Bladder Cancer: Innovative Approaches Beyond the Diagnosis

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Abstract: Bladder carcinoma (BC) is the most common urinary malignant tumor. In the light of the unsuccessful current therapies and their side effects, new pharmacological strategies are needed. In addition to the well known therapeutic possibilities described in the first section, we focused our attention on very recent and innovative tools to approach this target (new drug candidates from epigenetic modulators to endothelin receptor inhibitors, improved technological formulations, active principles from plants, and dietary components). Then, in the last paragraph, we analyzed the etiology of recurrent BC, with particular attention to cellular microenvironment. In fact, the incidence of recurrence is up to 90%, and 25% of tumours show progression towards invasiveness.

Keywords: Bladder cancer, gene therapy, endothelin receptors, targeted therapy, histone deacetylase inhibitors, plant-derived extracts, "field cancerization" recurrence, selective COX-2 inhibitors, Peroxisome Proliferator-Activated Receptor γ agonists.

1. BLADDER MALIGNANT TUMOR: AN OVERVIEW

Bladder carcinoma (BC) is the most common urinary malignant neoplasm with a ratio of male to female of 3.8 to 1.0 [1]. Most patients are elder than 60 years of age but BC may affect also younger patients. The most common risk factors are patient's age, active and passive tobacco smoking [2-4], and work-related exposure to chemicals [5], schistosomiasis [6], and familiarity [7]. Haematuria is the most common sign in BC, sometimes associated to strangury, bladder tenesmus, higher risk of urinary tract infections and pollakiuria. Pathologic staging [8, 9] depends on the presence or absence of invasion into the bladder wall.

Non-muscle Invasive (Superficial) Bladder Cancer (NMIBC)

Tumors which belong to stages Ta and T1 can be taken away through transurethral resection (TUR) which may also be used as a diagnostic tool to classify patients as having non-muscle invasive (superficial) bladder cancer (including carcinoma *in situ*, CIS, or rather high-grade tumours close to the mucosa) and muscle invasive tumours (Table **1**). Patients with TaT1 tumours may also be divided into three risk groups on the description of prognostic factors: low-, intermediate-, and high-risk group [10]. Notably, recurrence and progression risks are two independent variables; indeed, even though prognostic factors could indicate a high percentage for recurrence, the progression risk could still be low. Consequently, with the purpose of distinguishing the short- and long-term risk levels of both recurrence and progression in individual patients, the European Organization for Research and Treatment of Cancer proposed a scoring system with the corresponding risk tables [11]. This system has been built on the six most relevant clinical and pathological factors such as number of tumours, tumour size, prior recurrence rate, T category, presence of concomitant CIS and tumour grade.

TUR by itself could fully eradicate a TaT1 tumour, nonetheless recurrence occurs in up to 50% of patients, and progression to muscle invasiveness may affect up to 10-15% of patients; consequently, it is often necessary to perform a cystoscopy after 3 months [11-15]. In these cases it is important to evaluate adjuvant therapy as intravesical chemotherapy (mitomycin C, epirubicin, and doxorubicin) or immunotherapy (bacillus Calmette-Guérin, or BCG) instillations in order to prevent or decrease recurrence and/or progression. Moreover, therapy with BCG failed if: (*i*) muscle invasive tumour is detected during the follow-up; (*ii*) whenever high-grade, non-muscle invasive tumour is still detectable at both 3 and 6 months [16], although in patients with tumour at 3 months, an additional BCG course could exert a final response in more than 50% of cases, both in patients with papillary tumours and CIS [16, 17]; (*iii*) any worsening of the disease during BCG treatment, such as a higher number of recurrences, higher T or higher grade, or appearance of CIS, despite an initial response.

The subsequent follow up is based on the specific risk class for progression/recurrence. Patients with tumours at low risk of recurrence and progression should have a

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Table 1. **Stages and Grading of Bladder Carcinoma**

Primary Tumour Staging (T)	Patients may or may not show invasion of *muscolaris propria*; the former are diagnosed as MIBC (muscle invasive BC) and are staged T2-T4; the latter are diagnosed as NMIBC (non-MIBC) and are staged Ta and T1
TX	Primary tumor cannot be assessed
T0	In transurethral resection (TUR) or cystectomy specimen or biopsy there is no evidence of primary carcinoma after an initial cancer diagnosis
Ta	Noninvasive papillary carcinoma; it is characterized by the absence of *lamina propria* invasion
Tis	Carcinoma *in situ* (CIS); high-grade tumours but confined to the mucosa
T1	Tumor invades subepithelial connective tissue (*lamina propria*) but it doesn't involve *muscolaris propria*
T2	Tumor invades *muscolaris propria* bladder wall
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical fat
T3a	Microscopical invasion
T3b	Macroscopical invasion with presence of extravesical mass
T4	Tumor invades adjacent organs
T4a	Tumor invades prostate, uterus, or vagina
T4b	Tumor invades pelvic or abdominal wall
Regional lymph nodes involvement (N)	The nodal status depends on the number and size of positive lymph node; the evaluation involves the lymph node density, which is defined by the ratio of positive lymph nodes to the total number of nodes sampled
NX	Regional lymph nodes cannot be assessed
N0	Regional lymph node do not show the presence of metastasis
N1	Metastases of 2 cm or less at most, in a single lymph node in the true pelvis (hypogastric, obturator, external iliac or presacral)
N2	Metastases between 2 cm and 5 cm in size, in a single lymph node in the true pelvis (hypogastric, obturator, external iliac or presacral), or multiple lymph nodes, none more than 5 cm in size
N3	Metastasis in one or more common iliac lymph node, more than 5 cm in greatest dimension
Presence of Distant Metastases (M)	Metastases may invade several organs; the most affected are: lymph nodes (78%), liver (38%), lung (36%), bone (27%), adrenal gland (21%); intestine (13%)
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Presence of distant metastasis

cystoscopy at 3 months and, in case of negative results, the following cystoscopy is advised at 9 months and, afterwards, every year for 5 years. Patients with tumours at high risk of progression should have a cystoscopy and urinary cytology at 3 months. In case of a negative result, the following cystoscopies and urinary cytology should be repeated every 3 months for 2 years, every 4 months in the third year, every 6 months until 5 years, and every year thereafter. An annual exploration of the upper tract is recommended. Patients with intermediate-risk of progression (about one-third of all patients) should have an in-between follow-up scheme using cystoscopy and cytology, adapted according to personal and subjective factors.

Muscle-invasive and Metastatic Bladder Cancer

The standard treatment for patients with muscle-invasive (T2-T4a, N0-Nx, M0) BC is radical cystectomy, which involves the complete removal of the bladder and sometimes prostate and seminal vesicles in men, uterus and anterior part of vagina in women. Approximately 30% of patients with urothelial cancer present muscle-invasive disease; about half will relapse after radical cystectomy depending on the pathological stage of the primary tumour and the nodal status. However, this 'gold standard' only provides 5-year survival in about 50% of patients [18-22]. In order to improve these unsatisfactory results, neoadjuvant cisplatin-containing combination chemotherapy was introduced, that allows an over-

all survival by 5-7% at 5 years [23]. Notably, only cisplatin combination chemotherapy with at least one additional chemotherapeutic agent resulted in a significant benefit [24, 25]. Indeed, responses to single agents are usually short-lived and complete responses are rare. Moreover, no long-term disease-free survival has been reported with single-agent chemotherapy, and the median survival in such patients is only about 6-9 months. Cisplatin-containing combination chemotherapy has been the standard of care since the late 1980s. MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) has been proven superior to cisplatin monotherapy and CISCA (cisplatin, cyclophosphamide and adriamycine) [26, 27] and, more recently, to cisplatin/docetaxel [28]. MVAC and gemcitabine/cisplatin (GC) have prolonged survival up to 14.8 and 13.8 months, respectively [29-31]. High-dose intensity MVAC (HD-MVAC) with GCSF (granulocyte colony-stimulating factor) is less toxic and more efficacious than standard MVAC in terms of dose density, complete response and 2-year survival rate, although there is no significant difference in median survival between the two regimens [32, 33]. However, up to 50% of patients are unfit for cisplatin-containing chemotherapy, due to a weakened renal function, or because of a co-morbidity impairing high-volume hydration [34, 35].

