Fractionation of high molecular weight tannins in grape seed extract and identification of procyanidin B2-3,3’-di-O-gallate as a major active constituent causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells

Chapla Agarwal1,2, Ravikanth Veluri1, Manjinder Kaur1, Shen-Chieh Chou1, John A. Thompson1,2 and Rajesh Agarwal1,2

1Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, CO 80262, USA and 2University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, CO 80262, USA

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Several studies have documented the anticancer and chemopreventive efficacy of grape seed extract (GSE) against various malignancies including prostate cancer (PCA). GSE is a complex mixture of polyphenols including gallic acid (GA), catechin (Cat), epicatechin (Epi) and procyanidins–oligomers of Cat and Epi, some of which are esterified with GA. Initial studies to identify the GSE components cytotoxic to human prostate carcinoma (DU145) cells demonstrated that GA and several crude chromatographic fractions containing procyanidin dimers and trimers were biologically active. The focus of the present work was to purify 14 procyanidins from the fractions and to identify those with highest activity toward growth inhibition, cell death and apoptosis in DU145 cells. The most active procyanidin was identified by mass spectrometry and enzymatic hydrolysis as the 3,3’-di-O-gallate ester of procyanidin dimer B2 (Epi–Epi). B2-digallate exhibited dose-dependent effects on DU145 cells over the range 25–100 μM, whereas GA exhibited comparable activity at lower doses but was highly lethal at 100 μM. Structure–activity studies demonstrated that all three hydroxyl groups of GA are necessary for activity, but a free carboxylic acid group is not required even though esterification reduced the activity of GA. These data, and the fact that non-esterified B2 exhibited little or no activity, suggest that the gallloyl groups of B2-digallate are primarily responsible for its effects on DU145 cells. Taken together, these data identify procyanidin B2-3,3’-di-O-gallate as a novel biologically active agent in GSE that should be studied in greater detail to determine its effects against PCA.

Introduction

Prostate cancer (PCA) is the most invasive and frequently diagnosed malignancy in elderly males in USA, and is the second leading cause of cancer-related deaths in American men (1). It is estimated that in 2006, there will be ~234 460 new cases of PCA in USA of which ~27 350 men will die of this malignancy (1). Early stage of PCA or the malignancy that is confined only within the prostate is conventionally cured with surgical prostatectomy or radiation therapy; some low-risk patients, however, also choose watchful waiting (2,3). The disease that has spread beyond the prostate is generally first treated with hormonal ablation (4,5), and the addition of cytotoxic chemotherapy is used in selected hormone-refractory patients, which has shown a small (~2 months) survival advantage (6,7). Unfortunately, all these treatment approaches suffer from their own limitations together with the emergence of androgen independence during commonly used antiandrogen therapy (8), suggesting that additional approaches and strategies are needed to control initial phases of PCA development, as well as its progression to advanced and androgen-independent stage.

Since PCA is a chronic disease, its prevention and/or therapeutic intervention are emerging as attractive additional strategies for disease control (9). In recent years, several studies have shown that phytochemicals present in diet as well as those consumed as dietary supplements are effective anticancer and/or chemopreventive agents against several malignancies including PCA (9–12). Grape seed extract (GSE) is a complex mixture of various polyphenols, some of which are also present in green tea (13,14). The grape seed polyphenols are generally referred to as procyanidins, and are marketed in USA as ‘GSE with 95% standardized procyanidins as dietary supplement due to multiple health benefits (15,16). In addition to grape seeds, procyanidins are a diverse group of polyphenolics that are also abundant in black jack oak, horse chestnut, witch hazel and hawthorn, as well as in apples, berries, barley, bean hulls, chocolate, rhubarb, rose hips and sorghum (15–17 and references therein).

