Clancy et al., 2012). In addition, secretion

340   of matrix metalloproteinases (mainly MMP2 and MMP9) is also considered an appropriate

341   biomarker of cell transformation since MMPs play crucial roles in tumour invasion,

342   morphogenesis, angiogenesis, metastasis, and wound healing by remodelling the

343   extracellular matrix (Oum'hamed et al., 2004). Moreover, assessment of the anchorage-

344   independent growth of cells in semi-solid matrices is considered an intrinsic property of

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345 transformed cells (Borowicz et al., 2014). The paracrine signals secreted by cells

346 undergoing malignant transformation enhance the tumorigenic potential of nearby cells

347 cells (del Pozo Martin et al., 2015). Therefore, conditioned media obtained from these cell

348 cultures can be used to determine their ability to induce anchorage-independent growth of

349 tumour or stromal cells. In this way, the usefulness of an indirect soft agar approach has

350 recently been demonstrated (Bach et al., 2016).

351   As a summary, a battery of *in vitro* cancer-like phenotypic hallmarks must be able to

352 clearly predict the carcinogenic potential of a given environmental chemical. On this basis,

353 the lack of observed (i) morphological and proliferation changes, (ii) variations in the

354 secretion of matrix metalloproteinases, and (iii) anchorage-independent cell growth ability,

355 both directly and indirectly, demonstrate that the two selected HNMs should be considered

356 as non-carcinogenic DBPs.

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Conflict of interest statement

The authors report no conflict of interest. The authors alone are responsible for the content
and writing of the paper.

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Figure legends

Figure 1. BEAS-2B cell viability curves after 24 h of exposure to increasing concentrations of BNM and TCNM, ranging 1 to 50 μM. Data are presented as mean values of independent experiments ± SEM (n=3).

Figure 2. Long-term exposure to BNM and TCNM does not induce major changes in cancer phenotypic markers as cellular proliferation and cell morphology in BEAS-2B cells. **A)** Representative pictures of cell morphology taken 24, 48 and 72 h after seeding. **B-C)** BEAS-2B doubling time mean values ± SEM.

Figure 3. Analyzed HNMs do not induce cells anchorage-independent growth after a long-term exposure. A) Representative images of BEAS-2B colonies in soft agar. B) Mean number of colonies per well ± SEM. C) Mean colony size per treatment ± SEM (n=12). **P<0.01 compared with time-matched controls.

Figure 4. BEAS-2B cells long-term exposed to DBPs do not increase anchorage-independent growth capacity in HCT116 tumor cells. A) Representative images of HCT116 colonies exposed to BEAS-2B conditioned medium in soft agar. B) Mean number of colonies per plate ± SEM. C) Percentage of colonies bigger than 200 and 300 μm per well formed by HCT116 cells exposed to BEAS-2B CM ± SEM (n=12).

Figure 5. MRC5 fibroblasts grown in exposed BEAS-2B conditioned medium do not stimulate anchorage-independent growth capacity of HCT116 tumor cells. **A)** Representative images of HCT116 cells exposed to MRC5 CM in soft agar. **B)** Mean number of HCT116 colonies per well ± SEM. **C)** Percentage of big HCT116 colonies per well ± SEM (n=12).

Figure 6. Cells long-term exposed to HNMs do not acquire invasive capacity. **A)** BEAS-2B cells secretion of MMP 2+9 does not increase with long-term BNM nor TCNM exposure when compared to time-matched non-exposed controls. **B)** MRC5 fibroblasts do not show an increase in MMP 2+9 secretion when stimulated with exposed-BEAS-2B CM.
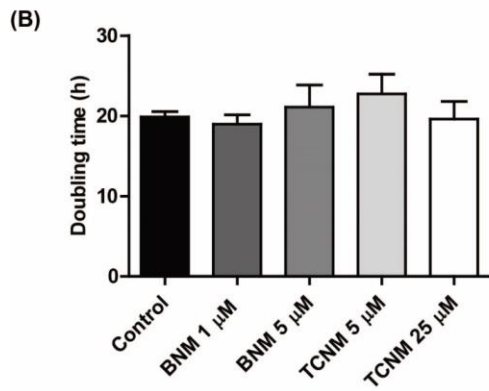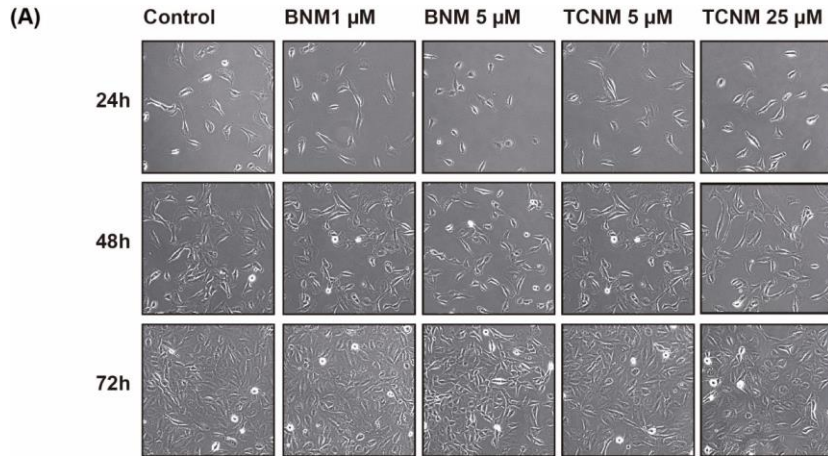
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540 Figure 1