2. NEW THERAPEUTIC APPROACHES: THE ROAD SO FAR

Novel pharmacological strategies are necessary in the light of the unsuccessful current therapies and their side effects to treat a considerable percentage of patients with BC. In addition to the current therapeutic possibilities discussed in some recent reviews [36-38], in this section we focused our attention on innovative tools (new drug candidates, improved technological formulations, plant extracts, and dietary components) to approach this target. The most interesting molecular targets and the results of the relevant clinical trials for invasive and metastatic BC presented at the annual oncology meetings (*i.e.*, Annual Meeting of American Society of Clinical Oncology) are reported in (Table **2**).

Cancer Gene Therapy

Cancer gene therapy provides several important tools to suppress the function of dangerous oncogenes, to return the expression of specific tumour suppressor genes, and to promote cancer reduction [39]. Among them, suicide gene therapy with adeno-associated virus (AAV) vector comprehends the specific targeting of chemotherapeutic agents to tumours by means of gene-directed enzyme prodrug therapy (GDEPT). On the basis of its non-pathogenicity, wide tropism, and prolonged transgene expression *in vivo*, herpes simplex virus is an interesting model because its thymidine kinase (HSV-TK) activates ganciclovir (GCV)-altered DNA synthesis in rapidly dividing cells. Apoptosis is involved in this process, but other non-apoptotic pathways could also occur ("bystander effect"). This strategy has been widely tested *in vitro* on different tumour cell lines and in animal/human models, especially to treat solid tumours, but data regarding the application of recombinant AAVs and HSV-TK in BC are few.

Researchers proposed an effective system for bladder-targeted gene therapy investigating the pharmacological activity of AAV-mediated HSV-TK/GCV system against BC cells and mice xenograft models [40, 41]. Recombinant AAV-HSVTK system limited BC growth *in vivo*. After the administration of GCV in a 3-day protocol T24 proliferation was reduced of 50-60% showing cells with a different morphology. It has been described that HSV-TK gene must enter the neoplastic cells to induce their death, although this phenomenon didn't seem to be realistic on the basis of the poor cell penetration efficiency reported for these gene delivery systems. As a matter of this, the researchers explored the sensitivity of T24/rAAV-HSV-TK cells to different concentrations and times of GCV exposure in addition to the degree of cell viability. Not only a time-dependent cell toxicity was enhanced with the duration of GCV exposure, but also the suppression of cell proliferation and increase in cell apoptosis in T24 cells were demonstrated. After infection, cells in G1 phase slowly increased, whereas cells in S phase decreased from 24 to 48 h and further along with the appearance of cells in sub G1 phase.

Endothelin Receptors Blockade: Is There a Real Alternative for BC Metastasis?

The endothelin (above all ET-1 isoform) system is involved in BC growth and metastases development. The mechanisms by which ET-1 promotes these effects are being progressively unravelled [42]. Overexpressed ET-1 receptor levels are associated with diminished disease-specific survival, promote signalling cascades in tumour at both primary and distant sites, and are positively correlated with the pro-invasive and pro-inflammatory mediators (IL-6, CCL2, COX-2, MMP2, and MMP9) as reported in a large number of experimental studies of BC and confirmed by immunohistochemistry in BC tissue microarrays [43]. ET-1 stimulates *in vitro* migration, invasion, and proteolytic activity of BC cells and, *in vivo*, favours lung metastatic colonization enabling an early inflammatory process characterized by macrophage influx and production of those pro-inflammatory mediators responsible of extravasation and dissemination of cancer cells to secondary sites [44].

Clinical data have demonstrated that using ET-1 receptor antagonists (Fig. **1**), alone and in combination with other drugs (docetaxel), could represent an innovative mechanism-based antitumor approach but results were often in contrast (*i.e.*, Atrasentan failed a phase III clinical trial for prostate cancer). In fact, the ET-1 antagonism has minimal effect on primary tumour growth although in patients with advanced cancers affects experimental metastasis at the early stage [45]. This discrepancy was explained by the administration of ZD4054 (Zibotentan, Fig. **1**), a selective ET-1 inhibitor, at two different stages of the BC development. Prior to injection of tumour cells, it diminished the early inflammatory response and the following rise of lung metastases due to a stronger influence on macrophages activity. Conversely, when its administration was initiated later, the therapeutic diminution of inflammation and metastases was inferior because these processes were no more affected by these cellular effectors [46].

Table 2.   The Most Promising Molecular Targets in BC

| Drugs	Targets/Effects	Trials
AAV-mediated HSV-TK/GCV systems	DNA synthesis	Preclinical
Replication-defective adenoviruses	p53 tumor suppressor expression, pathway defective cancer	Phase I/II
Anti-DR5 mAb Lexatumumab	Death receptors	Preclinical
PI3K inhibitors: Gefitinib and Wortmannin	PI3K pathway	Preclinical
BH3-mimetics: (-)-Gossypol	Bcl-2	Preclinical
Antisense oligodeoxynucleotides	Survivin, clusterin and other inhibitors of apoptosis proteins	Preclinical
Angiogenesis inhibitor: Sunitinib	vascular endothelial growth factor	Phase II
Endothelin-1 receptor antagonists: Zibotentan	Endothelin receptors	Preclinical and Phase I
Histone deacetylase inhibitors: Trichostatin A, Phenylbutyrate, Depsipeptide (FK228), SAHA, Valproic acid, KBH-A42	Histone deacetylases (HDACs)	Preclinical
DNMT inhibitor: Zebularine	DNA methyltransferases (DNMTs)	Preclinical
Farnesyl transferase inhibitors: R115777, Lonafarnib (SCH 66336)	Farnesyl transferase	Phase II
mTOR inhibitors: rapamycin analogs, Everolimus	Tyrosine kinases	Preclinical
EGFR inhibitors: small molecule tyrosine kinase inhibitors (Gefitinib and Sorafenib) and monoclonal antibodies (Trastuzumab)	Tyrosine kinases	Phase II
Ampelopsin	Cell cycle arrest in S phase	Preclinical
Sulforaphane	Mitochondria	Preclinical
Quercetin	Cell cycle arrest in G0/G1	Preclinical
Isorhapontigenin	Inhibiting anchorage-independent cell growth of cancer cell lines down-regulating XIAP (X-linked inhibitor of apoptosis protein) expression	Preclinical
Ursolic acid	Apoptosis	Preclinical
ß-Eleostearic acid	Apoptosis	Preclinical
Curcumin	Induction of HO-1 expression, G2/M arrest	Preclinical
Drimys angustifolia and *D. brasiliensis* essential oil	Apoptosis	Preclinical
Apaziquone	Cytotoxicity	Phase III
Alkylating agent: ICT2740	Cytotoxicity	Preclinical
Inducers of phase 2 enzymes: Oltipraz, CPDT	Activation of phase 2 enzymes	Preclinical
COX-2 inhibitors: Celecoxib	COX-2	Phase III
Vinflunine	Tubulin	Phase III
PPAR γ agonists: Ciglitazone	Peroxisome Proliferator-Activated Receptor γ (PPAR γ)	Preclinical
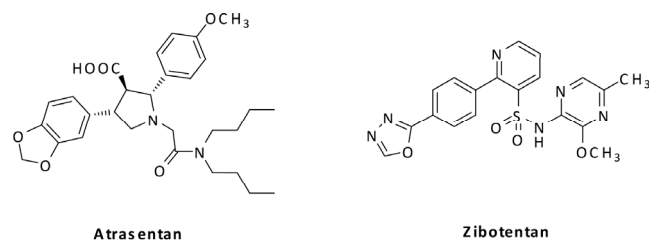
Fig. (1). Selective endothelin-1 receptor antagonists.