With regard to anticancer and cancer chemopreventive effects of GSE, several studies have shown that this mixture inhibits human breast carcinoma MCF-7, human lung cancer A-427 and human gastric cancer CRL-1739 cell growth, but enhances the growth and viability of normal human gastric mucosal and normal J774A.1 murine macrophage cells (18). Our studies have shown that GSE inhibits growth, induces cell-cycle arrest and causes apoptotic death of human breast carcinoma MDA-MB-468 and prostate carcinoma DU145 and LNCaP cells in culture (19–22). With regard to its anticarcinogenic effects in animal models, oral feeding of GSE or procyanidin-rich fraction from GSE has been shown to prevent azoxymethane-induced aberrant crypt foci formation in rats (23,24), and topical application of GSE significantly prevents phorbol ester-mediated tumor promotion in chemical carcinogen-initiated mouse skin (25,26). Our original observation of skin cancer preventive efficacy of GSE was further verified and confirmed by others in showing that GSE also inhibits ultraviolet B radiation-induced skin tumorigenesis (27). With regard to the in vivo efficacy of GSE against PCA, we recently reported that oral feeding of GSE strongly inhibits DU145 xenograft growth in nude mice (28). Together, these studies have convincingly documented the anticancer and chemopreventive efficacy of GSE against various epithelial cancers including PCA, and indicate the presence of biologically active phytochemicals in the crude mixture.

The composition of GSE has been investigated extensively, and is known to consist largely of gallic acid (GA), catechin (Cat), epicatechin (Epi) and procyanidin dimers and trimers composed of flavan-3-ol units with C4-C8 interflavan linkages (29–32). These compounds are also present as esters linked to GA at the ali-LNCaP units with C4-C8 (Figure 1) or C4-C6 interflavan linkages (29–32). The latter fraction produced a low level of activity; however, the effect was substantially less than with intact procyanidin dimers and trimers composed of flavan-3-ol units with C4-C8 (Figure 1) or C4-C6 interflavan linkages (29–32). The only major component of fraction I was GA that proved to be highly toxic to DU145 cells. Fraction II contained Cat and Epi, neither of which was active, and fraction III consisted mainly of dimers B1 (Epi–Cat) and B2 (Epi–Epi) and the gallate ester derivative of a flavanol monomer (33). The latter fraction produced a low level of activity; however, the effect was substantially less than with intact GSE consistent with earlier conclusions that dimers B1 and B2 are essentially inactive in DU145 cell assays (C. Agarwal, R. Agarwal, unpublished observations). Fraction IV contained two unidentified procyanidin dimers and a dimer-gallate ester and exhibited low activity similar to that of fraction III. None of the fractions II–IV were investigated further because fractions V–VIII were considerably more abundant in blackjack oak, horse chestnut, witch hazel and hawthorn, as well as in apples, berries, barley, bean hulls, chocolate, rhubarb, rose hips and sorghum (15–17 and references therein).

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active in our assay system. As summarized in the Figure 2, liquid chromatography/mass spectrometry (LC/MS) analyses of those fractions confirmed that each contained three or four major components characterized as trimers or gallate esters of dimers and trimers. High-performance liquid chromatography (HPLC)–UV analyses of these fractions in our earlier work (33) provided an estimate of the relative concentrations of 14 major components, taking into account an ~3-fold higher extinction coefficient at 280 nm for gallate esters relative to non-esterified (epi)catechin monomer units.

Fig. 1. Structures of GA, Cat, Epi, procyanidin B2 and procyanidin B2-3'-O-gallate. Flavanol rings A, B and C discussed in the text are shown for Cat. Position numbering begins on the C ring oxygen and continues clockwise around C and A rings as shown.

The goal of the present study was to purify 14 procyanidins detected in GPC fractions V–VIII and to assess the cytotoxic activity of each in DU145 cells. Initial biological screening of these compounds demonstrated that three of them were significantly more active than either intact GSE or any other isolated compound. Further work revealed that one compound, i.e. procyanidin B2-3,3’-di-O-gallate (hereafter referred as B2-G2), consistently produced the greatest activity in DU145 cells. GA also is known to be very active in these cells, so the effects of GA and several hydroxybenzoic acid derivatives were directly compared with B2 and B2-G2 in order to elucidate the structural components most important for inducing growth inhibition and death in prostate carcinoma cells.

Materials and methods

Materials

GSE was obtained from Traco Labs (Champaign, IL) and an ethyl acetate extract of the phenolic components prepared as described (33). GA, 4-hydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, propyl gallate and trifluoroacetic acid were purchased from Aldrich (Milwaukee, WI). Cat and Epi were from Sigma (St Louis, MO), procyanidin B2 was obtained from Indofine Chemical Co. (Hillsborough, NJ), and HPLC grade solvents and ammonium acetate were obtained from Fisher Scientific (Pittsburgh, PA).