(A)

	Control	BNM1 μM	BNM 5 μM	TCNM 5 μM	TCNM 25 μM
24h					
48h					
72h					

(B)
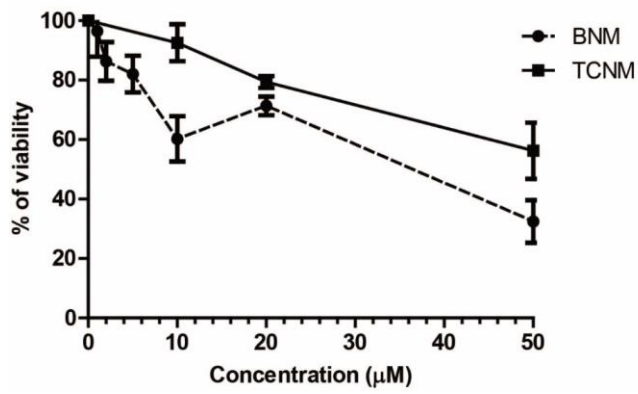
(C)

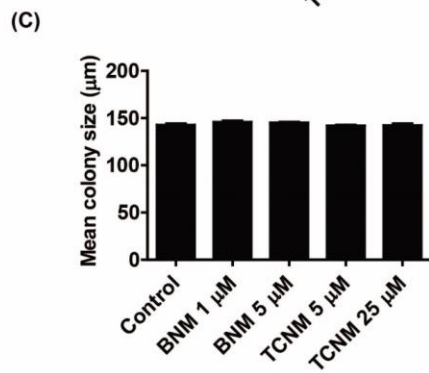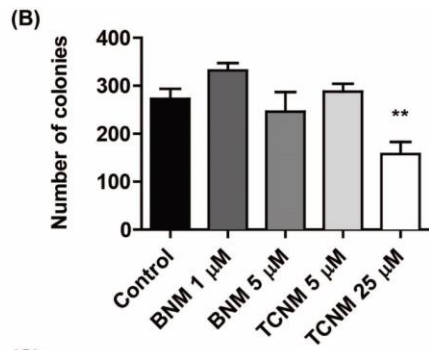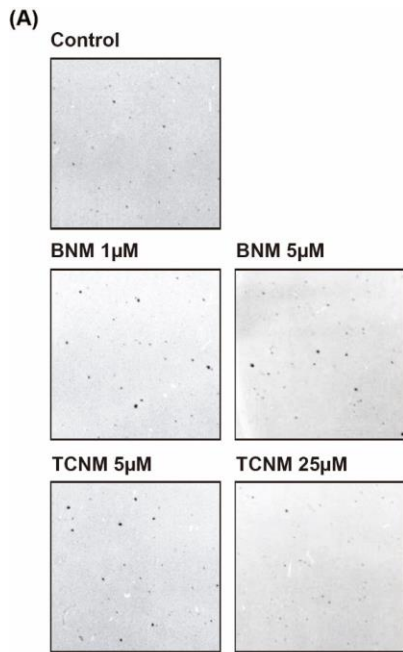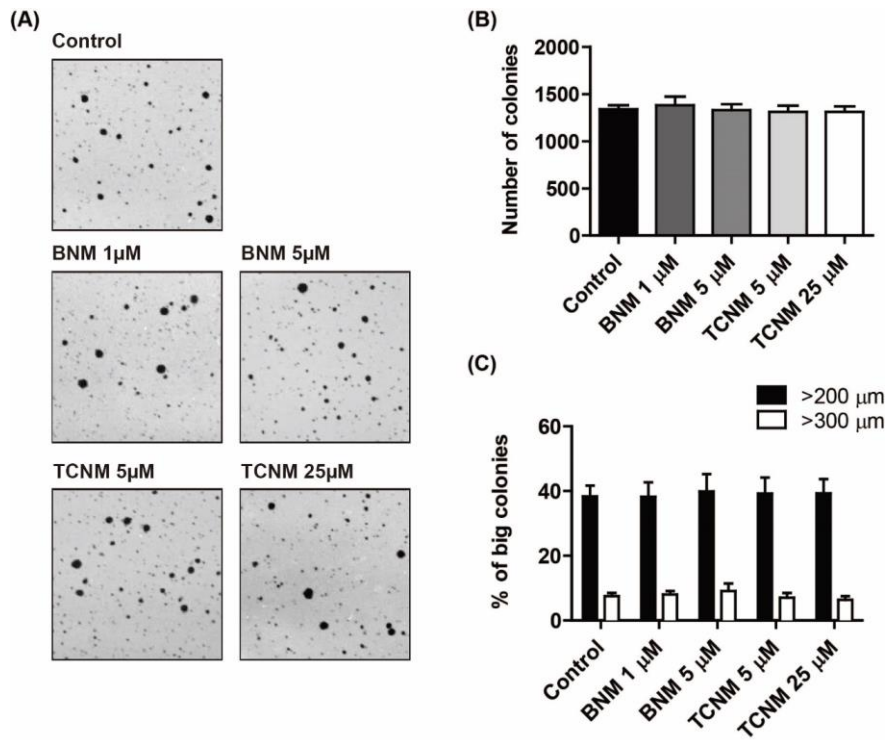|	DT ± SEM (h)
Control	19.88 ± 0.64
BNM 1 μM	18.96 ± 1.17
BNM 5 μM	21.09 ± 1.63
TCNM 5 μM	22.74 ± 1.49
TCNM 25 μM	19.61 ± 1.50