Histone Deacetylase Inhibitors (HDACi)

Extensive interest exists for epigenetic modifications in combination with cancer therapeutic strategies. Preclinical evidence showed that HDAC inhibitors (Fig. **2**) potentiate in a synergistic way chemotherapeutic agents whose mechanisms regard topoisomerase enzymes and DNA damage [47]. Trichostatin A (Sigma®) synergistically enhanced the antitumour effects of cisplatin and gemcitabine treatment in human HTB5, HTB9, T24, J82, UMUC14, and SW1710 cells. Interaction between the two drugs was calculated by the combination index based on the Cell Counting Kit-8 and on a clonogenic assay. The expression of cell cycle (p21WAF1/CIP1, cyclins, and pRb), apoptosis (caspase 3, 8 and 9, PARP, Bcl-2, Bad, and Bax), NF-κB, and survival (Akt, mTOR, and PTEN) proteins was performed by Western blot. Isobolic analysis confirmed strong addictive effects between gemcitabine and Trichostatin A, which caused a gemcitabine/Trichostatin A dose reduction. Both of them aimed at inducing cell cycle arrest and caspase mediated apoptosis, with the downregulation of the antiapoptotic NF-κB and Akt signaling pathways [48].

Instead, Valproic acid has been associated with Mitomycin C on human BC cells *in vitro* and this effect was compared to that of Valproic acid or Mitomycin C alone. HTB5 and HTB9 cells derived from low and high grade bladder tumors, respectively, were used. Treatment for 24-72 h with Valproic acid and Mitomycin C resulted in concentration and time dependent decrease in viability and proliferation. HTB9 cells showed marked sensitivity to Mitomycin C ($IC_{50} = 1$ μM) with respect to Valproic acid alone ($IC_{50} = 2.5$ mM) [49]. The effect on the chromatin structure induced by Valproic acid pre-treatment sensitized the BC cell lines favouring the therapeutic action of Mitomycin C, but animal model validation is further needed.

More in detail, other studies were performed to unravel the molecular effects of Valproic acid alone (0.125-1 mmol/l) on RT-4, TCCSUP, UMUC-3, and RT-112 BC cells without and with preincubation period of 3-5 days [50]. Valproic acid displayed time- and dose-dependent growth inhibition on BC cell lines justifying much stronger effects with a prolonged therapy. Conversely, in case of no preincuba-

tion, it is not possible to note any reduction in cancer growth at lower doses in TCCSUP, UMUC-3, and RT-112 cells. Moreover, this preincubation (3-5 days, at 1 mmol/l) was shown to modulate cell cycle progression affecting negatively the cell cycle-regulating proteins (cdk1, cdk2, cdk4, and cyclins B, D1, and E) and positively p27 and H3 acetylation.

Lastly, the HDAC inhibitor KBH-A42 significantly suppressed the proliferation of several tested human cancer cell lines. Among these, the UM-UC-3 bladder cancer cells were the least sensitive and growth inhibitory effects of KBH-A42 on UM-UC-3 cells were not mediated by apoptotic induction [51].

Targeted Therapy and New Technological Formulations

Intracellular drug delivery systems are proposed to transport anticancer drugs into tumour cells to reduce drug dosage and side effects [52]. Inadequate drug delivery to tumour cells is a major cause of failed intravesical therapy for NMIBC, partly due to the dilution of drug concentration by urine production during treatment and poor drug uptake by bladder tissues during instillation. A large number of submicron drug carrier systems have been recently proposed to enhance the delivery and capture of drugs by neoplastic tissues [53]. In recent years, medical therapies associated with nanomedicine have become more tailored to specific diseases and patients developing a new platform for anticancer strategies (prevention, early detection, imaging/diagnosis, and therapy) (Table **3**) [54].

Active Principles from Plant-derived Extracts

Nowadays there is an increased attention on traditional herbal medicines (Fig. **3**) on the basis of their known effectiveness in the treatment of pathologies for which they have been traditionally applied. Although the therapeutic potential, their activities could be affected by food-food, drug-drug, and drug-food interactions.

Ampelopsin: This chemotype, obtained from the root of the Chinese medicinal herb *Ampelopsis cantoniensis* or *grossedentata*, has anticancer effects on different human cancer cell lines, but its clinical use required repeated large dose application because of its water insolubility and its short half-life. To enhance its pharmacokinetic, Ampelopsin is formulated as a sodium salt (Amp-Na) and it has been tested on BC cells *in vitro* and BC xenograft model in mice [70]. Amp-Na can considerably inhibit the growth of S180 and EJ xenografts in mice and flow cytometry analysis revealed that tumour cells were principally arrested in the S phase, inhibiting the proliferation of BC cells. Pharmacokinetic analysis in normal mice demonstrated that it was excreted primarily through the urine; the absorption is mainly
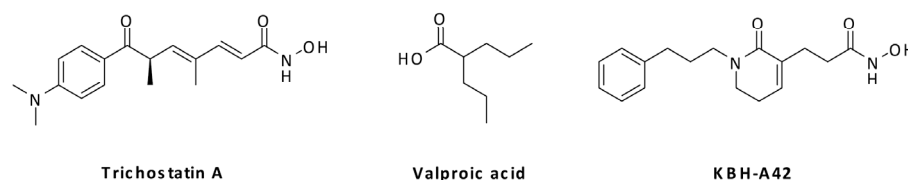

Fig. (2). Histone deacetylases inhibitors.

Table 3. **Drug Delivery Systems and New Drug Formulations for Targeted Bladder Cancer Therapies**