Chromatography

GPC separations were carried out by injecting methanolic solutions containing 200 mg of the ethyl acetate extract of GSE onto a 25 × 400 mm glass column prepared with Toyopearl HW-40S resin (Tosoh Biosep, Montgomeryville, PA). The compounds were eluted with methanol at a flow rate of 1.2 ml/min and monitored at 280 nm. Fractions were collected over the time intervals indicated in Figure 2, methanol evaporated under reduced pressure at 35–40°C and the dried residue stored at −20°C under argon until processed further. The principal components in GPC fractions V–VIII (Figure 2 and in ref. 33) were isolated by semi-preparative HPLC on a 10 × 250 mm Prodigy ODS-2 column (Phenomenex, Torrance, CA) eluted with a gradient consisting of water (A) and acetonitrile (B), each containing 0.1% trifluoroacetic acid at a flow rate of 3.0 ml/min. The initial solvent composition of 8% B in Awas raised to 20% B from 0 to 20 min and then to 50% B from 20 to 40 min. Each sample was evaporated to dryness at <40°C under vacuum and stored under argon at −20°C. The purities of isolated compounds were confirmed by analytical HPLC conducted as described for the preparative work except that a 4.6 × 250 mm Prodigy ODS-2 column was employed with a flow rate of 1.0 ml/min.

LC–MS studies

Compounds were analyzed in the negative ion mode with an Agilent-SL LC/MSD employing electrospray ionization (ESI). The HPLC column was a 2.0 × 150 mm Prodigy ODS-2 (Phenomenex) with the mobile phase described for semi-preparative HPLC except that the eluent contained 10 mM ammonium acetate instead of trifluoroacetic acid, and the flow rate was 0.20 ml/min. The nebulizer and collision gases were nitrogen and helium, respectively, with the former set at 20 psi. The drying gas was nitrogen at a flow rate of 8 l/min. Capillary and exit voltages were −120 and −40 V, respectively, and the instrument was scanned over the mass range 250–1500 Da. Tandem MS [MS/MS (MS²)] spectra were acquired with parent ions automatically selected by the system software. MS² spectra were recorded from pre-selected MS² ions.

Hydrolysis of gallate esters

Enzymatic hydrolysis of procyanidin gallate esters was carried out as described earlier (30). Briefly, each gallate ester (1.0 mg) was dissolved in 1 ml of 0.1 M acetate buffer at pH 5.0 and incubated with 1 mg/ml of Tannase (41 U/mg) (Wako Pure Chemical Industries, Osaka, Japan) at 37°C for 1 h. The reaction mixture was filtered through a 0.45 µm membrane and 5 µl injected onto the analytical HPLC system described above.

Cell growth and death assays

Human prostate carcinoma cell line DU145 was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 with 10% fetal bovine serum (Hyclone, Logan, UT) under standard culture conditions (37°C, 95% humidified air and 5% CO₂). RPMI 1640 and other culture materials were from Life Technologies (Gaithersburg, MD). DU145 cells were plated at 1 × 10⁵ cells/60 mm plates under standard culture condition. After 24 h, cells were fed with fresh medium and treated with dimethyl sulfoxide alone (control), or GSE, individual constituents or GA dissolved in dimethyl sulfoxide at desired doses. Dimethyl sulfoxide concentration was the same for all

![Image of structures and spectra](image-url)
C.Agarwal

such as B2-G2 (Figure 4a) or a trimer in which one of the flavanol possible structures, either the digallate ester of a procyanidin dimer additional 152 Da fragments yielding ions at m/z 577 and 407 induced dissociation caused successive expulsions of one or two ring from a retro-Diels-Alder reaction (34). In addition, collision-neutral fragment of 152 Da from the parent ion due to the loss of either prominent daughter ion at m/z 729 Da was formed by the loss of a
distinguished by collision-induced dissociation (Figure 4b). The
pounds differ by only 0.04 Da; however, the structures were readily
units is gallocatechin (or epigallocatechin). The masses of these com-