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544   Figure 2
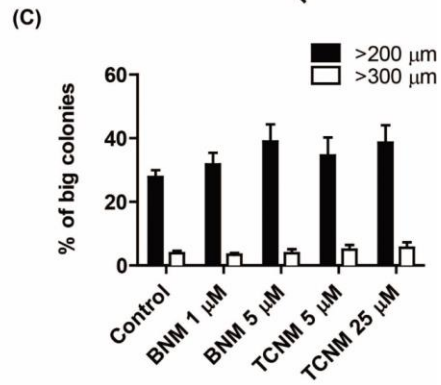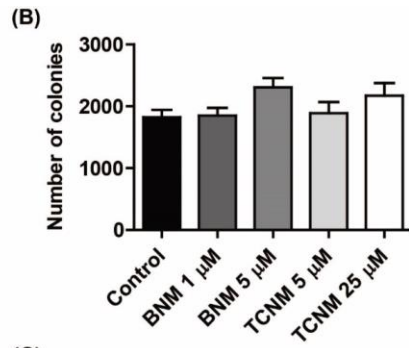
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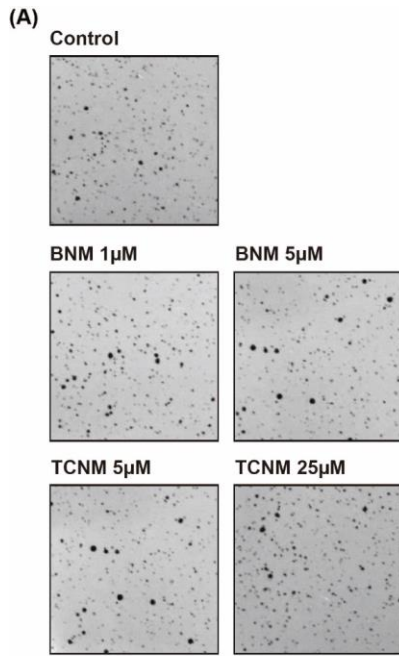
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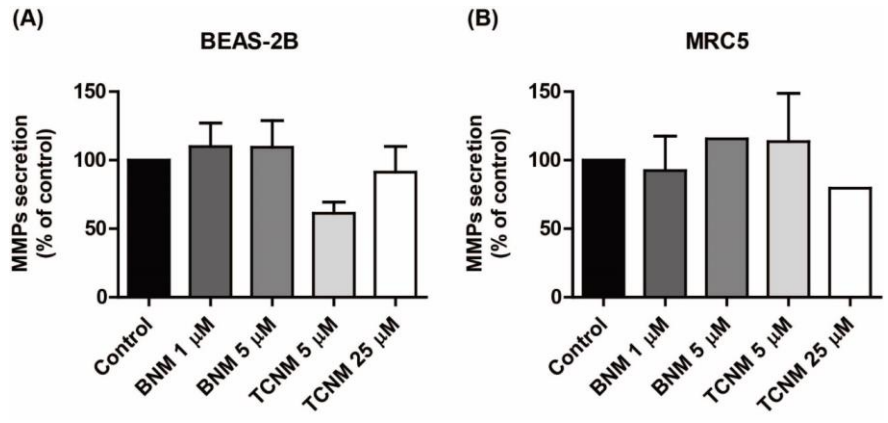
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550   Figure 3

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(A) BEAS-2B

(B) MRC5

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567   Figure 6

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