Drug(s)	Drug Delivery System or New Drug Formulation	Tumor Cell Line(s) or Model System(s)	Innovation and Results	Ref.
Small interfering RNA (siRNA) targeting human Amplified in breast cancer 1 (AIB1)	Inorganic amorphous calcium carbonate (ACC) hybrid nanospheres functionalized with Ca(II)-IP6 compound (NPACC/CaIP6)	Human BC T24 cells and female mice bearing T24 tumors	NPACC/CaIP6 protected the encapsulated siRNA from degradation. AIB1 knockdown mediated by ACC/CaIP6/siRNA complexes transfection resulted in cells proliferation inhibition, apoptosis induction and cell cycle arrest *in vitro*. NPACC/CaIP6 attenuated tumor growth and downregulation of PI3K/Akt signaling pathway *in vivo*	[55]
Duplex dsP21-322 and its chemically modified variants (RNAa-based drugs)	Lipid nanoparticles (LNP) for intravesical delivery	Human BC KU-7 and T24-P cell lines. An orthotopic murine model of BC	p21 induction, cell-cycle arrest, and apoptosis *in vitro*. LNP formulation also improved duplex stability. Intravesical delivery of LNPdsP21-322-20F into mouse bladder with established orthotopic human BC extended their survival	[56]
Cisplatin	The hydrophobic PCL core can be used to load superparamagnetic iron oxide nanoparticles (SPIONs), with pendant dicarboxylic groups in the hydrophilic shell	UMUC3 cells	SPIONs-loaded, cisplatin-conjugated polymeric nanoparticles (Pt−Fe−PNs) are mucoadhesive and release 30% of the cisplatin in the first 4 h followed by a slow sustained release over 4 days. These Pt-Fe-PNs induced cytotoxicity against UMUC3 bladder cancer cells with IC$_{50}$ of 32.3 μM	[57]
Cisplatin	Highly biocompatible and stable coordination polymer (Prussian Blue) nanoparticles with a hollow interior and a microporous framework (HPB)	T24 cells	LC$_{50}$ >1000 μg mL^{-1}. The particles of size of around 100 nm meet the passive tumor-targeting conditions of enhanced permeability and retention and tend to accumulate at tumor tissue	[58]
Epirubicin	Poly(ethyl-2-cyanoacrylate) nanoparticles (EPI-NP)	Human BC T24 and RT4 cells	Two EPI-NP formulations were developed. Their penetration and accumulation in pig urothelium were studied	[59]
Paclitaxel	Nanoparticle albumin bound paclitaxel (Abraxane®, ABI-007)	Patients eligible for this study all had high grade transitional cell carcinoma of the bladder with cystoscopic biopsies consistent with stage T1, Ta or Tis NMIBC	Increased solubility and lower toxicity compared to docetaxel in systemic therapy	[60]
Paclitaxel	Gelatin nanoparticles	Tumor bearing pet dogs with pathologically confirmed transitional cell carcinoma	Drug release *in vitro* and *in vivo* was performed obtaining the pharmacokinetics of paclitaxel gelatin nanoparticles in plasma, urine, and tumors. These nanoparticles showed low systemic absorption and favorable bladder targeting	[61]
Docetaxel	Mucoadhesive formulations based on hyperbranched polyglycerols (HPG), hydrophobically derivatized with C$_8$/C$_{10}$ alkyl chains in the core and modified with methoxypolyethylene glycol (MePEG) and amine groups in the shell	Low-grade (RT4, MGHU3) and high-grade (UMUC3) human urothelial carcinoma cell lines and KU7 cells	*In vitro* the formulation potently inhibited BCr proliferation, whereas *in vivo* it inhibited tumor growth in an orthotopic model of BC	[62]

(Table 3) contd….

Drug(s)	Drug Delivery System or New Drug Formulation	Tumor Cell Line(s) or Model System(s)	Innovation and Results	Ref.
Paclitaxel	Unimolecular micelles based on hydrophobically derivatized hyperbranched polyglycerols (dHPGs) for use as mucoadhesive intravesical agents against NMIBC	Human urothelial carcinoma cell lines (RT4, MGHU3, UMUC3) and KU7 cell line	The release profiles were characterized by a rapid-release phase followed by a slower sustained-release phase. Moreover, paclitaxel/HPG-C$_{10}$-PEG was stable in acidic urine. *In vitro*, all formulations decreased BC proliferation with low cytotoxic. *In vivo*, the mucoadhesive HPG-C$_{10}$-PEG formulation was more effective in reducing orthotopic tumour growth than Taxol	[63]
Mitomycin C (MMC)	Cationic nanoparticles of chitosan (CS), poly-ε-caprolactone coated with chitosan (CS-PCL) and poly-ε-caprolactone coated with poly-L-lysine (PLL-PCL)	MB49 mouse urinary bladder carcinoma and G/G mouse urinary bladder cell lines	Longer residence time, higher local drug concentration and prevention of drug loss during bladder discharge. Complete drug release was obtained with only CS-PCL nanoparticles. On the other hand, CS and PLL-PCL nanoparticles did not completely liberate MMC	[64]
(-)-Epigallocatechin-3-gallate (EGCG)	Nanogold particles (pNG)	C3H/HeN mice subcutaneously implanted with MBT-2 murine BC cells	EGCG-pNG inhibited tumor cell growing by means of cell apoptosis. Additionally, VEGF downregulation was also determined	[65]
Paclitaxel, Daunorubicin	Micelle nanocarrier (telodendrimers) functionalized with PLZ4 (from high throughput screening of combinatorial cyclic peptide libraries) and the imaging agent DiD dye (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine)	Orthotopic invasive dog BC xenograft model in mice	More efficient in targeted drug delivery *in vitro* and *in vivo*. PLZ4 facilitated the uptake of micelles together with the cargo load into the target cells	[66, 67]
Paclitaxel (PTX), Docetaxel (DTX)	Diblock copolymer (methoxy poly(ethylene glycol)-block-poly(lactic acid) (MePEG-PDLLA) and methoxy poly(ethylene glycol)-block-poly(caprolactone) (MePEG-PCL) nanoparticles	Freshly excised pig bladder sections	PTX or DTX loaded in MePEG-PDLLA micelles produced higher urothelial tissue levels and greater bladder wall exposures	[68]
Mitomycin C (MMC) + oral sodium bicarbonate	Urine alkalinization improves drug stability and cellular uptake	Patients with NMIBC	This study did not demonstrate the efficacy of urinary alkalinization before early single-dose MMC instillation following TuRBT in improving the effectiveness of MMC instillation	[69]

achieved through the mice bladder epithelium (absolute bioavailability of 69.10%, half-life ~11.01 min, low local toxicity).

Sulforaphane (SFN): It is a component of the isothiocyanate family present in cruciferous vegetables [71]. Epidemiological studies confirmed that *Cruciferae* intake is associated with limited risk of various cancers including BC [72, 73]. As reported in a recent and complete study, its administration induced a reduction of the average tumour volume in SFN extract-treated mice, a decrease in tumour cell angiogenesis and improved immune action. Moreover, the expression of cyclooxygenase 2 (COX-2) is inhibited by SFN extract, thus promoting apoptosis without cell specificity *in vitro* [74]. Its primary targets are mitochondria, thanks to the activation of caspases pathway. The skill of SFN to reduce BC UM-UC-3 xenografts was also confirmed [75].

Quercetin: This flavonoid is present in many fruits and vegetables and exerts antioxidant, cardioprotective and anti-cancer properties. Among its anti-tumour effects, it inhibits cell proliferation, promoting cell cycle arrest or cell death by blocking different enzyme systems (including ecto-5'-NT/CD73). Quercetin has already demonstrated growth inhibitory effects through decrease of cell viability, inhibition of colony formation, enhancement of apoptosis, and cell cycle arrest in G0/G1 in T24, EJ, and J82 BC cell lines. T24, a more malignant BC cell line, presents a low ATP- and ADP-hydrolysis activity and a mayor ecto-5'-NT (nucleotidase)/CD73 activity while RT4, a less malignant BC cell line, presents higher ATP- and ADP-hydrolysis activity than AMP-degrading activity. This different expression pattern of ectonucleotidases justifies the involvement of ecto-5'-NT/CD73 in tumorigenesis of the urinary bladder. Several
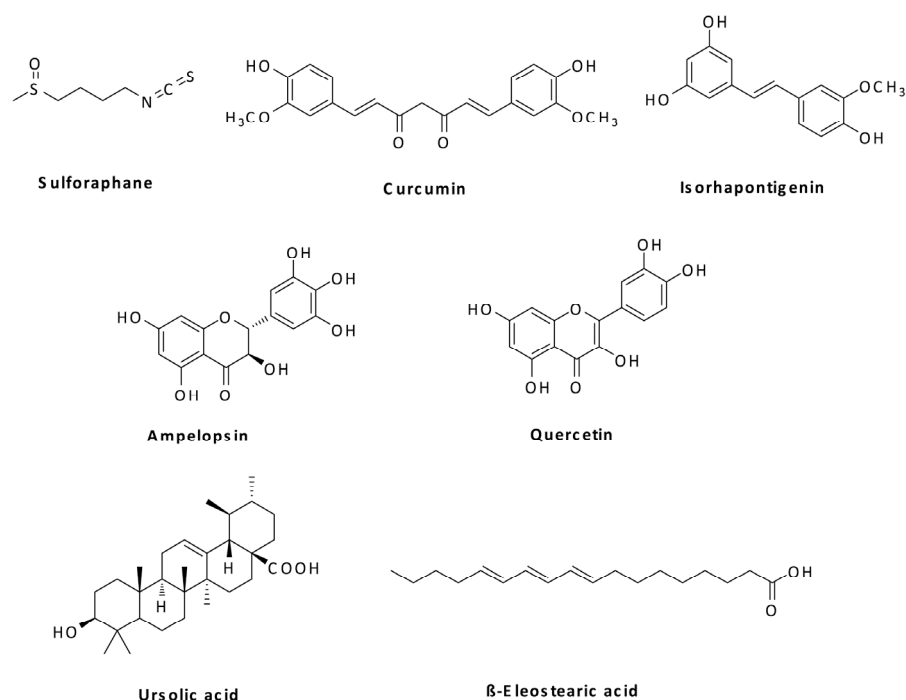
Fig. (3). Active principles found in plant-derived extracts.