Results

Biological activities of procyanidins isolated from GSE

Each of the GPC fractions V–VIII (Figure 2) was shown previously by LC–MS analyses to contain three or four major components (33). A total of 14 compounds have now been purified by semi-preparative HPLC and their biological activities examined in DU145 cells. As shown in Figure 3, compounds VII-b, VII-d and VIII-c were most effective in inhibiting cell growth and/or causing cell death during 72 h incubations. At equivalent doses of 100 μg/ml, intact GSE produced 74% growth inhibition, whereas compounds VII-b, VII-d and VIII-c caused 90, 83 and 88% inhibition (P < 0.001), respectively (Figure 3a). Similarly, at the same dose and treatment time, all three compounds were more effective than GSE at killing cells (Figure 3b). Compounds VII-c and VIII-b were also active (although not more active than GSE) in both cell growth inhibition and cell death assays (Figure 3). Studies to examine the apoptotic nature of these effects demonstrated that VII-b, VII-d and VIII-c also produced the strongest apoptotic responses accounting, respectively, for 5.2-, 5.8- and 3.7-fold (P < 0.001) increases in apoptotic cell populations compared with a 3.3-fold increase by GSE (Figure 3c). Many of the other procyanidins exhibited apoptotic activity of a similar or lesser magnitude than GSE. Among the 14 compounds isolated from GSE, therefore, our data distinguish VII-b, VII-d and VIII-c as the most cytotoxic and potent inducers of apoptosis in PCA cells.

The relative potencies of these three compounds in DU145 cells were re-evaluated at the same concentrations and compared with intact GSE. As shown in Table I, after 72 h incubations, compound VII-b exhibited the greatest potency of the three procyanidins, causing substantially more cell growth inhibition (88%, P < 0.001), cell death (56%, P < 0.001) and apoptosis induction (5.6-fold, P < 0.001) than VII-d and VIII-c. The latter two compounds also caused growth inhibition and cell death, but only to a degree comparable with intact GSE although induction of apoptosis by these compounds was somewhat more pronounced than with GSE. These data establish that compound VII-b is the most active of the 14 procyanidins in the DU145 cell assays.

Identification of VII-b

The chemical structure of VII-b was investigated by negative ion ESI LC–MS (Figure 4). The [M–H]– ion at m/z 881.4 Da suggests two possible structures, either the digallate ester of a procyanidin dimer such as B2-G2 (Figure 4a) or a trimer in which one of the flavanol units is galloatechin (or epigalloatechin). The masses of these compounds differ by only 0.04 Da; however, the structures were readily distinguished by collision-induced dissociation (Figure 4b). The prominent daughter ion at m/z 729 Da was formed by the loss of a neutral fragment of 152 Da from the parent ion due to the loss of either a galloyl group or the flavanol B ring together plus portions of the C ring from a retro-Diels-Alder reaction (34). In addition, collision-induced dissociation caused successive expulsions of one or two additional 152 Da fragments yielding ions at m/z 577 and 407

(425–H2O). Collision-induced dissociation of the m/z 729.3 ion (i.e. MS2 analysis shown in Figure 4c) further demonstrated losses of 152 Da fragments yielding ions at m/z 577, 559 (577–H2O) and

Fig. 3. Biological activities of GSE components isolated by GPC and semi-preparative HPLC. DU145 cells were cultured under standard conditions and treated with dimethyl sulfoxide (C), GSE (G) or indicated constituents (detailed in Table I and Figure 2) in dimethyl sulfoxide at 100 μg/ml dose for 72 h. Cells were then collected and counted for (a) total live and (b) dead cells or (c) processed for annexin V-propidium iodide staining followed by fluorescence-activated cell sorting analysis as described in Materials and methods. The data shown in each case are mean ± standard error of three–four samples. #, P < 0.05 and *; P < 0.001; versus dimethyl sulfoxide control.

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541 (577–2H2O). Interflavan bond cleavage occurred as well producing ions at 289 and 441 Da corresponding, respectively, to Epi (or Cat) and the gallate ester of one of these flavanols. The dimer digallate structure is expected to fragment with multiple losses of 152 Da fragments both from losses of galloyl groups and retro-Diels-Alder reactions as mentioned above. On the other hand, the alternative trimer structure mentioned is not expected to undergo more than one 152 Da loss. In addition, interflavan bond cleavage within the trimer would not produce an ion at m/z 441 Da seen in the MS 3 spectrum. These data, therefore, are consistent only with a procyanidin digallate structure for compound VII-b.