studies have elucidated that ecto-5'-NT/CD73 acts as a pro-liferative factor and as a linker protein implicated in cell growth and invasiveness in different tumours; thus its inhibition would be a potential target for future BC treatment. Quercetin exerted an antiproliferative effect on T24 cell line with an IC_{50} of 47.7 µM and inhibited ecto-5'-NT/CD73 activity after treatment for 24, 48, and 72 hours by a direct action on the enzyme also at lower doses (30 µM). The effects of similar dosages (10 and 30 µM) on proliferation of RT4 showed that this cell line was resistant to Quercetin, differently from T24 cells. Taken together, these data reinforce a possible association between ecto-5'-NT/CD73 inhibition and BC proliferation. Another finding of the present work is the enhancement of ADP hydrolysis by Quercetin treatment. The increased ADPase activity together with a lower AMPase activity may induce an AMP accumulation with consequent lower adenosine production. While extracellular adenosine is known to activate proliferative pathways, extracellular AMP has the opposite effect; thus the consequence of this AMP accumulation could be one reason for the decrease of cancer cells proliferation when treated with Quercetin [76].

Isorhapontigenin: *Gnetum cleistostachyum*, another Chinese herb, has been used for treatment of BC for years. A new polyhydroxy stilbene derivative, Isorhapontigenin, has been recently isolated and it has proven to possess pro-apoptotic and anti-cancer effects in human BC RT112, UMUC3, and T24T cell lines [77], by inhibiting anchorage-independent cell growth of cancer cell lines, down-regulating XIAP (X-linked inhibitor of apoptosis protein) expression, and inducing apoptosis. XIAP overexpression in cancer tissues is associated with cancer progression, metastasis, and resistance to cancer therapy. The down-regulation of XIAP by Isorhapontigenin is caused by direct inhibition of the expression and binding activity of nuclear transcription factor

SP1 with XIAP promoter SP1 binding sites, leading to the inhibition of *XIAP* gene transcription. Moreover, Isorhapontigenin has many biological effects due to an antioxidant pathway with regard to the inhibition of phosphorylation of PKC, ERK1/2, JNK, and P38, further leading to direct or indirect inhibition of NF-κB and AP-1 activation.

Ursolic acid (UA): This pentacyclic triterpenoid, commonly found in medicinal herbs, exhibits anticancer effects (inhibition of tumorigenesis, invasion, metastasis and angiogenesis, and promotion of tumor cell apoptosis and differentiation). As a matter of this, researchers pointed out the role of UA in AMP-activated protein kinase (AMPK) downstream signaling effectors (JNK, p53 and mTOR complex 1) to mediate important cellular functions such as cell growth and proliferation, apoptosis, ceramide production, and ATP consumption. AMPK, considered as the master energy sensor, provides cell energy content and plays an important role in cell survival or death in response to various pathological stress [78]. More in detail, the same authors focused their attention on the possible function of endoplasmic reticulum (ER) stress in the anti-BC mechanism induced by UA. Several stress stimuli (*i.e.*, anti-cancer drugs) alter ER physiological functions inducing apoptosis through the involvement of different transcription factors, Bcl-2 family members, upregulation of TRAIL receptors, activation of pro-apoptotic ASK1–JNK signaling, and recruitment of caspases. UA treatment enhances, at least in part, ER stress-associated apoptosis mechanisms [79].

β-Eleostearic acid: The β-Eleostearic acid, abundant in some plant seeds, has been studied because of its structural similarity with the conjugated linolenic acid known to promote health in humans as other conjugated fatty acids. Established evidence confirms that this class of natural compounds can selectively kill tumor cells also by apoptosis

through the modulation of the cellular production of reactive oxygen species (ROS). Accumulation of a large amount of ROS leads to lipid peroxidation, protein and enzyme inactivation, and DNA damage. In addition, ROS formation mediates the apoptotic action also *via* PPARγ activation. β-Eleostearic acid might be also considered as a PPARγ agonist. β-Eleostearic acid inhibited BC T24 cells growth in a dose-dependent manner (incubation for 12-48 h at concentrations of 10-80 μmol/L) with generation of ROS and induction of apoptosis as evidenced by flow cytometry analysis. At low ROS concentration, mitogenic and proliferative effects were characteristically observed, whereas at high ROS concentrations, macromolecular damage and cell death took place. The expression of caspase 3 and Bcl-2, investigated by immunoblot analysis, indicated that β-Eleostearic acid mediated induction of apoptosis by down-regulating Bcl-2 and activating of caspase 3. In view of a dual therapeutic approach for tumours targeting ROS and PPARγ signaling pathways, these studies in T24 cells suggest that ROS generation is critical for cell killing by means of PPARγ activation and apoptosis induction [80].

Curcumin: the major active phenolic compound in spice turmeric was shown to inhibit cell proliferation (inducing G2/M arrest, increasing apoptosis after the treatment with gemcitabine, paclitaxel, TNF and TRAIL, thanks to the silenced NF-κB activation) [81, 82]. As reported in another study, Curcumin, given orally to mice, enhances the antitumour effect of the chemotherapeutic drugs BCG [83] and gemcitabine [84], without being alone a good anti-cancer agent in a murine orthotopic BC model [85]. Usually, thanks to its structural electrophilic features, it was shown to have anti-inflammatory and anti-cancer effect. Low doses of Curcumin induce heme oxygenase-1 (HO-1) expression, acting as an antioxidant and anti-inflammatory agent [86]. This phase II enzyme is activated by Nrf2 transcription factor and contributes to cell cytoprotection, but it has been demonstrated that HO-1 is overexpressed in several tumors. The expression of HO-1 in BC cells derived not only by Curcumin but also by gemcitabine and irradiation treatment, and suppression of HO-1 improves the antitumoral action of gemcitabine *in vivo* [87]. In clinical studies, the overexpression of HO-1 is associated with the high tumour grade, the use of siRNA to knockdown HO-1 enhances the sensitivity of BC cells to Doxorubicine *in vitro*, and therapy with intravesical anthracyclines improves HO-1 activation in the poor disease-free patients [88]. On the opposite, the induction of HO-1 expression by Curcumin *in vivo* is considered advantageous in chemoprevention, but damaging in antitumoral treatment. For this reason, in order to improve the anti-invasive action of Curcumin in a BC multi-drug therapy, the administration of a HO-1 inhibitor should be recommended.

Drimys angustifolia and D. brasiliensis essential oil: Researchers evaluated the cytotoxic effects of the volatile oils extracted by the leaves of these two species, proposing a nanoemulsion formulation and assessing the *in vitro* cytotoxicity on T24 cell lines [89]. The oil chemical composition was evaluated by GC/MS (bicyclogermacrene and cyclocolorenone were the most abundant compounds for the *D. angustifolia* oil and *D. brasiliensis* oil, respectively). The nanoemulsions were prepared and fully characterized. Only

the *D. brasiliensis* oil was efficient in reducing the cell viability of T24 at three different concentrations 125, 250, and 500 μg mL^{-1} (33.2% ± 2.8, 60.3%± 1.6 and 80.5% ± 8.8, respectively), as assessed by MTT assay and confirmed by cell counting. Finally, *D. brasiliensis* oil incubation caused an enhancement of annexin-V and propidium iodide population, characteristic of late apoptosis induction under cytometry analysis.

Metabolism Enzymes Modulators and Substrates (Figure 4)

CYP1A1 substrates: CYP1 family (CYP1A1, 1A2, 1B1) mediated oxidation of important xenobiotics (polycyclic aromatic hydrocarbons and aromatic amines) to carcinogenic metabolites during Phase 1. Its contribution to the development of BC is well established due to the overexpression and/or polymorphism of this protein in human exfoliated urothelial cells of BC. For these reasons, these CYPs could be molecular targets for the development of new tumourselective therapeutic strategies. A potential approach for BC is to take advantage of the oxidation functionality of CYP1A1 to activate selectively cytotoxic drugs within the tumour. Researchers synthesized a truncated chloromethylpyrroloindoline (ICT2700) belonging to the natural duocarmycin family, which possessed the chemical possibility to be CYP-activated to a potent DNA (adenosine) alkylating agent, ICT2740 (its active metabolite). Other examples are exemplified by Apaziquone (currently in Phase III) [90], NSC686288 [91], and Banoxantrone [92], which were used against BC. ICT2700 showed a significant *in vitro* cytotoxicity in RT112 (CYP1A1 positive) but not in EJ138 (undetectable CYP1A1) human BC cell lines. Patient selection for ICT2700 therapy would be greatly dependent on the corresponding identification of tumor CYP1A1 expression to reach the goal of the personalized medicine [93].

CPDT: People with a deficit of Phase 2 enzymes or knock-out models (*i.e.*, glutathione *S*-transferase, GST; NAD(P)H:quinone oxidoreductase 1, NQO1) are more susceptible to bladder cancer, while the overexpression of Phase 2 enzymes through gene transfection protects cultured cells against carcinogen-provoked DNA injury. One of the principal mechanisms due to the activation of an individual Phase 2 enzyme affects the Keap1-Nrf2-ARE signaling system. Oltipraz, belonging to the recognized class of inducers of Phase 2 enzymes, the dithiolethiones, [94] has been the most extensively investigated also if it suffered from significant side effects in humans. One of its analogs, 5,6-dihydrocyclopenta[*c*][1,2]-dithiole-3(4*H*)-thione (CPDT), was considered the most potent activator of rat Phase 2 enzyme activities and their corresponding proteins in many tissues, being the bladder epithelium the most susceptible in a dose-dependent manner (induction of GST and NQO1 by CPDT was 4.2 and 4.8 fold higher than the one of Oltipraz under the same conditions of therapy, respectively). It has been detected an important increase in the expression of both enzymes in the bladder also at the lowest dose tested (0.98 μmol/kg/day) with an enhancement of GSH content. As mentioned above, Nrf2 is the key transcriptional factor of many Phase 2 genes, and its activation by CPDT reveals the role of this compound in chemoprevention in primary normal human bladder epithelial cells and rat BC NBT-II cells *in*
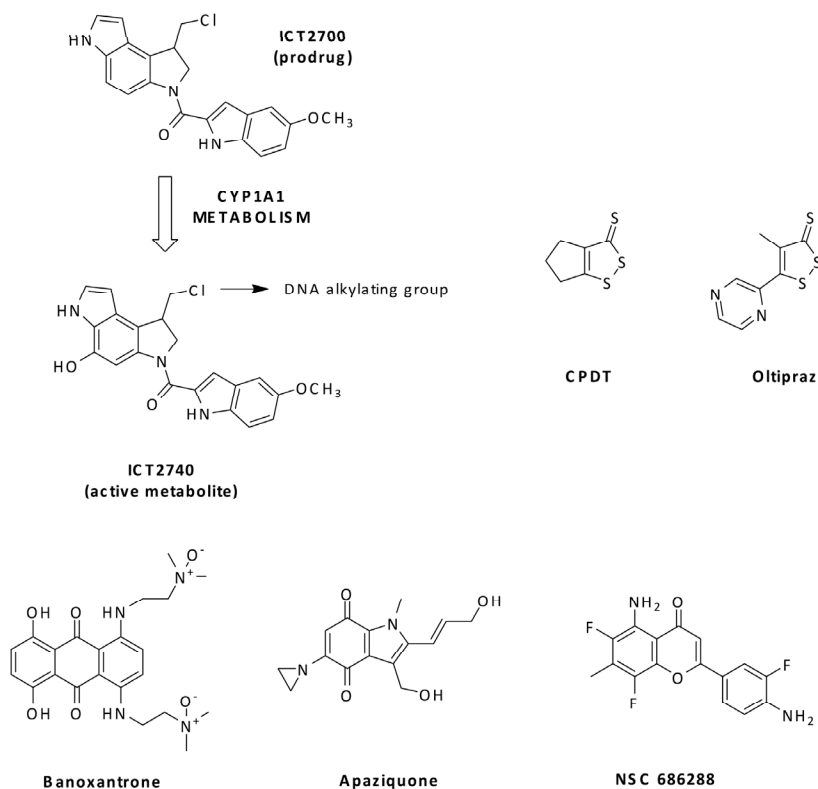
Fig. (4). Metabolism enzymes modulators.

vitro. Another Phase 2 enzyme, UGT1A, was not responsive to CPDT in the bladder *in vivo* and in human bladder epithelial cells *in vitro,* but its induction was enhanced in NBT-II cells [95].

Celecoxib: The important role played by COX-2 pathway in the development of BC is supported by several epidemiologic studies [96]. Indeed, Celecoxib (Fig. **5**) can prevent neoplastic transformation of urothelium and reduce urothelial tumor burden *in vivo* both alone and associated with BCG [97].
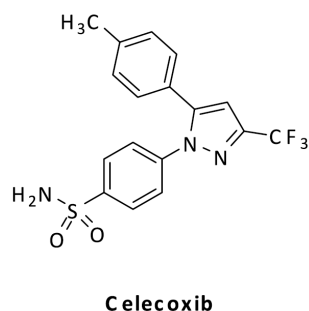

Celecoxib

Fig. (5). Selective COX-2 inhibitors: Celecoxib.

Sabichi and colleagues conducted a phase IIb randomized placebo-controlled trial of Celecoxib (200 mg twice a day) for a minimum of 12 months to prevent recurrence in 146 patients following transurethral resection of established NMIBC and adjuvant BCG [98]. In addition, an ongoing phase III randomized controlled Bladder COX Inhibition Trial (BOXIT) of the same daily dose of Celecoxib (albeit 400 mg once a day in BOXIT and 200 mg twice a day in ref. [98]) versus placebo for 24 months is being conducted in a

similar patient population (intermediate and high-risk NMIBC). A number of clinical trials testing celecoxib in patients with established cancer have not reported issues relating to its acceptance.

Selenium and other micronutrients: SELEBLAT is a phase III randomized, double-blind, placebo-controlled, multicenter, academic trial that consists in 200 μg/day selenium-yeast supplementation for 3 years with a successive follow-up period of 3 years in order to prevent recurrence of NMIBC. SELEBLAT was initiated in Belgium financed by the Agency for Innovation by Science and Technology [99]. The first purpose of SELEBLAT is to define the recurrence free interval that is from the starting date of trial to the date of recurrence in patients with superficial transitional cell BC. The second goal is to explain the progression-free interval that is from the starting date of trial to the date of progression. In SELEBLAT can be included both males and females who are at least 18 years, and who underwent a TUR of a histologically confirmed transitional cell malignant tumor of the bladder, stage pTa, pT1 or carcinoma *in situ*. Patients have been recruited from September 2009 to August 2012.