To identify the procyanidin portion of VII-b, the digallate was treated with an esterase that produced GA and procyanidin B2 (Figure 4d) as determined by comparing the HPLC retention and MS/MS data with authentic standards and by observing peak area enhancements when co-injecting authentic GA or B2 with the hydrolysis products. None of the other procyanidin dimers co-elute with B2 (26,31). For comparison, compound VI-d proposed previously to be a dimer monogallate from MS data (Figure 2) (33) also was converted to GA and procyanidin B2 upon enzymatic hydrolysis. The HPLC peak area ratio of GA:B2 in this case was ~50% of the ratio obtained from hydrolysis of the digallate as expected. These data confirm the structure

![Fig. 4.](image-url)
of VII-b, the most active procyanidin isolated from GSE, as B2-G2, shown in Figure 4a.

Comparison of B2-G2 with GA and hydroxybenzoic acid derivatives

The activity of B2-G2 was compared with GA as the latter was shown previously to be highly active (33). Treating cells with 25, 50 or 100 μM amounts of each compound resulted in increased inhibition of cell growth (Table II). GA and B2-G2 exhibited 33–81% (P < 0.001) and 32–63% (P < 0.001) inhibition at 25–50 μM doses, respectively. At 100 μM GA, all the cells were destroyed within 24 h; therefore, no data could be obtained at the highest concentration. On the other hand, 100 μM B2-G2 was less toxic and caused 80% (P < 0.001) cell growth inhibition comparable with that with 50 μM GA. Unlike the high toxicity of GA, the effects of B2-G2 were dose dependent even at a 100 μM concentration, causing 44% cell death (P < 0.001) and a 5.1-fold increase in the apoptotic cell population relative to vehicle controls. Non-esterified B2 produced detectable levels of cell death or apoptosis, but the effects were small compared with GA and B2-G2. These data suggest different modes of action in DU145 cells for GA and B2-G2 and suggest that the latter is more likely to exhibit useful antitumor properties.

Additional studies were conducted to examine structural features essential for the activity of GA. As shown in Table II, three hydroxybenzoic acid derivatives with fewer than three phenolic hydroxyl groups caused little or no effect on cell growth with the exception that 100 μM 3,4-dihydroxybenzoic acid produced a modest 22% inhibition. All three of these compounds induced <15% cell death, which are small effects compared with GA at equimolar doses. The propyl ester of GA, Propylgallate (PG), on the other hand, yielded dose-dependent inhibition of cell growth (28–73%), although the effect was less than for either GA or B2-G2 at equivalent doses (Table II). The highest dose of PG (100 μM) produced 20% cell death, which is comparable with that observed with GA and B2-G2 at a 50 μM dose (Table II). These data indicate that the phenolic hydroxyl groups of GA are essential for activity, whereas the presence of a free carboxylic acid group on GA does not appear to be essential. Of course, we cannot rule out the possibility that PG was converted to GA by cellular esterases.

Discussion

With increasing body of evidence implicating diets rich in fruits and vegetables with lower incidence of cancer, in recent years, efforts have been directed toward identifying and characterizing the phytochemicals present in these diets with potential anticancer activity. Though the concept of eating fruits and/or vegetables, i.e. consuming phytochemicals naturally, would be more effective in the fight against cancer, the evaluation of biological efficacy of individual components is an important step toward drug discovery and development. This approach is further necessitated by the fact in general that most phytochemicals have low bioavailability and may be present in only very small amounts in plant materials. Thus, once an active constituent is identified, there are a number of options available to increase its bioavailability and to make it commercially available in larger amounts. With this rationale in mind, the purpose of our investigations is to further isolate and characterize the individual high molecular weight procyanidins responsible for both anticancer and cancer preventive effects of GSE in numerous cell culture and animal models of cancer (18–22). In addition, probing the structural components of these compounds responsible for activity may provide insights into mechanisms of action.