The investigational medicinal product is selenium (200 μg/day as safe and cheap selenium yeast). First statements of this trial for the BC prevention reported that the administration of selenium yeast for six months increases the levels of selenium in the plasma in a dose-dependent fashion. Another trial, SELENIB, that is analogous to SELEBLAT chemoprevention study, is based on the administration of selenium and vitamin E and is ongoing in the UK. It aims to include 500 patients with NMIBC receiving each day for 5 years a sup-

plement of 200 μg high selenium yeast or placebo and 154 mg of α-tocopherol or placebo [100].

Other trials have been involved in chemoprevention [101, 102]. The time to tumour recurrence was enhanced by high doses of vitamins or *Lactobacillus casei* powder. Also the administration of retinoids was evaluated with several results. The rate of recurrence was reduced neither by vitamin B6 nor by difluoromethylornithine [103]. Generally, prospective cohort and case-control studies have shown that there are contradictory results with regard to the association of BC with fruits and vegetables and there are also inconsistent epidemiologic data about the protection of specific groups of fruits and vegetables against BC, even if clinical studies have demonstrated that groups like brassica and allium promote glutathione *S*-transferases minimizing oxidative stress. Surely, it is possible to correlate certain micronutrients with a reduced risk of BC. The Spanish bladder cancer study [104] not only exposed that a great intake of folate and B-vitamins were protective against BC, but also evaluated the role of meat cooking and related carcinogens in a large population-based case-control study, showing that there was an association between a big intake of processed meat, especially red meat, and the increased occurrence of BC. Indeed, a minor risk of BC was seen in individuals in the highest quartile of dietary vitamin B12 intake.

Vinflunine: It is a third-generation semisynthetic vinca alkaloid given by fluorination of vinorelbine. As the other vinca alkaloids, VFL (Fig. **6**) exerts its antineoplastic effects by interacting with tubulin that is involved in mitosis and coordinating chromosomal segregation, even if the binding affinity of VFL to tubulin is rather lower than the affinity of other vinca alkaloids to the latter. The higher antitumor activity of VFL *in vitro* and *in vivo* and its exceptional safety profile are probably due to the fact that VFL reaches high intracellular concentrations while still allowing the other vinca alkaloids to bind the unassembled tubulin. In cell cultures, VFL reduces the microtubule network of interphase cells and induces G2-M arrest, leading to amassing in mitosis at the metaphase/anaphase transition and apoptosis. On a transurethrally implanted murine BC cell line, VFL showed anti-tumour activity against superficial BC model higher than that of vinorelbine with a good general tolerance. Two single-agent phase II trials were performed with regard to transitional cell carcinoma (TCC): thanks to the fact that VFL, tested in patients pre-treated with platinum, has shown to have moderate response rates and encouraging disease control rates, the first phase III trial for second-line TCC of the urothelium was planned with the aim at investigating its activity and first results were presented at the 2008 ASCO conference. In conclusion, therapy with VFL seems to be a plausible option for patients with TCC progressing after first-line platinum-containing chemotherapy [105].

Ciglitazone: Among the insulin-sensitizing drugs used for the treatment of type II diabetes, there are thiazolidinediones (TZD), such as rosiglitazone, troglitazone, pioglitazone and Ciglitazone (Fig. **7**). These TZD were shown to be high-affinity ligands of Peroxisome Proliferator-Activated Receptor γ (PPAR γ), that plays an important role in regulation of metabolism and inflammation and it is also involved in carcinogenesis of BC as described in our previous review [36].

Only two studies reported on Ciglitazone-inhibitory effects *in vitro* on metastatic bladder TCC [106], on RT4 (derived from a well differentiated grade I papillary tumour in which the epithelial marker E-cadherin is detected while there is no expression of the mesenchymal marker N-cadherin) and T24 (derived from an undifferentiated grade III carcinoma, in which N-cadherin replaced the E-cadherin) BC cell lines, and *in vivo* in nude mice of high grade bladder tumor xenografts. Ciglitazone therapy induced G2/M phase cell cycle arrest and apoptosis only in T24 high grade BC cells with a PPARγ activation-independent mechanisms. Specifically, Ciglitazone mediated up-regulation of TRAIL and a down-regulation of c-FLIP and surviving, leading TRAIL-refractory T24 cells to be responsive to TRAIL with a resulting cell death, probably due to the down-regulation of inhibitors of apoptosis proteins, the ROS-mediated up-regulation of DR5 or the caspases 3 and 8-mediated β-catenin cleavage [107].
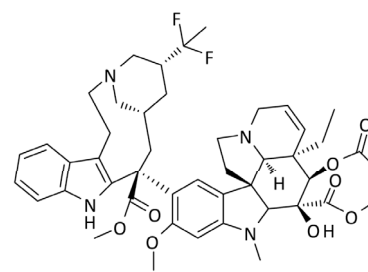
Vinflunine

Fig. (6). Vinflunine.
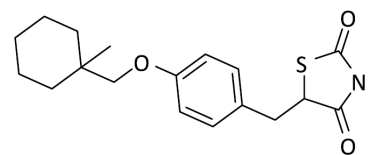
Ciglitazone

Fig. (7). Ciglitazone.

3. THE PROBLEM OF RECURRENCE AND RELAPSE IN BC

The etiology of primary BC, with particular attention to genetic factors, had been extensively analyzed elsewhere [7]. Anyway, an aspect of BC is its high frequency of recurrence. Most patients are affected by this condition more than once in their lifetime and the incidence of recurrence ranges between 50% and 90%, with up to 25% of tumours showing progression towards invasiveness [108-110]. The main risks for BC patients are recurrence, progression to higher stage/grade, and metastases formation. Moreover, conventional treatments are largely ineffective in recurrence prevention in all tumours [111] and recurrence is a relatively fast phenomenon: ~80% of patients are affected within two years since their first BC treatment; consequently, despite surgical and/or chemical therapy, about half of them with invasive tumours have a fatal outcome as a consequence of metastases [112-115]. Furthermore, it should be recalled that BC is also

a heterogeneous disease, being this fact strictly correlated with prognosis [116], despite the relatively simple organization of bladder urothelium. In fact, urothelium is an epithelium consisting of 3-6 layers of cells, further divided into three anatomical regions: a basal layer, mainly composed by less differentiated cells, involved in proliferation; one or more intermediate layers, showing more differentiated cells; a superficial layer, harbouring terminally differentiated, mono- or bi-nucleated, umbrella-shaped cells characterized by a highly specialized cell membrane forming plaques that cover the entire cavity of the bladder [117]. Molecularly, the level of cell differentiation is identifiable by cytokeratins expression: cytokeratin-20 is typical of highly specialized cells, cytokeratin-7 is more frequent in intermediate layers, cytokeratin-5 and -6 are typical of the basal sheet [118-120].

Relapse and recurrence both indicate that more than one BC forms in a different bladder section, either synchronously (multifocal tumours) or metachronously. However, there is a significant difference between these two words: "relapse" means that the disease was not completely cured, possibly because of an ineffective treatment [121] and so the *same disease* shows up twice; "recurrence" means that the patient is affected by the *same kind of disease* twice, but the two episodes are not correlated. These two words refer to two different theories about how BC affects patients more than once in their life, namely the "field cancerization" hypothesis and the "intraluminal seeding" and/or "intraepithelial spread" hypothesis. According to the former theory, tumours arising are not genetically related, thus they are not coming from the same progenitor cells, and consequently they are not clones. Hence, multiple tumours may show up independently and more than one tumour focus may be present at any time in bladder. The latter theory is based on the fact that there is only one cell (or, at most, a few of them) as a progenitor of all foci, and this cell or any of its offspring is able to invade the surrounding tissues either by moving inside the bladder lumen, or by sliding throughout the epithelium. Then, the cell is able to fix in a new place and grow, creating a new tumour focus. According to this theory, then, all foci should be genetically related, since they share common progenitor cell(s), and considered as separated parts of the same clonal unit. The possibility for tumour cells to move inside the bladder lumen and/or to invade the surrounding tissues is not trivial: for example, the chance to have upper urinary tract cancers secondary to a BC is significantly increased (15-22 fold) in presence of concomitant vesicoureteral reflux [122, 123]. Similarly, also tumoral cell spreading inside the urothelium is supported by available data [124, 125].

Theoretically, the discrimination between these two theories is simple: if two tumours are genetically related, they should share (at least some of) the genetic markers they harbour [7], supporting the clonal origin of multifocal BC; if not, this will support the field cancerization hypothesis. Indeed, a large literature accumulated during the years, crediting either one of these two hypotheses [126]. Actually, in many reports containing data from different patients, it is possible to identify both genetically related and unrelated foci (although the former are usually more numerous than the latter), thus the general feeling is that both mechanisms may act in BC. It is possible that the major source of contradiction is the incomplete knowledge of tumour genesis, es-

pecially regarding the pre-neoplastic lesions that only ultimately lead to malignancy. It should be remembered that cellular transformation is a multi-step process; according to some Authors, at least 4-6 genetic alterations are required for malignancy [127]. Some of the genes involved in BC are largely represented in tumour samples; however, their combination is largely focus-specific [7, 128]. It is possible that, even if the foci are genetically related, they may share only a part of their mutated genes, and consequently the analysis of a subset of these genes may lead to incorrect conclusions. Also, it is well established that the clonal expansion relies heavily on the cellular micro-environment surrounding the transformed cell(s) [129, 130]. In addition, even the genetic status of apparently healthy, contiguous tissue should influence BC reformation. Indeed, genotoxic stresses applied to the entire urothelium were shown to create a favourable genetic background for BC occurrence, for example in people living in the area surrounding Chernobyl, Ukraine [131]; a similar role is played by chronic whole bladder inflammation in patients affected by schistosomiasis [132].

Another source of contradiction comes from the definition itself of "field cancerization". This theory was first described by Slaughter in 1953 [133] in oral squamous cell carcinoma and was afterwards used also for other epithelium-related tumours, such as oral cavity [134], larynx, lung [135], ovary [136], cervix [137], colon and skin [138]. In most reports, the field cancerization is described as a large-scale change in the cells of the whole urothelium, and each cell has the potential to evolve into a mature tumour independently; therefore, these tumours are genetically unrelated. However, according to some Authors, this is not the right way of considering this theory [139]. Clusters of less than 200 contiguous cells, showing a genetic alteration, may grow to create "patches" [138] and each patch, being composed of genetically related cells, may be considered as a clone. In human bladder, these patches may be up to 1 cm^2 in size [140]. However, it was also established that genetically-related foci can be also more than 7 cm apart in the urothelium [141]. Thus, the pre-neoplastic lesion may grow and displace the surrounding normal epithelium, becoming a "field", which is then defined as one or more epithelial cells showing multiple genetic alterations, of monoclonal origin, yet not necessarily characterized by invasiveness or metastatic behaviour [134]. Hence, due to genetic clone divergence, the more the patch grows, the more distant portions of them may appear genetically different, despite their common origin [142]. This situation does create a "field" of pre-transformed cells sharing part – but not all – of their genetic alterations. This, coupled with the relative limitation of technical analysis, might explain the heterogeneity of multifocal tumours when they occur. In any case, even if the foci may be considered as all clonally related, their differences should be taken into account because they might influence the efficacy of chemical therapeutic agents, because of a differential response to treatment.

Most of the abovementioned hypotheses are based on the existence of cancer stem cells (CSC) in most, if not all, tumours [143, 144], and therefore also inside the mutated urothelium [128, 145]. To date, these cells had not yet been isolated inside the bladder, but some indirect proofs of their existence were recently collected. Some populations of het-

erogeneous cells, that are able to form colonies and self-renew [146, 147] and that show the expression of stem cell-related genes, were isolated [148-150]. Such cells, if not killed/removed during BC therapy, may be the cause of BC recurrence [151]. These data, coupled with the recent discovery of normal stem cells inside the urothelium [152], strongly support the existence of bladder CSC. Single bladder stem cells are able to form clones that may expand and replace the surrounding clones showing lower fitness. Interestingly, the size of their colonies is in the same range of that of tumoral foci (*i.e.*, up to 4.7 mm wide) [152]. Thus, it is quite easy to envisage that some of them, hit by one or more mutations, may change from unipotential to multipotential [152], acquire pre-neoplastic characteristics and create the cellular and genetic background for a field of genetically related clones having typical pre-carcinogenic lesions.

The above considerations lead to another problem: why one clone should have more fitness than another, if both are yet under cell-cycle control and both share most of their genetic alterations? Also, it is known that pre-neoplastic bladder cell lines may stay dormant for long times [153]; why are they dormant, and why do they activate at a given time? As stated before, microenvironment is one of the variables that are implicated in cancer recurrence [154] and it is known that invasion relies also on lymph node stromal cells that create an adequate environment for the growth and further expansion of transformed cells [155, 156]. It was also explained that the presence of a "human" microenvironment is important as well in the development of orthotopic xenografts of breast cancer [157] and follicular lymphoma [155, 156]. Instead, a normal tissue microenvironment is known to inhibit tumour growth initiated by the Rous sarcoma virus [158, 159]. More recently, the same was demonstrated also for epithelia: cells growing as a monolayer behave differently from those organized in acini, and the more acini are organized, the more suppressive is the tissue surrounding mutated cells [160]. It has been also shown that it is possible to isolate apparently normal cells containing the same mutations found in the corresponding tumours [161, 162]; all together, these discoveries would explain the abovementioned dormancy of pre-cancerogenic cells, while complex cell-cell interactions might be responsible for the activation of such cells, for their migration towards a non-suppressive environment, and for the selection of cells able to survive inside a different microenvironment (invasion of surrounding tissues) [160, 163]. These data may be easily contextualized in BC: clonal expansion of mutated cells may occupy large areas of the bladder, supported by pre-carcinogenic stem cells which are controlled somehow in their expansion by the surrounding tissues. However, the more the patch grows, the more divergent are the cells composing it; consequently, two circumstances may occur: (i) newly mutated cells acquire new genetic characteristics, and (ii) these cells are themselves surrounded by pre-carcinogenic cells, which likely exert a lesser inhibition control over each other. The occurrence of one or more foci of fully carcinogenic cells, as a consequence of further physiological or genetic changes, would be then only a logical consequence.

4. CONCLUSIONS

BC is still one of the principal causes of disease and death. Far from being a simple cancer model, recent studies demonstrated that it hides various levels of complexity and more effort is needed to understand its etiology and to develop innovative management. New lines of research in chemical treatment are promising and alternative pharmacological tools are emerging (endothelin receptor antagonists, epigenetic modulators, active principles isolated from plants, gene therapy, selective COX-2 inhibitors, PPAR γ agonists), although the complete comprehension of what happens, at the molecular and cellular levels, in pre-neoplastic tissue is still an issue. Some of these mechanisms started to be unveiled in the very last years, and results are promising. Moreover, interesting results are coming from the technological development of well known drugs (*i.e.* functionalized nanoparticles, liposomes, sustained-release dosage forms) in order to overcome solubility or targeting issues in the view of the high rate of recurrence with BC.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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