In earlier work, GA was found to be highly active in DU145 prostate carcinoma cells; however, separating GSE into GA and seven flavanol-containing fractions by GPC revealed that fractions containing mainly procyanidin trimers and gallate esters of dimers and trimers also were active (33). The main components of these fractions have now been isolated and their activities examined individually. The results demonstrate that B2-G2 (Figure 4a) is the most active of the 14 compounds tested for inhibition of the growth and/or enhancement of the death of DU145 cells. Monogallate esters of a putative C4-C6 dimer (VII-d) and a procyanidin trimer (VIII-c) also were active, but were not investigated further as B2-G2 exhibited substantially greater activity.

The digallate ester of procyanidin B2 has previously been identified as a component of GSE (30) and several diverse biological properties have been determined. For example, B2-G2 was shown to be cytotoxic in human melanoma cell line RPMI 7951, whereas non-esterified B2 did not affect cell viability (35). Interestingly, Epi 3-O-gallate was ~6-fold more toxic than B2-G2 in these cells in contrast to our results with DU145 cells showing little activity for the former compound. Others demonstrated that B2-G2 is an effective inhibitor of rat squa- lene epoxidase and attributed this effect to its antioxidant activity (36). The antioxidant properties of several procyanidins were evalu- ated recently in a chemical system (37); radical scavenging activity of B2-G2 was substantially greater than that of non-esterified B2 and comparable B2-3'-O-gallate. In other studies, B2-G2 was found to be a potent inhibitor of DNA polymerase α, whereas B2-G was ~3- fold less active and B2 was inactive in that assay (37). There are examples in the literature, therefore, of B2-G2 activity relevant to anticarcinogenesis.

The finding that GA is highly active in DU145 cells prompted initial studies to elucidate the structural components of this trihydroxybenzoic acid required for activity. Mono- and dihydroxybenzoic
acids exhibited little or no effect on DU145 cells, demonstrating the importance of a third hydroxyl group for activity. Esterification decreased the effects of GA on cell growth, as PG was less active than GA at all doses. As mentioned earlier, it is possible that the effects of PG were due to GA formed intracellularly by esterases and additional work will be necessary to resolve this point. PG was also less active than B2-G2 at equivalent doses; however, when the comparison is based on gallate equivalents, the activities were comparable as 100 μM PG produced effects similar to 50 μM B2-G2. These data and the fact that non-esterified B2 exhibited little or no activity indicate that the galloyl groups of the B2-G2 diester are primarily responsible for its effects on DU145 cells. As mentioned for PG, the intracellular release of GA from B2-G2 is possible. Whether the intact procyanidin diester or released GA is responsible for the effects of B2-G2, our data support the latter compound as the single component of GSE most likely to exhibit effective anti-PCa activity due to a more favorable dose–response profile as compared with GA.

The results of our work indicate that B2-G2 warrants further investigation with respect to its mechanism of action in vitro. Since B2-G2 seems to be the most active component present in GSE, based on our previous findings with GSE, we speculate that growth inhibitory and apoptotic effects of B2-G2 in DU145 cells might also be due to its effect on cell-cycle regulatory molecules involving an alteration in cyclin–CDK–CDKI axis and activation of caspases (21). However, further mechanistic studies are required to completely and convincingly elucidate the biological effects of B2-G2 in both cell culture and animal PCA models. It was determined recently that epigallocatechin-3-gallate, the monomer analog of B2-G2, undergoes auto-oxidation with the concomitant production of reactive oxygen species in cell culture (38). It is likely that B2-G2 also undergoes auto-oxidation to some extent; however, the monomer is inactive in DU145 cells, so substantial differences in the fate and/or actions of these gallate esters in vitro are expected. The effectiveness of B2-G2 against PCA also must be evaluated in vivo, including the critical issues of absorption and bioavailability. Whereas pure B2-G2 can be isolated from GSE by leased GA is responsible for the effects of B2-G2, our data support the monomer is inactive in DU145 cells, so substantial differences in the fate and/or actions of these gallate esters in vitro are expected. The effectiveness of B2-G2 against PCA also must be evaluated in vivo, including the critical issues of absorption and bioavailability. Whereas pure B2-G2 can be isolated from GSE by

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References


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